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Characterization of the human mitochondrial Arginyl-tRNA synthetase and contribution to the général understanding of pathologies linked to mutations on mitochondrial aminoacyl-tRNA synthetases

Ligia Elena Gonzalez Serrano

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ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

Architecture et Réactivité de l'ARN – UPR 9002 du CNRS

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présentée par :

Ligia Elena GONZÁLEZ SERRANO

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**Caractérisation de l'ArgRS mitochondriale
humaine et contribution à la compréhension des
pathologies liées aux mutations des
aminoacyl-ARNt synthétases mitochondriales**

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**Characterization of the human mitochondrial
Arginyl-tRNA synthetase and contribution to the
general understanding of pathologies linked to
mutations on mitochondrial aminoacyl-tRNA
synthetases**

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Abbreviations

A Ampere
Å Angstrom
aa Amino acid
aaRS Aminoacyl-tRNA synthetase
APP3 Aminopeptidase P
Asp Aspartic acid (aspartate)
ATP Adenosine triphosphate
BHK21 Baby hamster kidney cells
Ci Curie
CNS Central nervous system
DNA Deoxyribonucleic acid
E. coli Escherichia coli
EF Elongation factor
ER Endoplasmic reticulum
HEK293T Human embryonic kidney cells
Icp55 Intermediate cleaving peptidase
IM Inner membrane
IMP Inner membrane peptidase
IMS Intermembranes space
IPTG Isopropyl β -D-1-thiogalactopyranoside
kDa kilo Dalton
KO Knockout
lactate elevation
LBSL Leukoencephalopathy with brainstem and spinal cord involvement and
MARS Multi aminoacyl-tRNA synthetase complex
min Minute
MIP Mitochondrial processing peptidase (Oct1, ocatapeptidyl aminopeptidase)
mM Millimolar
MPP Mitochondrial processing peptidase
MRI Magnetic resonance imaging
MRS Magnetic resonance spectroscopy
mt Mitochondrial
MTS Mitochondrial targeting sequence (or signal)
MVA Modified Vaccina Ankara
MW Molecular weight
NGS Next-generation sequencing
nm Nanometer
OM Outer membrane
PCH6 Pontocerebellar hypoplasia type 6
PCR Polymerase chain reaction
RC Respiratory chain
RNA Ribonucleic acid
SDS-PAGE Sodium dedocyl sulfate-polyacrylamide gel electrophoresis
SOD2 Superoxide dismutase 2
UV Ultraviolet
 μ g Microgram

Introduction

1. Mitochondria: more than the energy machinery of the cell

Mitochondria play an important role in energy metabolism, converting the energy released by the oxidation of nutrients into ATP. They also carry out terminal metabolic pathways of energy molecules such as β -oxidation of fatty acids, the Krebs cycle, among other functional diversities. In addition to the listed functions these organelles also play a major regulatory role in calcium homeostasis, cell proliferation, apoptosis, formation of iron-sulfur clusters, among others (Kirichok *et al.*, 2004; Mandal *et al.*, 2011; Meisinger *et al.*, 2008). Recent investigations have assigned to mitochondria functions in the immune response (Angajala *et al.*, 2018). The participation of the mitochondria in all these vital functions makes this organelle essential for the survival of the cell.

Mitochondria have their own genetic information, which in humans is a circular DNA encoding 13 proteins, 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs). However, their functions also depend on the importation of numerous proteins coded by the nuclear genome. These imported proteins are involved in the maintenance and expression of the mitochondrial genome and in the metabolic activities (**Figure 1**).

The mitochondrial translation of the genetic information is the essential process for the expression of genetic information encoded by the mitochondrial DNA (mt-DNA). The genetic code determines the rules for the equivalence codon/amino acid and the transfer RNA (tRNA) acts as an interpreter at the ribosome level. It ensures the association between the codon of the mRNA and the amino acid fixed on the tRNA by codon/anticodon complementarity. Thus, the fidelity of the translation is based on this complementarity (named "genetic code") but also on the correct association of each amino acid onto the cognate tRNA (named "second genetic code"). This last association is called aminoacylation reaction. It consists on covalently fixing an amino acid on the 3'-end of the corresponding tRNA. This reaction is catalyzed by the aminoacyl-tRNA synthetases (aaRS). The specificity of the attachment of each amino acid onto the cognate tRNA(s) is ensured by elements present on the aaRS and the tRNA (Giegé and Frugier, 2003; Giegé *et al.*, 1998; Hasegawa *et al.*, 1991; Ibba *et al.*, 1999; Saks *et al.*, 1994).

Mitochondrial dysfunctions are involved in a wide and diverse spectrum of human disorders as cancer, muscular dystrophies, neurodegenerative and neurodevelopment disorders. Mutations in the imported mitochondrial aminoacyl-tRNA synthetases have been related with pathologies that mainly target the central nervous system.

This present introduction will be divided in four main parts: 1) Mitochondria: more than the energy machinery of the cell; 2) the mitochondrial translation machinery; 3) the import and processing of nuclear-encoded mitochondrial proteins; and 4) the mitochondrial translation machinery and human diseases.

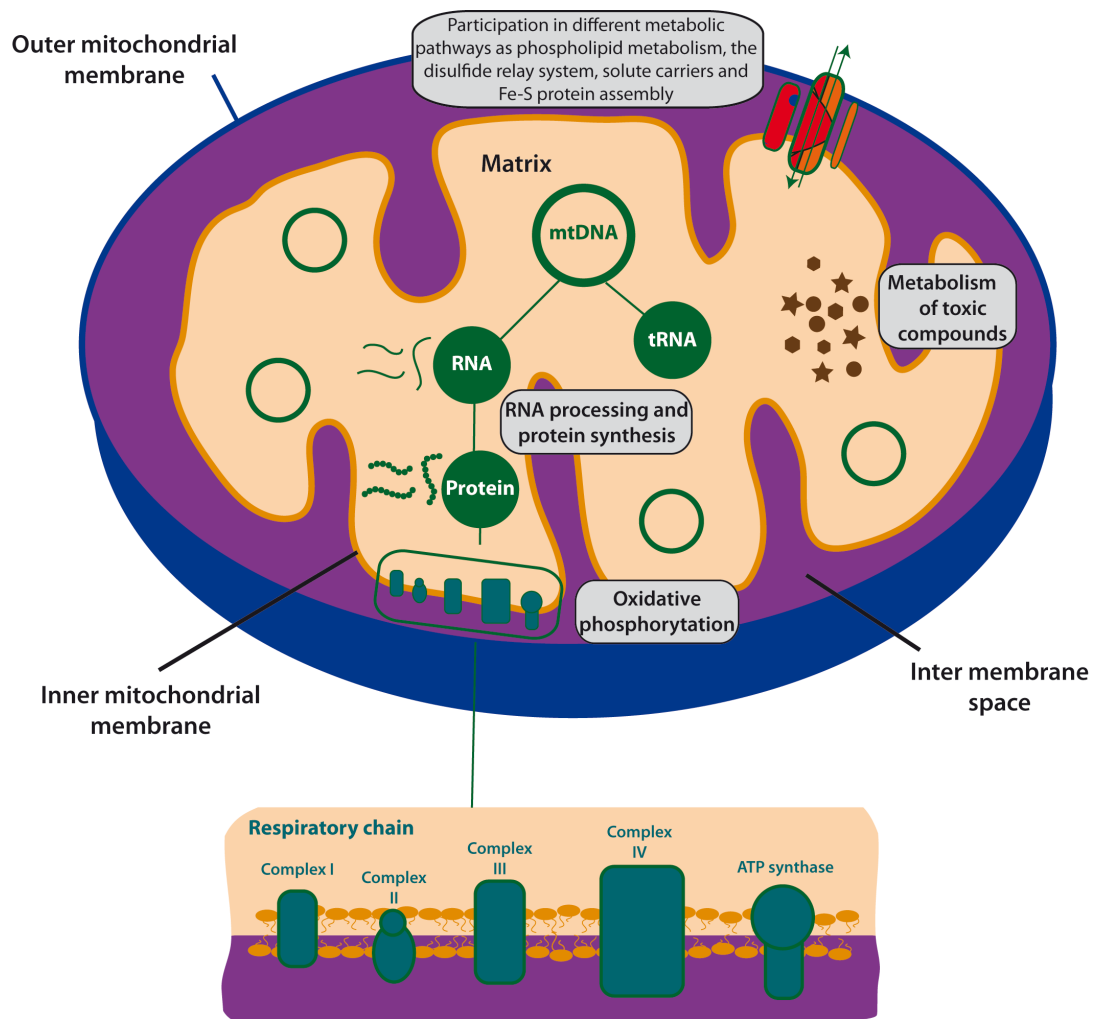


Figure 1. Schematic representation of the mitochondrial components and major mitochondrial functions.

1.1. Origin of mitochondria

The origin of mitochondria seems well established with the theory of endosymbiosis (Gray *et al.*, 2001). An alpha proteobacterium would be the original endosymbiote whose evolution would have been accompanied by a massive transfer of genes to the nuclear genome of its host (Gray *et al.*, 1999). This hypothesis is supported by the existence in the nuclear genome of numerous pseudogenes homologous to fragments of human mt-DNA. Analysis of mitochondrial proteomes revealed that only part of the proteins would be of alpha proteobacterial origin, another part would have a eubacterial origin and the remainder would have no eubacterial homologues. Various endosymbiotic models for the origin of mitochondria and chloroplasts from alpha Proteobacteria and Cyanobacteria imply transfers of bacterial genes into the nuclear genome of the eukaryotic host (reviewed in (Dyall *et al.*, 2004; Gray *et al.*, 1999; Kurland and Andersson, 2000)). Horizontal transfer, deletion and gene duplication phenomena might explain the remaining proteins with orphanage origin. There are data indicating that transfers from mitochondrial to nuclear genomes are an ongoing evolutionary process (Kurland and Andersson, 2000).

Finally, the maintenance of only some genes in the mitochondrial genome, at least in mammals, remains an open question and could be due to the extremely hydrophobic nature of the corresponding proteins, which would interfere their import through the mitochondrial membranes (Anderson *et al.*, 1981).

1.2. Morphology of mitochondria

The word “mitochondria” comes from the Greek civilization, *mitos* meaning “filament”, *chondros* meaning “seed” and was proposed after the observation of their appearance by optical and electronic microscopy (Logan, 2006). Mitochondria are compartmentalized organelles of approximately less than 10 µm; this size varies depending on the cell type (Okie *et al.*, 2016). The number of mitochondria per cell varies from few hundreds to several thousands depending as well on the cell type (Kuznetsov and Margreiter, 2009).

Mitochondria are delimited by two lipid membranes creating two separate mitochondrial compartments: the internal matrix space and the intermembrane space (**Figure 1**). The matrix contains hundreds of enzymes including those required for the

citric acid cycle and β -oxidation. The inner membrane is folded into numerous cristae, increasing the surface area (Palade, 1953). The outer membrane is permeable to all molecules less than 5 kDa, however most of those molecules cannot pass through the inner membrane because of its high selectivity (Vander Heiden *et al.*, 2000).

1.3. Functions: Metabolic pathways hosted in the mitochondria

Mitochondria are widely known for its main function, the synthesis of cellular energy in form of ATP using the ATP synthase. However, another essential function of mitochondria is the Iron-sulfur (Fe-S) cluster biogenesis (Lill, 2009). A mitochondria reduced to its simplest function will indeed maintain Fe-S metabolism, but not necessarily the respiratory chain and ATP synthesis (Beilschmidt and Puccio, 2014). Mitochondria participate in addition to other functions necessary for cellular homeostasis and maintenance. Mitochondria are critical in the metabolism of lipids, carbohydrates and amino acids (Fernie *et al.*, 2004). They are also involved in the synthesis of some phospholipids, including phosphatidic acid, cytidine diphosphate diacylglycerol, phosphatidyl glycerol and cardiolipin (Voelker, 1991), thus autonomously creating lipids necessary for their membrane structure. In addition, mitochondria are involved in the synthesis of urea, steroids, heme compounds and Ca^{2+} signalling (Kirichok *et al.*; Ohta, 2003). Mitochondria also play a role in cell differentiation and cell proliferation (Mandal *et al.*, 2011). Mitochondrial function and biogenesis is tissue-specific, for example, liver mitochondria contain specific enzymes for the detoxification of ammonia (Degli Esposti *et al.*, 2012; Fernandez-Vizarra *et al.*, 2011).

1.4. Crosstalk of the mitochondria within the cell

Mitochondria are far from being an isolated organelle. The relationship between the mitochondria and the cell is symbiotic. The nucleus handles much of the machinery that replicates and maintains the mitochondrial structure, while the mitochondria improve functions that the cell cannot perform efficiently. An example of this is the 32 molecules of ATP formed from oxidative phosphorylation *versus* the 2 molecules of ATP released from glycolysis. The mitochondria need to work with the rest of the cell to maintain cellular and mitochondrial homeostasis (Fosslien, 2001). Mitochondrial

homeostasis refers to the maintenance and regulation of mitochondrial morphology and function through some events such as: mitochondrial biogenesis, the orchestrated process of transcription and translation of proteins in the nucleus and cytoplasm; balance of fission and fusion events; proper distribution of mitochondria in the cell through transport; and, mitophagia, which is the degradation of damaged mitochondria (Suen *et al.*, 2008).

1.5. Mitochondrial Genome

The mitochondrial matrix contains several copies of mt-DNA as well as all the elements necessary for its replication and expression. While the mitochondrial genome of *Arabidopsis thaliana* is large (125 000 bp) (Initiative, 2000), the human mitochondrial genome is a small double-stranded circular DNA of 16 569 bp (Anderson *et al.*, 1981). It presents maternal inheritance and is transmitted by mitochondria present in the oocytes. The two strands have different densities, distinguishing a heavy strand richer in guanine, from a light strand richer in cytosine. Each strand has its own origin of replication, which can be bidirectional and asynchronous (Clayton, 1991), or unidirectional (Falkenberg *et al.*, 2007). Human mt-DNA is highly compact with no introns and contains only one major non-coding region (NCR) known as the control region (comprising ~900 bp in mammals). The control region contains the origin of replication of one DNA strand, the origins of transcription for both DNA strands, and the Displacement loop (D-loop), necessary for replication and transcription of the genome. Human mt-DNA codes only for 13 proteins (all subunits of the respiratory chain), 2 ribosomal RNAs and 22 transfer RNAs, both of which are essential for mitochondrial protein synthesis (Clayton, 1991; Falkenberg *et al.*, 2007).

1.6. Respiratory chain at the crossroad

Mitochondria are known as the "energy powerhouse of the cell" because approximately 90% of ATP production takes place in the internal mitochondrial membrane. ATP is produced by the respiratory chain complexes (I-V) (**Figure 2**). Complex I (ubiquinone NADH dehydrogenase) is in charged of the oxidation of NADH and pumps protons (H^+) into the intermembrane space while reducing the ubiquinone. Complex II (succinate dehydrogenase) oxidizes the succinate into malate, thus

releasing reducing equivalents (electrons) that are transported to complex III through the ubiquinone. Complex III (ubiquinol-cytochrome-reductase) receives electrons, releasing H^+ in this process. Complex IV (cytochrome-C oxidase) reduces O_2 to H_2O , producing H^+ . As each complex moves electrons along the chain, protons are pumped from the matrix into the intermembrane space. The generated proton gradient is used to direct the synthesis of ATP by the V Complex (F1F0 ATP synthase), which phosphorylates ADP to ATP (Alberts *et al.*, 1989). This proton gradient is commonly known as the mitochondrial membrane potential ($\Delta\psi_m$) and represents the energy available to drive changes in the ATP / ADP ratio and reactive oxygen species, as well as control the sequestration of mitochondrial calcium (Graier *et al.*, 2007).

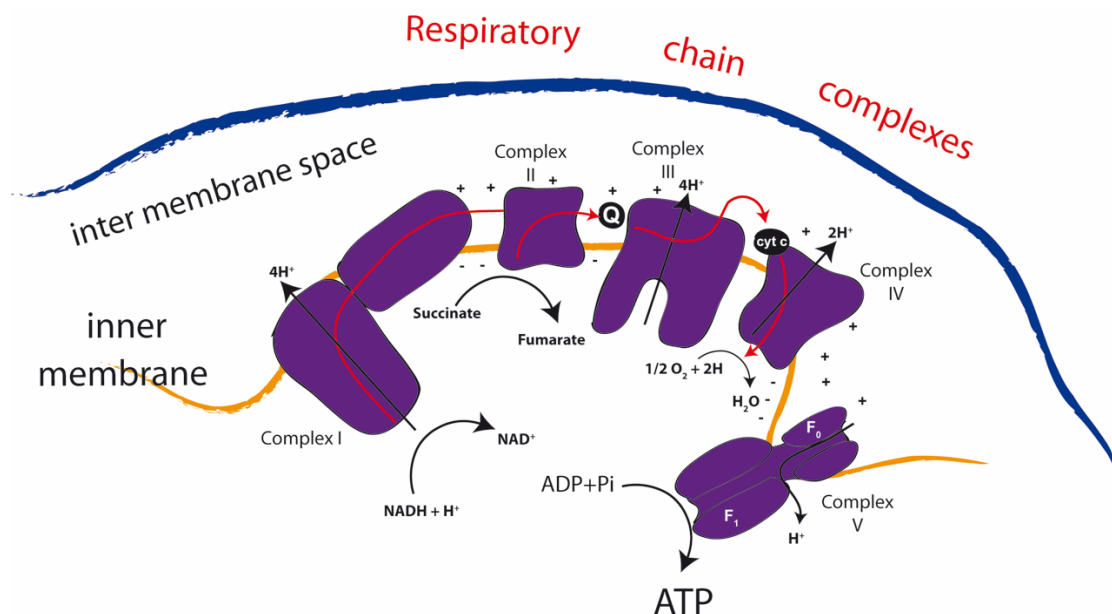


Figure 2. The respiratory chain complexes. Organization of the complexes along the mitochondrial inner membrane. Complex I: NADH: Ubiquinone reductase; Complex II: Succinate-coenzyme Q reductase; Complex III: Coenzyme Q: cytochrome *c* oxidoreductase; Complex IV: Cytochrome *c* oxidase; Complex V: ATP synthase. **Q**: Coenzyme Q; **cyt c**: cytochrome *c*.

2. Mitochondrial translation machinery

Among the 37 genes present in the human mitochondrial genome, only 13 code for proteins, all components of the respiratory chain. The other subunits of this respiratory chain (85 proteins) as well as the other mitochondrial proteins (estimated at ~1500 (Meisinger *et al.*, 2008)), including the mt-aaRS, are imported from the cytosol (Taylor *et al.*, 2003). Mitochondrial translation machinery is intimately associated with the

inner mitochondrial membrane where mitochondrial ribosomes are also located (Liu and Spremulli, 2000). Its mechanism is similar to eubacterial systems but with some particularities. The mitochondrial genetic code presents variations compared to the "universal" code. In mammalian mitochondria genomes, the stop codon UGA is decoded by the tRNA^{Trp}, the isoleucine codons AUA and AUU by the tRNA^{Met} and the arginine codons AGA and AGG become stop codons. So, there are four stop codons instead of three, and there are only four arginine codons instead of six (Florentz and Sissler, 2003). Given the limited number of 22 mt-tRNAs available to decode the sixty existing codons (one per amino acid, except two for leucine and two for serine), a codon-anticodon interaction limited to two of the three positions could allow greater flexibility in mRNA/tRNA recognition. Mitochondrial ribosomes are distinguished by their low rRNA content (33% versus 66% in bacterial ribosome and 60% in cytosolic ribosome), and by the loss of some structural regions composed of RNA, which seems to be compensated by a higher protein content (O'Brien, 2003).

2.1. Co-evolutionary events in the mitochondrial translation machinery

The dual genetic origin of the macromolecules constituting the translation machinery has raised numerous questions as to their properties, mechanism, and specificities of their partnerships, regulation of their expression and mechanisms of co-evolution. Indeed, the mitochondrial genome evolves 15–20 times more rapidly than the nuclear genome (Adrion *et al.*, 2016; Brown *et al.*, 1979; Castellana *et al.*, 2011), generating highly variable sequences so that the RNAs coded by the mammalian mitochondrial genomes are peculiar. All have lost some information as compared to their bacterial homologs. For instance, mRNAs miss 3' and 5' untranslated regions, ribosomal RNAs are significantly shorter than bacterial counterparts and tRNAs present a range of peculiarities, from the absence of a few nucleotide signature motifs to the absence of full structural domains (Helm *et al.*, 2000; Willkomm and Hartmann, 2006). All of these "non-canonical" RNAs are, however, recognized by nuclear encoded proteins, requiring accommodation rules of the two sets of macromolecules.

The evolutionary origin of the different nuclear-encoded mitochondrial proteins remains however intriguing. Sequence alignments of the mt-aaRSs have indeed not identified sequence stretches or signature motifs that could originate from alpha-

proteobacterial ancestors, favouring various horizontal gene transfers events along evolution (Brindefalk *et al.*, 2007). In general, it has been postulated that the conservation of a functional translation in mitochondria requires a continuous adaptation of proteins in response to reduced or degenerated RNAs. This is illustrated by a gain of plasticity, demonstrated for example when studying the human mt-AspRS and compared to the homologue AspRS from *E. coli*. Even if both proteins share a common 3D architecture, the mammalian mt-aaRS showed a gained in plasticity, enabling it to handle any tRNA^{Asp} and especially the non-canonical mt-tRNA^{Asp} (Neuenfeldt *et al.*, 2013). The enlarged plasticity of the mitochondrial enzymes as compared to that of aaRSs from other origins might be the result of an evolutionary adaptation of the nuclear-encoded protein to the rapidly evolving mitochondria-encoded tRNAs (**Figure 3**). Evolutionary-induced changes in intrinsic properties of proteins may represent an alternative to other strategies, such as those reported for the mitochondrial ribosome, where the strong restriction in RNA sizes is compensated by extension of the number and size of the nuclear encoded proteins (Adrion *et al.*, 2016). This particularity will be further discussed below.

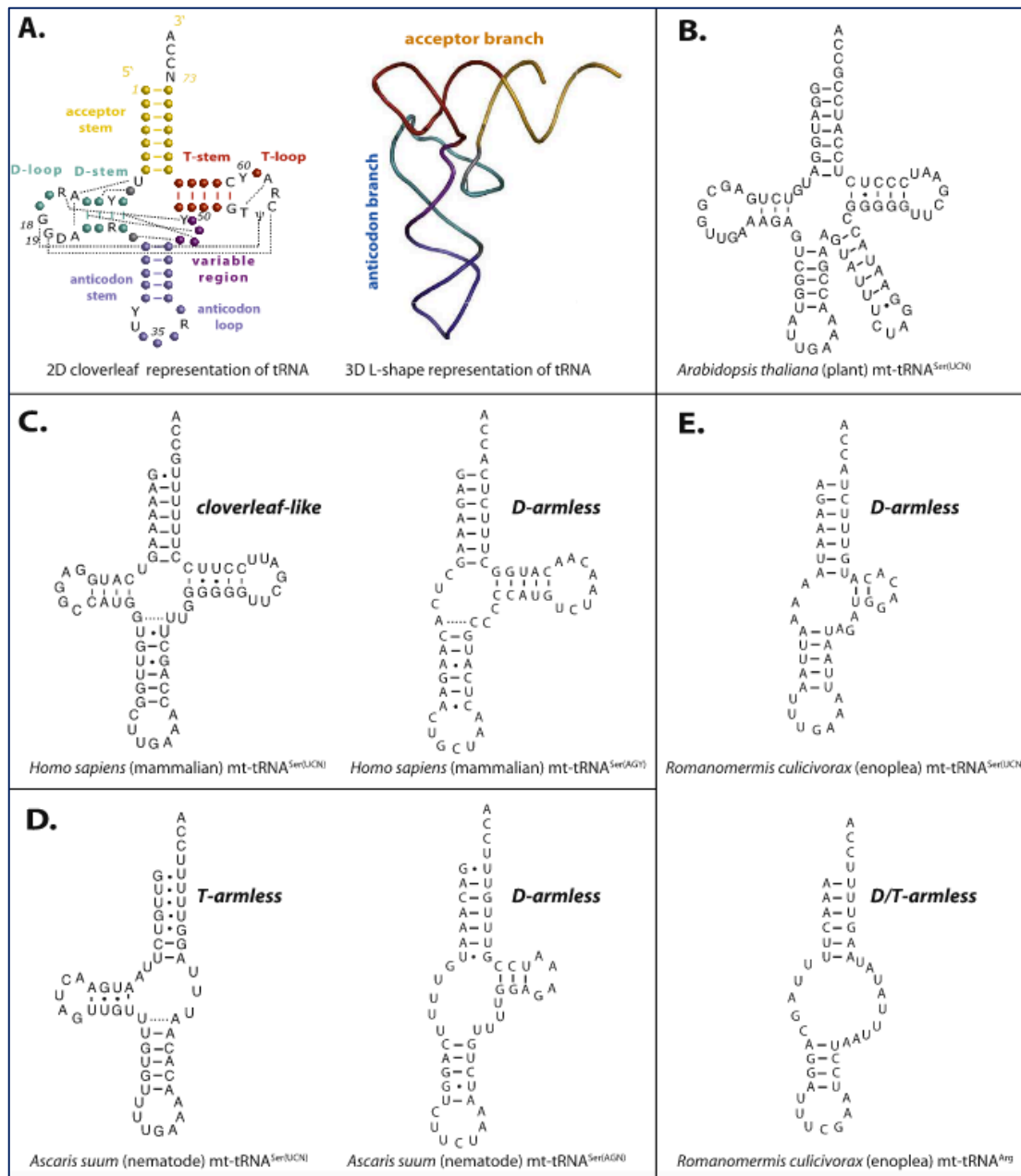


Figure 3. Atypical structural properties of mitochondrial tRNAs (Figure taken from J.L et al., 2014) A) 2D and 3D representation of canonical tRNA. tRNA's domains are colored and named. Conserved and semi-conserved nucleotides are indicated. Long distance interactions are shown with dashed lines. B) Mt tRNA^{Ser(UCN)} from *Arabidopsis thaliana*, as a representative of the three tRNAs accepting serine in plant mitochondria. Of note, this mt-tRNA displays all canonical features and a long variable region, which is typical for canonical tRNA^{Ser}. C and D) Mt-tRNA^{Ser(UCN)} and mt-tRNA^{Ser(AGY)} in *Homo sapiens* (as a representation of mammals) and *Ascaris suum* (as a representation of Nematodes), respectively. E) The extreme case of Enoplea (group of Nematodes) is illustrated by the T-armless tRNA^{Ser(UCN)}.

2.2. The mitoribosome

The mitochondrial ribosome is the central player in the mitochondrial translation machinery. Over the last few years, many studies have been carried out to gain a better understanding of its structure (Amunts *et al.*, 2015; Greber *et al.*, 2015). Mammalian mitoribosomes, like other ribosomes, are composed of a large and a small subunit, mt-LSU and mt-SSU, respectively. The mammalian mt-SSU is composed of a 12S mt-rRNA and 30 proteins, of which 15 are mt-specific in humans. The mammalian mt-LSU is composed of 16S mt-rRNA and about 53 proteins, of which 22 are mt-specific proteins (Mai *et al.*, 2017).

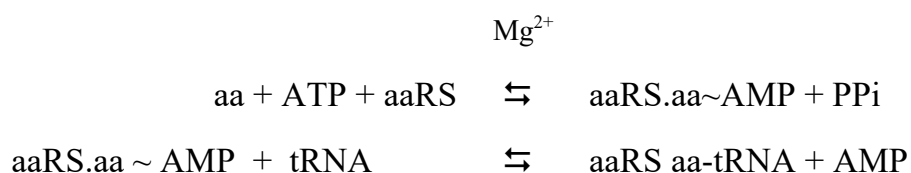
Despite its endosymbiotic origin, the size and the composition of the mammalian mitoribosome differ from those of the bacterial ancestor. The mitoribosome has a smaller fraction of RNAs and a higher amount of proteins. The RNA:protein ratio evolved from 2:1 in bacteria to 1:2 in the mammalian mitochondria (Agrawal and Sharma, 2012). Interestingly, structural data indicates that some of the new proteins will replace the missing RNA content, and additionally others proteins will occupy new positions in both subunits of the mt-ribosome (Sharma *et al.*, 2003). Beyond that, it was been also shown that certain amino acids from proteins replace nucleotides, directly interacting with nucleotides of the ribosome, thus creating amino acid/nucleotide interactions to maintain the structure of the RNA (Hosseini *et al.*, 2018). Indeed, recent studies focused the attention in deciphering how to fold and protect the mitochondrial rRNA, which is drastically reduced in size, but also with significantly fewer G residues (Hosseini *et al.*, 2018). The authors show that new mt-proteins physically substitute for the truncated RNA helices, maintain mutual spatial orientations of the helices, compensate for the lost RNA-RNA interactions, reduce the solvent accessibility of bases, and replace guanines conserved in bacteria by forming specific amino acid-RNA interactions. They also highlight that G residues (known to be the most sensitive nucleotide to oxidation) are primarily lost at positions highly exposed of the mitoribosome, indicating that the loss of G nucleotides result from selected evolution to the highly oxidizing environment present in the matrix of mammalian mitochondria (Hosseini *et al.*, 2018). Altogether, it reveals that the mitoribosome is a nice illustration of the transition from an RNA-centric world to a RNA-protein world and of an ingenious mechanism of adaptation of nuclear-encoded proteins to mt-DNA encoded

RNAs. In this situation, the new mt-proteins contribute directly to the strengthening of the rRNA fold.

In addition, the high-resolution cryoEM structure of mammalian mitoribosomes revealed the unexpected incorporation of mitochondrial mt-tRNA^{Phe} (in porcine) and mt-tRNA^{Val} (in human) into mt-LSU at the place where 5S rRNA occurs in other ribosomes (Amunts *et al.*, 2015; Greber *et al.*, 2015). Strikingly, these two tRNAs are present in highly transcribed units of mt-DNA, together with the two mt-DNA-encoded rRNAs. The group of Zofia Chrzanowska-Lightowlers has investigated further this observation and demonstrate that when steady-state levels of mt-tRNA^{Val} are reduced, human mito-ribosome biogenesis displays an adaptive response by switching to the incorporation of mt-tRNA^{Phe} (Rorbach *et al.*, 2016). They further suggest that the replacement of 5S rRNA (exclusively nuclear-encoded in mammals) by an mt-DNA encoded mt-tRNA support the absence of a functional requirement for RNA import into mammalian mitochondria (Chrzanowska-Lightowlers *et al.*, 2017; Rorbach *et al.*, 2016).

2.3. The aminoacylation process

Before an amino acid can be used for the protein synthesis, it must be transferred to a specific (cognate) tRNA molecule, through a reaction named aminoacylation. This accurate process is of particular importance as it allows the implementation of the genetic code. It is catalyzed by aminoacyl-tRNA synthetases (aaRSs), via a two-steps reaction:



The first step, the activation step, described in 1955 by Hoagland consists of forming a transitional reaction with the amino acid and an intermediate ATP molecule (Hoagland, 1955), which will then be transferred to the tRNA in a second step. The transfer of the activated amino acid takes place on the 2' or 3' OH of the terminal adenosine of the tRNA (Hoagland *et al.*, 1958; Hoagland *et al.*, 1957). Some aaRSs

require the presence of the tRNA for the amino acid activation step, this is the case for the class I aaRS: ArgRS, GluRS, GlnRS and LysRS (Arnez and Moras, 1997).

2.4. The mitochondrial tRNAs

The human mitochondrial genome encodes for 22 tRNAs, one per amino acid specificities, and two additional ones for leucine and serine, respectively. This set of tRNAs is reduced but sufficient to ensure mitochondrial protein synthesis. The genes coding for the tRNAs are distributed on the two strands of the mitochondrial genome with 14 tRNAs on the “heavy” strand and the other 8 on the “light” strand. “Heavy” tRNAs, encoded by the "light" strand, have a higher proportion of G•U base pairs. The "light" tRNAs have fewer Gs and have a high proportion of A, U and C, causing a series of typical structural characteristics such a biased base-pair content, thus, leading to a high ratio of CpA and UpA dinucleotides (Helm *et al.*, 2000). Moreover, due to the extreme compaction of the mitochondrial genome, these tRNAs are generally smaller than their cytosolic counterparts (from 62 to 78 nucleotides as compared to 75 to 95 to "classical" tRNAs).

The transcription of mtDNA is bidirectional and independent for each strand. It is performed by a T3/T7 RNA polymerase and generates three polycistronic transcripts. At the end of transcription, mt-tRNAs must undergo maturation phenomena essential to their function. Primary transcripts are cleaved by RNase P (at the 5' end of the tRNA) and RNase Z (at the 3' end of the tRNA) (reviewed in e.g. (Levinger *et al.*, 2004a)). These two cleavages are accompanied by the addition of the 3' CCA trinucleotide (essential for aminoacylation) by the CCA-adding enzyme (Mörl and Marchfelder, 2001) and by several post-transcriptional nucleotide modifications (Suzuki *et al.*, 2011). The total set of mammalian mt-tRNA with their post-transcriptional modifications was published; the authors identified 15 species of modified nucleosides at 118 positions in 22 species of mt-tRNAs (Suzuki *et al.*, 2011; Suzuki and Suzuki, 2014) (**Figure 4**). Although generally less modified than conventional tRNAs, the mt-tRNAs contain three mitochondria-specific modifications: 5-formylcytidine (f⁵C), 5-taurinomethyluridine (τ m⁵U), and 5-taurinomethyl-2-thiouridine (τ m⁵s²U) (Suzuki *et al.*, 2011). Some of the modifications are present at unusual positions such as adenine methylation in position 9 identified for 13 mammalian mt-tRNAs and which, in the case

of human tRNA^{Lys}, has been demonstrated to be essential for classic secondary "cloverleaf" folding (Helm *et al.*, 1999).

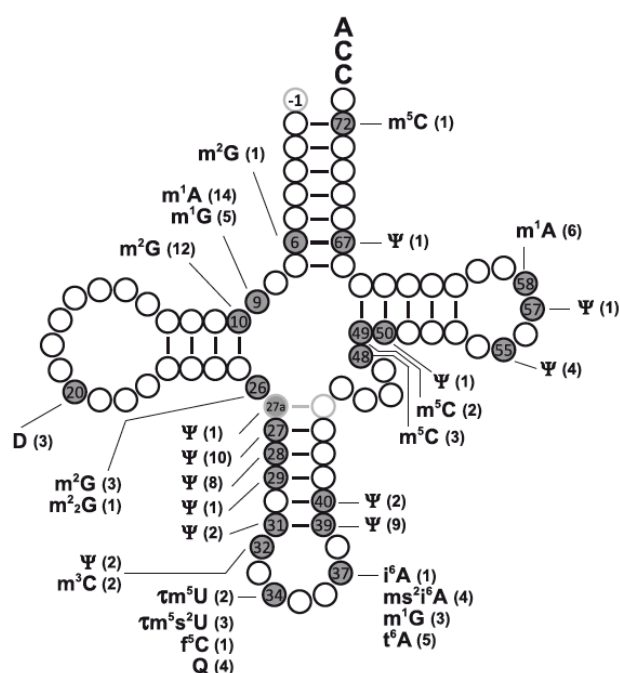


Figure 4. Summary of post-transcriptional modifications in bovine mt-tRNAs (Figure taken from T. Suzuki and T. Suzuki, 2014). Species and numbers of post-transcriptional modifications identified in 22 bovine mt-tRNAs are mapped on the schematic secondary structure of tRNA. The modified positions are depicted by gray circles with a symbol indicating each modification. At each position, the number of tRNAs that bear the modification are shown in parenthesis. Positions 27a and 43a, indicated by light gray circles, are unique to mt-tRNA^{Ser(UCN)}. G₋₁ is specific to mt-tRNA^{His}.

2.5. Mammalian aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases (aaRS) are housekeeping enzymes, ubiquitously expressed. They are modular enzymes, constituted, at least, by the catalytic domain and the tRNA anticodon-binding domain. Those are possibly surrounded by additional modules for structural or functional purposes (Delarue and Moras, 1993; Guo *et al.*, 2010).

In human, cytosolic and mitochondrial aaRSs are encoded by two different sets of nuclear genes; except GlyRSs and LysRSs that are encoded by single genes, but are generated either from two translation initiation sites or by alternative mRNA splicing, respectively (Bonfond *et al.*, 2005a). So far no gene has been identified for the mitochondrial glutamyl-tRNA synthetase (mt-GlnRS), and it has been demonstrated that Gln-tRNA^{Gln} is produced by transamidation of Glu-tRNA^{Gln} by the GatCAB aminoacyl-tRNA amidotransferase (Echevarría *et al.*, 2014).

2.6. Two classes of aminoacyl-tRNA synthetases

The presence of conserved motifs within their catalytic domains, the differences in their structures, and the presence of functional properties (face of the recognized tRNA and amino acid binding site) made possible to divide aaRSs into two classes (Cusack *et al.*, 1990; Eriani *et al.*, 1990) (**Table 1**).

2.6.1. Class I aminoacyl-tRNA synthetases

AaRSs from class I are mainly monomeric, except the TyrRS and the TrpRS, which are homodimeric. Their catalytic site includes a Rossmann fold, and two short common consensus sequences made of catalytic residues: HIGH (His-Ile-Gly-His) and KMSKS (Lys-Met-Ser-Lys-Ser). It is worth noting that close derivatives of these motifs have been described in some systems, like in the case of the ArgRS from yeast, where an -MSTR motif was found instead of the “KMSKS” (Delagoutte *et al.*, 2000). The Rossmann domain was initially identified as a nucleotide-binding motif (Eriani *et al.*, 1990). It is in charge of the ATP binding and it is composed of 5-stranded parallel β sheet connected by α helices. Both histidine residues of the HIGH motif are involved in the recognition of the amino acid and the ATP. The lysine residues of the KMSKS motif are involved in the interaction with the ATP and are located near the accepting end of the tRNA. This motif is located on a mobile loop, which is able to change its conformation according to the substrates to which the aaRS is bounded. The subdivision of class I aaRS into subclasses is based on their sequence homologies corresponding to a classification of the amino acids chemical nature. Thus, subclass Ia corresponds to hydrophobic amino acids, subclass Ib to long side chain amino acids and subclass Ic to aromatic amino acids (Burbaum and Schimmel, 1991).

2.6.2. Class II aminoacyl-tRNA synthetases

Class II aaRS have more diverse oligomeric states than class I aaRS, ranging from homodimer to heterotetramer (with the exception of monomeric mitochondrial PheRS (Sanni *et al.*, 1991)). The separation of the aaRS into two classes was established after the resolution of the structure of the SerRS from *E. coli* in 1990 (Cusack *et al.*, 1990), and then that of the AspRS from *S. cerevisiae*, which revealed the presence of a new structural motif for ATP recognition (Ruff *et al.*, 1991). Three sequences are

present in the catalytic domain, which fold into a 9-strand antiparallel β sheet bounded by α helices. Each motif contains a strongly conserved core with an invariant residue motif 1 (...**P**...), motif 2 (... ϕ **R**XE/D...) and motif 3 (...GXGXGXER ϕ ϕ ϕ ϕ ...), where ϕ , X or **bold** characters stand respectively for hydrophobic, non-conserved, or strictly-conserved residues. The motif 1, in addition to its role in the formation of the catalytic site, participates in the dimerization of the enzyme. The motifs 2 and 3 also contribute to the formation of the active site and each one of them have an arginine residue in a particular structural context. Class II aaRS have also been subdivided into subclasses according to their sequence homologies. Sub class IIa share the same organization in β sheet of their C-terminal domain with the exception of the SerRS. The three aaRSs of subclass IIb keep the same organization of their tRNA anticodon-binding domain. Finally class IIc is constituted by the aaRS not fulfilling criteria of classes IIa and IIb (Arnez and Cavarelli, 1997; Cusack *et al.*, 1990; Eriani *et al.*, 1990).

	Class I	Class II
Characteristics		
Motifs	HIGH KMSKS	M-1 ...P... M-2 ...(Gx) ₃ ER... M-3 ...FRxE...
Active site	Parallel β sheet "Rossmann fold"	Antiparallel β sheet
ATP conformation	Extent	Compact
tRNA binding	Minor groove	Major groove
CCA-3' conformation	Compact	Extent
Aminoacylation site	2' OH	3' OH
Sub-Classes		
	Ia	IIa
	ValRS α IleRS α MetRS* α_2 CysRS α ArgRS α	SerRS α_2 ThrRS α_2 ProRS α_2 GlyRS* $\alpha_2\beta_2$ HisRS α_2 AlaRS* α_4
	Ib	IIb
	GluRS α GlnRS α LysRS1 α	AspRS α_2 AsnRS α_2 LysRS1 α_2
	Ic	IIc
	TyrRS α_2 TrpRS α_2	PheRS* $\alpha_2\beta_2$

Table 1. Principal characteristics and classification of the aminoacyl-tRNA synthetases.

* aaRS whose oligomeric structure is not preserved in evolution.

2.7. Cytosolic versus mitochondrial aaRS.

Although cytosolic and mitochondrial aminoacyl-tRNA synthetases share the same main function, many specific features differentiate them.

2.7.1. Cytosolic aaRSs: Macromolecular organisation and alternative functions.

2.7.1.1. The multisynthetases complexe

The formation of complexes involving several aaRSs has been observed in archaea and eukaryotes (Laporte *et al.*, 2014; Mirande *et al.*, 1982). In mammals 9 of the 20 synthetases (AspRS, LysRS, ArgRS, GlnRS, MetRS, LeuRS, IleRS and GluProRS) are involved with 3 multifunctional proteins (p43, p38 and p18 renamed AIMP 1, 2 and 3) in the formation of a supramolecular complex (Park *et al.*, 2005). All three AIMP (ARS-Interacting Multi-Functional Protein) participate in cell regulation as "signal" molecules. The multisynthetasic (MARS) complex is known and studied by different approaches to discover its network of interactions and structural organization (Bandyopadhyay and Deutscher, 1971). The three AIMPs would connect the subdomains and stabilize the entire structure. AIMP1 is located in the middle of the complex and is associated with ArgRS via its N-terminal. AIMP2 binds to many components and its role is critical for the assembly of the complex (Kim *et al.*, 2002) (**Figure 5**). The complex arrangement is organized in two sub-domains depending on the affiliation of the proteins to AIMP2 (Kaminska *et al.*, 2009). AIMPs are mutually dependent for their cellular stability (Han *et al.*, 2006).

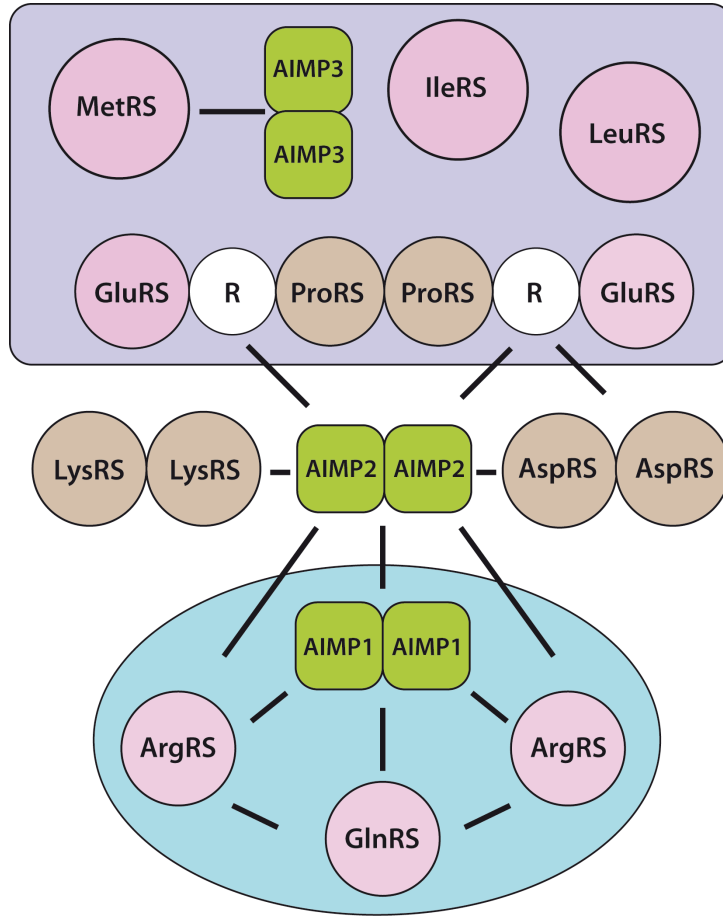


Figure 5. Schematic representation of the human cytosolic multi-aaRS complex. (Figure adapted from (Kaminska *et al.*, 2009)). Class I aminoacyl-tRNA synthetases are indicated in pink, and Class II aminoacyl-tRNA synthetases are shown in brown. The three auxiliary proteins AIMP1, AIMP2 and AIMP are shown in green, the linker domain (WHEP domain) of multifunctional GluProRS (R) is shown in white.

The role of this complex is still poorly understood, but two hypotheses have been proposed. First, it was suggested that the complex could improve translation efficiency by directing the flow of tRNAs into targeted cellular compartments (Ray *et al.*, 2007). Then, it could serve as a molecular reservoir (“depot hypothesis”) where anchored proteins display canonical functions and undergone a functional switch towards alternative/versatile activity after release (Han *et al.*, 2006; Han *et al.*, 2008; Ray *et al.*, 2007)

2.7.1.2. *Alternative functions*

The aaRSs are a family of enzymes whose participation in protein biosynthesis has been clearly established and analyzed from both structural and functional aspects (Rajendran *et al.*, 2018; Schimmel, 1987; Sissler *et al.*, 2017). However, in the past few years, this functional aspect has been largely extended to new activities that may be close to or completely different from the classical aminoacylation reaction (Francklyn *et al.*, 2002; Ibba *et al.*, 2005; Martinis *et al.*, 1999; Park *et al.*, 2005).

Alternative cellular and/or extracellular activities occur by means of alternate/moonlighting functions of the cytosolic aaRSs. The functional switch from canonical to non-canonical role is achieved either by alteration of the polypeptide fold, alteration of surface properties (such as e.g. post-translational modification(s)), or by atypical cellular organization (different cellular location, relocated to the nucleus or secreted, different macromolecular organization, different partnership, ...) (reviewed in e.g. (Han *et al.*, 2008; Ray *et al.*, 2007). Although catalytic domains may be involved in alternative functions, most of these are performed by structural domains acquired during evolution (Guo *et al.*, 2010). Additionally, some alternative functions are performed by splice variants or paralogs rather than by the aaRS implicated in the aminoacylation activity (Pang *et al.*, 2014).

Examples of alternate functions described for aaRSs are: import to the nucleus of some aaRSs in order to control the quality of the tRNAs in the nucleus (Nathanson and Deutscher, 2000), the modular structure of ThrRS of *E. coli* that regulates its own gene expression (Caillet *et al.*, 2003; Romby *et al.*, 1996), role of the C-terminal part of the TyrRS as a cytokine, and participation of the N-terminal part of the TyrRS in the angiogenesis (Wakasugi *et al.*, 2002a; Wakasugi *et al.*, 2002b), involvement of the LysRS in the HIV viral cycle (Duchon *et al.*, 2017), and involvement in some types of cancer (Shin *et al.*, 2008), among others. As this field of research is currently expanding, many new functions will most certainly be added in the coming years.

2.7.2. Mitochondrial aaRSs: structural and functional peculiarities.

2.7.2.1. Structure

Mt-aaRSs maintain the characteristic motifs that define them as class I or II synthetases (Bonfond *et al.*, 2005b). Striking divergences are, however, observed when considering their modular organization. Modular design of the aaRSs is a result of a patchwork assembly of different functional modules during evolution. While a very large number of aaRSs crystallographic structures are known, less information is available regarding the resolution of mammalian mt-aaRSs and especially of the human mt-aaRSs structures (**Figure 6**). Presently, only four mt-aaRSs have been crystalized and their structure established. The mt-PheRS shows the largest architectural changes with a particular oligomeric organization. While bacterial and cytosolic PheRSs are heterotetrameres ($\alpha_2\beta_2$), the mt-PheRS is a monomeric enzyme made of a fusion of α and β subunits (Bullard *et al.*, 1999). Crystallographic structures of the mt-PheRS have been resolved in the absence and presence of tRNA, showing that the binding of tRNA^{Phe} is accompanied by rearrangement involving a $\sim 160^\circ$ hinge-type rotation from a 'closed' to an 'open' state of PheRS, with global repositioning of the anticodon binding domain upon tRNA binding (Klipcan *et al.*, 2008). Other peculiar example is the mt-SerRS. Mammals possess two mt-tRNA^{Ser} isoacceptors, within which one is D-armless. mt-SerRS has acquired additional N- and C- terminal extensions (compared with its bacterial homolog) so that to be able to recognize and aminoacylate both mt-tRNA^{Ser} isoacceptors (Chimnaronk *et al.*, 2005). The resolution of the mt-AspRS crystallographic structure revealed a common 3D structure with the corresponding of *E. coli* AspRS. However, functional differences in thermodynamics and biophysical properties, in stability and in substrate selectivity, were detected (Neuenfeldt *et al.*, 2013). It was also shown that rearrangements between the insertion domain (bacterial insertion domain specific to all bacterial-type AspRSs) and the catalytic domain improve accessibility and binding of the tRNA end to the catalytic site (Neuenfeldt *et al.*, 2013). The structure of TyrRS was shown similar to eubacterial TyrRS, having a canonical tyrosine binding domain and adenylate binding residues typical of class I

synthetases. However, its electrostatic surface potential distinguishes it from all other known TyrRS. On the other hand, human mitochondrial TyrRS has two insertions in its structure, absent in eubacterial homologues. The first insertion, is located near the recognition site of the mitochondrial tRNA^{Tyr} accepting end and could be involved in the stabilization of the accepting stem. The second insertion, located on the "back" side of the synthetase (the side interacting with the tRNA being considered as the "front" side) is more important, with about twenty amino acids, and found in sequence in all mitochondrial TyrRSs (Bonnefond *et al.*, 2007).

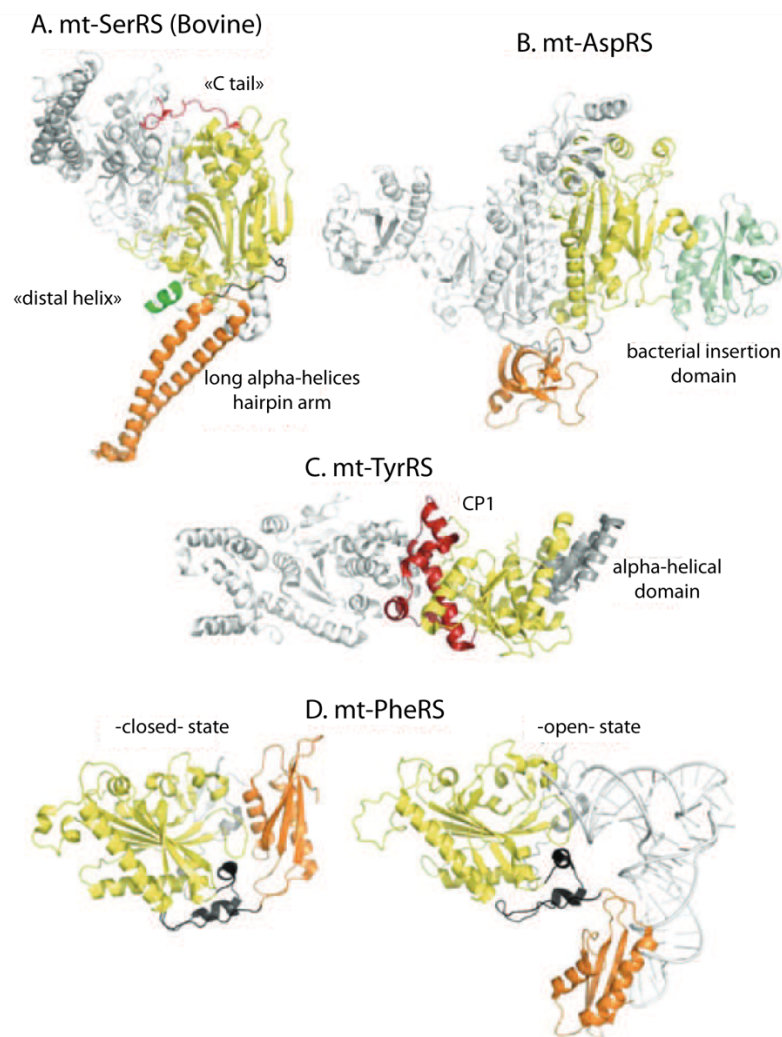


Figure 6. Known crystallographic structures of mammalian mt-aaRSs. (Figure taken from H. Schwenzler *et al.*, 2013). A) Bovine mt-SerRS, where the specific “distal helix” and “C-tail” are emphasized in green and red, respectively. In addition, the bacterial-type N-terminal long α -helices hairpin arm is shown in orange. B) Human mt-AspRS, where the bacterial insertion domain is highlighted in light green. C) Human mt-TyrRS, where the CP1 and the α -helical domains are indicated in red and gray, respectively. Note that the S4-like domain is missing in this structure. D) Human mt-PheRS in the -closed- state, and in the -open- state, complexed within *Thermus thermophilus* tRNA^{Phe} (in white). Binding of tRNA engenders a drastic conformational change of mt-PheRS through ~ 160 .

2.7.2.2. Functional peculiarities

2.7.2.2.1. *Low aminoacylation efficiency*

It has been shown that mt-aaRSs have reduced catalytic activity compared to their cytoplasmic or eubacterial counterparts. For the mt-PheRS, mt-TrpRS and mt-LeuRS the catalytic efficiency (k_{cat}/K_M) for amino acid is 100 times lower than for *E. coli* enzymes, and 250 times lower for ATP (Bullard *et al.*, 1999; Bullard *et al.*, 2000; Jørgensen *et al.*, 2000). When using total tRNA extracts from *E. coli*, mt-LeuRS has a specific activity 250 to 400 times lower than its known counterparts, and mt-PheRS 20 to 30 times lower than other class II synthetases (Bullard *et al.*, 1999). These reduced activities could reflect a low efficiency protein synthesis in the mitochondria or suggest the intervention of an additional factor that would improve aminoacylation by structuring mitochondrial tRNA for example.

Mt-AspRS is one of the most extensively studied systems in the host laboratory. Mt-AspRS shares 43% of identical residues, the same modular organization (including the bacterial-type insertion and C-terminal extension domains), and a same architecture as *E. coli* AspRS, a representative bacterial homolog (Neuenfeldt *et al.*, 2013). However, and despite the fact that the two enzymes are likely descendants from a common ancestor, numerous functional idiosyncrasies/discrepancies were reported, including a reduced catalytic efficiency (Bonfond *et al.*, 2005a; Sissler *et al.*, 2005), a reduced identity set for cognate tRNA recognition (see below; (Fender *et al.*, 2006)), and an increased sensitivity to inhibitors (adenylate analogues, (Messmer *et al.*, 2009)).

2.7.2.2.2. *Broad substrate spectrum*

Another special feature of the mammalian mt-aaRSs is their broad spectrum of action. Thus, in addition to aminoacylating human mt-tRNAs, mt-aaRS are able to aminoacylate *in vitro* cytosolic and eubacterial cognate tRNAs (Kumazawa *et al.*, 1991). Mt-AspRS was found to have a broad charging spectrum including, in addition to its cognate tRNA, tRNA^{Asp} from *E. coli*, *T. thermophilus*, and *S. cerevisiae*, which all possess the full set of aspartate identity elements and classical structural features

(Fender *et al.*, 2006). This unilateral nature of aminoacylation reflects the singularity of the mt-tRNAs, which are only recognized by the mt-aaRSs. Nonetheless this *in vitro* condition is an artificial situation extremely unlikely to occur in nature. It is very doubtful that a human mt-aaRS face a bacterial tRNA, or an archae tRNA. However, this is an important point to make because it illustrates that mt-aaRSs have released their specificity (probably to be able to recognize their “bizarre” tRNA). While the other aaRSs (which are not mitochondrial) remained "constrained" and as a result, maintain strict recognition of the cognate and homologue tRNA substrates.

2.7.2.2.3. *Simplified set of identity elements*

Identity rules were defined as a series of positive (determinants) and negative (anti-determinants) elements that respectively trigger specific aminoacylation and prevent mischarging (Florentz *et al.*, 2001; Giegé *et al.*, 1998). Studies of the human mt-aaRSs have revealed that they recognize a simplified set of identity elements within their cognate tRNAs. For instance, it was shown that the human mt-TyrRS does not recognize the first base pair N1-N72 as an element of identity, despite this represent a classical identity element of the tRNA^{Tyr} (Bonfond *et al.*, 2005b). Indeed, the human mt-TyrRS can recognize the G1-C72 base pair typical of eubacterial and mitochondrial tRNA^{Tyr} as well as the C1-G72 reverse base pair present in eukaryotic and archaea tRNA^{Tyr}. As another example, the host laboratory has explored the identity set of the human mt-tRNA^{Asp} and confirmed the identity role of the GUC anticodon as in other aspartylation systems. The authors have, however, revealed the non-involvement of the position 73 (so-called "discriminator base"). While for all aspartate aminoacylation systems, G73 is universally conserved and known as a primordial identity element, this base is no longer involved in the identity of human mt-tRNA^{Asp}. Mt-AspRS has adapted to the "degeneracy" of mt-tRNA^{Asp} and the loss of G73 as an identity element has been shown to be compensated within the human mt-AspRS catalytic site by a selected mutation of an essential amino acid involved in the recognition of the discriminatory base (Fender *et al.*, 2006).

The unveiling of simplified (reduced) identity sets raises a fundamental question concerning the fidelity and quality of protein synthesis within mitochondria. This reduction could in fact induce incorrect aminoacylations of tRNA (mischarging), as the mt-SerRS, which *in vitro* incorrectly misacylates the mt-tRNA^{Gln} (Shimada *et*

al., 2001). It was shown that the recognition of mt tRNA^{Gln} by mt-SerRS *in vitro* is 3700 times lower than that of mt tRNA^{Ser(UGA)}, suggesting that kinetic discrimination arise from competition between mt-SerRS and mt-GlnRS occurs in the mitochondrion to maintain the fidelity of mitochondrial translation (Shimada *et al.*, 2001). Further investigations considering this eventuality showed that the EF-Tu elongation factor has a lower affinity for Ser-tRNA^{Gln} than for Ser-tRNA^{Ser} and Glu-tRNA^{Gln}, suggesting that EF-Tu would have a critical role in maintaining translation accuracy by controlling the quality of aa-tRNAs (Nagao *et al.*, 2007).

3. Import and processing of nuclear-encoded mitochondrial proteins

Mitochondria require an efficient system of importation of fully functional proteins. Several research teams have investigated this process of protein importation. It has been described for a wide range of mitochondrial proteins (Neupert and Herrmann, 2007; Pfanner and Geissler, 2001; Wiedemann *et al.*, 2004). Although some mt-aaRS systems have been studied in plants (Duchêne *et al.*, 2005; Duchêne *et al.*, 2001) and yeast (Rinehart *et al.*, 2005), the data is restricted when regarding the mechanisms of import for the human mt-aaRSs.

3.1. Cytosolic expression of mitochondrial precursor proteins

Nuclear-encoded mitochondrial proteins are translated as precursors in the cytosol. Post-translational and co-translational mechanisms for translocation of mitochondrial precursor proteins are widely accepted (Schleiff and Becker, 2011; Wiedemann *et al.*, 2004). In the case of proteins translocation is post-translational, cytosolic chaperones guide the precursor protein, thanks to the presence of a protein sorting sequence, to the Tom20, Tom22 and Tom70 receptors of the translocase of the outer mitochondrial membrane (TOM) complex (Chacinska *et al.*, 2009). Concerning co-translational translocation, it is mRNA that is brought to the mitochondria, instead of the protein (Mossmann *et al.*, 2012).

3.1.1. Mitochondrial targeting sequence

Most of the mitochondrial proteins directed to the mitochondrial matrix, the inter-membrane space as well as the inner membrane, are first synthesized in the form of precursor proteins exhibiting an N-terminal extension, which in most of the cases is cleaved after import into the mitochondria (Neupert, 1997). These extensions, named mitochondrial targeting sequence (MTS), are usually made of a sequence from twenty to fifty amino acids. Although no consensus sequence has been described yet, they all form α amphiphilic helices, enriched with basic, hydroxyl and hydrophobic residues (Mossmann *et al.*, 2012). On the other hand, most of the mitochondrial proteins located in the outer membrane and the transport proteins located in the internal membrane present an N- or C- terminal signal or an internal signal that will not be cleaved (Rapaport, 2003).

3.1.2. Translocase machinery of the mitochondrial outer membrane

The nature of the addressing signal together with the chaperone proteins will determine the sort of outer membrane translocation complex receptor to be used to the importation process. The TOM complex consists of 7 different subunits, which are grouped into 3 categories: (i) the receptors Tom20, Tom22 and Tom70, (ii) the tunnel of the TOM complex consisting in Tom40 and, (iii) three small proteins Tom5, Tom6 and Tom7 (Schleiff and Becker, 2011; Van Wilpe *et al.*, 1999). Proteins with an N-terminal signal interact with the chaperone Hsp70 and are recognized by the Tom20/22 complex. Then, with the assistance of Tom5 they cross the pore of Tom40 (Dietmeier *et al.*, 1997). Proteins carrying an internal signal (which will not be cleaved) are recognized by the chaperone Hsp90, who brings them to the Tom70 receptor (Young *et al.*, 2003) (**Figure 7**).

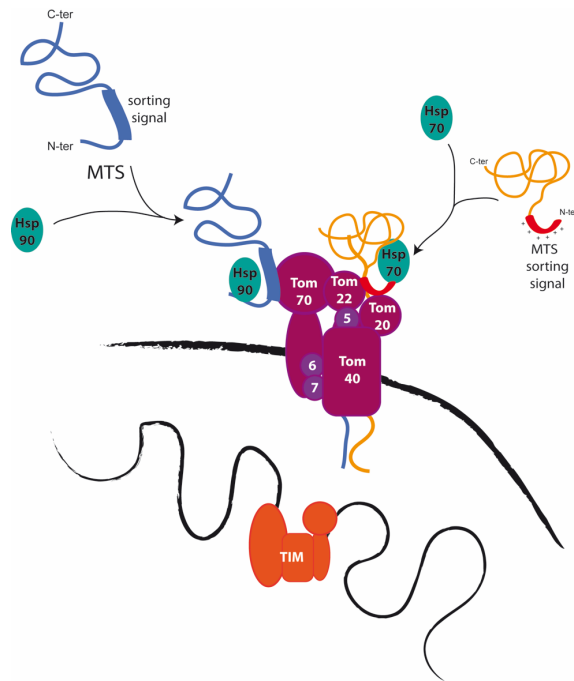


Figure 7 Schematic representation of TOM complex. Mitochondrial precursor proteins are translated by the cytosolic chaperones (Heat shock protein hsp90 and hsp70) to the central mitochondrial entry gate, the translocase of the outer membrane (TOM complex). Internal sorting signal (left) and N-terminal MTS (right), made of positively charged amino acid (represented by the + sign).

The passage through the outer membrane represents the first import step for the majority of mitochondrial proteins. However, for the proteins located within the outer membrane and having a simple topology (as a single transmembrane segment), only the passage through the TOM complex is required. The other proteins of the outer membrane with more complex topology (like Tom40 which presents multiple β strands) cross the TOM complex and are carried in the inter-membrane space by the SAM (Sorting and Assembly Machinery) complex, which will then allow their insertion into the outer membrane (Mossmann *et al.*, 2012).

3.1.3. Translocase machinery of the inner mitochondrial membrane

The Translocator Inner Membrane (TIM) complex consists in the assembly of three membrane proteins: (i) the receptor Tim50 exposing a large domain to the inter-membrane space, (ii) Tim23 constituting the pore of the TIM complex and (iii) Tim17 being closely associated with Tim23 and which, is supposed to regulate the activity of Tim23 (Bolender *et al.*, 2008). The transit of proteins through Tim23 directly depends on the membrane potential that exists through the inner mitochondrial membrane. Indeed, the latter, in addition to activating the Tim23 pore, exerts an electrophoretic

effect on the positively charged pre-sequences and thereby draws the pre-sequence to the matrix side (**Figure 8**).

It is at this stage that the import of some proteins from the internal membrane ceases. Actually, these proteins have a hydrophobic signal that directly follows the pre-sequence retaining them in the inner membrane. However, another part of the proteins of the inner membrane and of the matrix are drawn by the Presequence translocase-Associated Motor (PAM), a ATP dependent motor protein complex (Pfanner and Geissler, 2001).

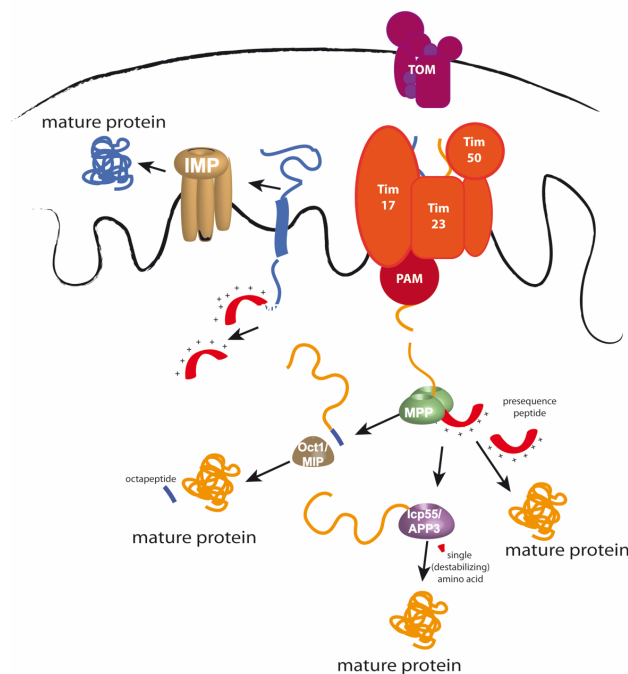


Figure 8. Schematic representation of TIM complex and some proteolytic processes. The N-terminal targeting and sorting signals of mitochondrial precursor proteins are proteolytically removed upon import. This processing step is carried out by several proteases localized in the mitochondrial matrix and inner membrane: The mitochondrial processing peptidase (MPP), the octapeptidyl aminopeptidase 1 (Oct1), the inner membrane peptidase (IMP), the intermediate cleaving peptidase 55 (Icp55), and the Aminopeptidase P 3 (AAP3).

3.1.4. Peptidases implicated in the mitochondrial targeting sequence processing

The proteolytic cleavage of N-terminal MTS occurs after the internalization of the precursor protein into mitochondria and although some studies have been done, the mechanism is still not completely clear. Several peptidases have been identified that cleave with different specificity the N-terminal part. The mitochondrial processing peptidase (MPP) removes the MTS (Mossmann *et al.*, 2012). Additional cleavages by Oct1 (octapeptidyl aminopeptidase 1 (Gakh *et al.*, 2002b)) or Icp55 (intermediated cleaving peptidases) can subsequently occur to remove newly exposed residues in the

N-terminal (**Figure 8**). In addition, the membrane bound inner membrane peptidase (IMP) removes, after MPP cleavage, the newly exposed hydrophobic sorting signal of proteins translocated to inter-membrane space (Mossmann *et al.*, 2012). The second known membrane bound peptidase Pcp1 (processing of cytochrome c peroxidase) cleaves internal hydrophobic sorting signals. After the cleavage the mature protein is then handled by the chaperone proteins mt Hsp70, Hsp60 and Hsp10 to reach its final three-dimensional conformation and be functionally active (Bukau and Horwich, 1998).

It turns out that cleavage site selection follows very loose consensus sequences making predictions difficult. It is assumed that the precursor proteins are translocated through the mitochondrial membrane as unfolded polypeptide chains. This facilitates the correct recognition of the MTS and the physical translocation through the narrow import machinery. Thus, after processing and sub-mitochondrial sorting, a chaperone complex will refold the protein in its active form (Voos, 2013).

4. Mitochondrial translation machinery and human diseases

Mitochondrial diseases are a class of heterogeneous disorders produced by mutations of either mt-DNA or nuclear DNA. Clinical manifestations can vary from mild to profound, from highly tissue selective to multi-systemic, making clinical diagnosis a difficult task. One of the remains unresolved issue is why different mt-DNA or nuclear mutations result in largely heterogeneous and often tissue-specific clinical presentations. Mitochondrial tRNA (mt-tRNA) mutations are frequent causes of mitochondrial diseases both in children and adults. However numerous mutations of nuclear genes coding for ubiquitously expressed mitochondrial proteins have been reported in association with very tissue specific clinical manifestations suggesting the existence of yet unknown factors/actors that determine the tissue specificity of the mitochondrial translation.

Several studies have reported mutations in most of the components involved in the mitochondrial translation process (Rötig, 2011). However, due to the dual genetic origin of these, the inheritance pattern is not the same when it concerns proteins encoded by the nuclear genome or when it concerns molecules encoded by the mitochondrial genome (Mai *et al.*, 2017).

During my PhD, I contributed to an opinion review on the recent advances in the pathologies related with mutations in the human mitochondrial aminoacyl-tRNA synthetases and in the human mt-tRNAs (Sissler *et al.*, 2017). Some aspects discussed in the review will also be considered in other parts of the introduction.

4.1. Inheritance of mitochondrial diseases caused by mutations on the mt-DNA

There are several unique properties of the mt-DNA that is worth recalling for the comprehension of related disorders: (i) there are multiple copies (up to thousands) of mt-DNA in each cell; (ii) mt-DNA is maternally inherited; (iii) the phenomenon of homoplasmy and heteroplasmy. Homoplasmy implies that all mt-DNA are identical, which could be all wild type or all mutated. Heteroplasmy is a mixture of mutated and wild type mt-DNA in a single cell. In this last situation, the clinical manifestations usually occur above a given threshold of mutated *vs* wild-type mt-DNA (named "threshold effect") and thus can vary between different tissues. In women with heteroplasmic mt-DNA mutations, there is a bottleneck in the female germ line, which means that the transmission of heteroplasmy level from mother to offspring is often random and unpredictable. This explains the heterogeneity in heteroplasmy level, clinical phenotype and severity frequently observed within the same pedigree (Alston *et al.*, 2017).

4.2. Inheritance of mitochondrial diseases caused by mutations on the nuclear-DNA

Most of the genes encoding the mitoproteome are in the nuclear genome and follow Mendelian inheritance patterns. *De novo*, X-linked, dominant and recessive inheritance cases have been reported in the literature. One representation of the variability in the inheritance pattern is *POLG* gene (Mai *et al.*, 2017). Mutations in the *POLG* gene have been identified as one of the most common causes of inherited mitochondrial diseases in children and adults. Mutations in this gene have been reported presenting both recessive and dominant genotypes (Tang *et al.*, 2011).

Homozygosis, heterozygosis, dominant and recessive inheritances are useful concepts when it comes to understand the probability of an individual inheriting mitochondrial disorders. As a remind, somatic cells contain two alleles for each gene.

In the compound heterozygous the two alleles of a gene are not identical at the two chromosomal loci, contrary to the homozygous compound where the alleles for a gene are identical on both chromosomes (Sissler *et al.*, 2017). A dominant mutation will produce a phenotype in individuals who present mutations in one copy of the alleles, which can come from just one parent. In the case of recessive mutations, both alleles (one from each parent) must be mutated to manifest the disease.

4.3. Mt-tRNAs and mt-aaRSs in human pathologies

While over the past 30 years more than 260 pathological mutations have been identified within human mt-tRNA, mRNA and rRNA genes, it is only very recently that mitochondrial diseases have been associated with mutations in genes encoding other macromolecules of mitochondrial translation machinery, including the EF-G1 (Coenen *et al.*, 2004), EF-Ts (Smeitink *et al.*, 2006) and EF-Tu (Valente *et al.*, 2009; Valente *et al.*, 2007) translation factors and the MRPS 16 ribosomal protein (Miller *et al.*, 2004), among others.

Numerous studies have attempted to unravel the molecular impacts of the mutations on the various properties of the affected tRNAs and lead so far to a mosaïcicity of impacts. The consecutive genotype-phenotype relationship to pathological mutations in tRNA genes is very complex. Indeed, if the same mutation can be the cause of different pathologies, the same pathology can also be linked to different mutations. This can be explained by both a random distribution of mutated mt-DNA among different tissues as well as by variable co-existence within the same mitochondria or cell of healthy DNA and mutated DNA (heteroplasmy). In most cases, the effects of mutations are mild and affect either a single step of the tRNA life cycle or a combination of several of them. However, an initial impact is frequently observed on structural properties of affected tRNAs, followed by sub-sequent cascade effects on downstream functions (Florentz and Sissler, 2003; Levinger *et al.*, 2004b; Wittenhagen and Kelley, 2003). The pathological mutations of mt-tRNA and the diseases they cause are listed on the webservers "mitomap" (www.mitomap.org) (Brandon *et al.*, 2005) and "mamit" (mamit-trna.u-strasbg.fr) (Pütz *et al.*, 2007). Alterations of the mt-DNA are responsible of various severe disorders as e.g. encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and Leber hereditary optic neuropathy (LHON). Some reviews

have compiled the data concerning these pathologies caused by mutations on the mt-tRNAs (Boczonadi *et al.*, 2018; Florentz *et al.*, 2003; Rötig, 2011).

Concerning mutations in the nuclear genes encoding the mitochondrial aminoacyl-tRNA synthetases, over the last years they have been increasingly related to a variety of mostly paediatric and some adult onset tissue specific disorders. Mutations on *DARS2*, the nuclear gene coding for mt-AspRS, was the first case reported in patients with a cerebral white matter disorders called Leukoencephalopathy with Brain stem and Spinal cord involvement and Lactate elevation (LBSL) (Scheper *et al.*, 2007). Nowadays, mutations in each of the 19 human mt-aaRSs genes have been reported in human disease (Sissler *et al.*, 2017). Despite being ubiquitously expressed, mutations in these genes show an unexpected variety of phenotypes, including many neurological disorders affecting the white matter or causing epileptic encephalopathies (Konovalova and Tyynismaa, 2013; Sissler *et al.*, 2017). In an effort to broaden knowledge of mutations on the mt-aaRS the hosting laboratory developed a knowledge-based repository coupled with a web server (<http://misynpat.org>) that offers an analysis and follow-up tools: to retrieve literature and data related to each mt-aaRS (e.g. references, affected genes, related disorders, allelic compositions in patients); to locate the mutated amino acid on 3D representation (crystallographic structure or homology model) and/or generate a model of the mutant; and finally, to place the mutated position in multiple sequence alignments of aaRS sequences, ranging from bacteria up to human, thus allowing the evaluation of the conservation of a mutation (Moulinier *et al.*, 2017). Furthermore, several investigations have been carried out in order to understand the molecular mechanisms underlined to these disorders. Some of the major's outcomes from those studies are that it seems that whatever the mutation in mt aaRSs is, no common combination of molecular steps correlates mutations with phenotypic expressions. Interestingly, the molecular impact of mutations is not necessarily at the level of the aminoacylation function of synthetase. Pathology-related mutations can have a direct effect on the mitochondrial translation machinery by impacting one or more biogenesis steps and/or the functioning of mt aaRS. Alternatively, they may have an indirect effect by affecting the following steps and/or subsequent product activities (DNA-coded subunits translation of 13 mt of respiratory chain complexes, respiratory chain complex activities and ATP synthesis). In addition, although a dominant effect on the brain and neural system is observed, sporadic manifestations also occur in the skeletal muscle, kidney, lung and/or heart (Schwenzer *et al.*, 2014b).

The strengths of my thesis work were oriented towards the clinical overview of the pathologies produced by mutations on the mt-aaRSs. This is in part represented in article #1, where one of the major points was the comparison of injuries in the anatomical organs systems by pathologies caused on the one hand by tRNAs and on the other by aaRSs (Sissler *et al.*, 2017). Additionally, an exhaustive study was conducted on the clinical manifestations produced by diseases linked to mutation on the mt-aaRSs. The contents of this comprehensive analysis are presented in chapter I of this thesis.

Article # 1

Recent Advances in Mitochondrial Aminoacyl-tRNA Synthetases and Disease

Marie Sissler, Ligia Elena Gonzalez Serrano, Eric Westhof

2017

Trends in Molecular Medicine

Opinion

Recent Advances in Mitochondrial Aminoacyl-tRNA Synthetases and Disease

Marie Sissler,^{1,*} Ligia Elena González-Serrano,¹ and Eric Westhof¹

Dysfunctions in mitochondria – the powerhouses of the cell – lead to several human pathologies. Because mitochondria integrate nuclear and mitochondrial genetic systems, they are richly intertwined with cellular activities. The nucleus-encoded mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs) are key components of the mitochondrial translation apparatus. Mutations in these enzymes predominantly affect the central nervous system (CNS) but also target other organs. Comparable mutations in mt-aaRSs can lead to vastly diverse diseases, occurring at different stages in life, and within different tissues; this represents a confounding issue. With newer information available, we propose that the pleiotropy and tissue-specificity of mt-aaRS-associated diseases result from the molecular integration of mitochondrial translation events within the cell; namely, through specific crosstalk between the cellular program and the energy demands of the cell. We place particular focus on neuronal cells.

Human Mitochondrial Translation: Relevance to Disease

Human mitochondria host numerous essential metabolic pathways, including cellular energy production in forms of ATP via **oxidative phosphorylation** (OXPHOS, see [Glossary](#)), carried out by proteins of **respiratory chain complexes**. Thirteen of these proteins are encoded within **mitochondrial DNA** (mt-DNA) and are synthesized via the mitochondrial translation machinery, while 84 additional proteins are encoded by the nuclear genome [1]. This skewed genomic distribution also applies to molecules of the mitochondrial translation machinery, given that all required RNAs (two rRNAs and 22 tRNAs) are encoded by mt-DNA, and all the necessary proteins (ribosomal proteins, tRNA modifying enzymes, translation factors, aminoacyl-tRNA synthetases, etc.) are encoded by the nuclear genome, synthesized within the cytosol, and imported into mitochondria [2,3]. Noteworthy, the nuclear and mitochondrial genomes accumulate mutations at different rates [4,5], which can lead to a large-scale reduction or alteration of mt-DNA-encoded RNA components that still need to be recognized by nucleus-encoded proteins [6]. From the more than 1000 proteins imported within mitochondria, only some are necessary for translation [1]. Presently, the network of interactions between proteins involved in translation with those involved in non-translation is poorly characterized.

Pathologies associated with mitochondrial dysfunctions involve a large number of genetic causes with different inheritance mechanisms – either maternally inherited mutations in RNAs and proteins encoded by mt-DNA, or Mendelian inherited mutations of proteins encoded by the nuclear genome [7–9] ([Box 1](#)). Mutations in proteins involved in mitochondrial metabolic processes and directly affecting mitochondrial central functions such as ATP production have been known and studied for some time [10,11]. However, mitochondrial disorders linked to

Trends

Mutations in nucleus-encoded mt-aaRSs impact on tissues with high energy demand, notably the CNS.

Disease-associated mutations occur at positions rarely conserved in phylogeny, but some occur at positions that are highly conserved in mammals.

These observations point to complex molecular origins with either direct and/or indirect effects on the efficiency of mitochondrial translation and/or alternative functions played by mt-aaRSs.

We propose that the pleiotropy and tissue-specificity of these disease-associated mutations reflect the integration of mitochondrial translation processes within cellular homeostasis; these may occur through specific crosstalk between the cellular program and cellular energy demands, especially in neuronal cells.

Among the multiple communication pathways between mitochondria and the nucleus, the mitochondrial unfolded protein response may play a role in the integrated response.

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Box 1. Genetics of mt-aaRSs and mt-tRNAs Mutations

All mutations reported have been identified by genomic sequencing. Mutations in mt-tRNA occur in mt-DNA and follow the maternal inheritance of mitochondria. The copy number of mitochondria in human cells is tightly regulated and varies enormously depending on the cell type [68]. Mutations in mt-DNA generally affect only a proportion of mt-DNA copies (termed heteroplasmy, in contrast to homoplasmy where all mt-DNA copies are identical [28,29]). The appearance of the disease state depends on the proportion of mutated versus wild-type mt-DNAs and on the amount of available functional gene products (biochemical threshold). The affected organ system(s), in this situation, depend(s) on the random segregation of mutated mt-DNA during mitotic division (mitotic segregation). By contrast, mt-aaRS mutations occur in nuclear DNA and present a Mendelian pattern of inheritance. Up to now, the observed mutations exhibit **recessive inheritance**. Thus, reported mutations lead to autosomal (i.e., affecting any chromosome other than a sex chromosome) recessive disorders. Patients are mainly **compound heterozygotes**, although some have been reported to be **homozygotes**.

Clearly, missense mutations in structurally/functionally key regions of the protein (evolutionary conserved positions; **Box 4**) are lethal and are not available for study. However, in the heterozygous compound state a disease might be observed when mutations affecting both alleles lead to mild functional defects, or when a 'strong' mutation (e.g., a premature stop) is coupled to a splicing defect in the second allele that leaves some remaining/residual functional activity [30,67]. In short, the cumulative effects of two mutations should maintain sufficient gene products with some degree of activity. The overall threshold level for the degree of activity that must remain so as to be observable may depend on the onset of the disease stage (**early-onset** vs **late-onset disease**) and on the cell type. In addition, the window around the threshold may vary with the mutational effects and the type of mt-aaRS. For example, in LBSL patients, the window starts when the symptoms become detectable, reaching a level that is still compatible with a life-expectancy of >20 years [30]. By contrast, for mt-ArgRS mutations, which cause severe early-infantile epileptic encephalopathy, the window is narrow around the threshold [69].

mutations in various components of the mitochondrial translation machinery potentially affecting ATP production indirectly, are being reported at an increasing rate [12–14]. Mitochondrial tRNAs (mt-tRNAs) and mt-aaRSs constitute two families of molecules that are raising particular interest in disease because of their functional connection (one being a substrate of the other in the **aminoacylation** reaction) [15,16]. In the following we first illustrate the diversity of affected anatomical systems related to mutations within these two types of molecules. We then focus on mt-aaRSs as key players in the mitochondrial translation machinery (**Figure 1**, Key Figure). An overview of the structure and function of aaRSs is provided in **Box 2**. The first description of a series of mutations in an mt-aaRS causing a particular **leukodystrophy** dates back to 2007 [17]. In only 10 years, mutations in each of the nuclear genes coding for the 19 mt-aaRSs have now been correlated to human diseases, with pleiotropic clinical manifestations [18–20]. Our stance follows two tracks: first, we propose that the effects of mutations on the translation machinery are subtle and difficult to identify, essentially appearing within a neuronal context in the CNS; second, that alternative biochemical pathways – some of which are yet to be discovered – may be involved in differentially modulating mt-aaRS functions. We also attempt to organize the available data, analyzing reported mt-aaRS mutations in the context of protein architecture and in terms of evolutionary amino acid conservation of affected positions. We aim to delineate the boundaries that define the potential molecular origins of these diseases and suggest directions for future research along several lines that should be integrated to promote possible therapeutic strategies.

Diseases Associated with mt-aaRSs and mt-tRNAs

The diversity of pathologies linked to mutations in mt-aaRSs and mt-tRNAs display a marked bias for the CNS (**Figure 2**). Indeed, mutations in all mt-tRNAs and in 17/19 mt-aaRSs (the exceptions being mt-TyrRS and GlyRS) are reported to cause CNS damage [21,22]. This apparent convergence does not, however, hold true when considering the clinical manifestations. For example, mutations in mt-AspRS and tRNA^{Asp} involve pathologies linked to the CNS; nevertheless, mutations in mt-AspRS result exclusively in **LBSL disease** [23], while mutations in mt-tRNA^{Asp} lead to **sporadic bilateral optic neuropathy** [21], **mitochondrial myopathy** [24], **myoclonic epilepsy**, and **psychomotor regression** [25].

Glossary

Aminoacylation: an enzymatic reaction involving the attachment of an amino acid to its isoaccepting tRNA carrying an anticodon complementary to the codon specifying that amino acid according to the genetic code. It is a two-step reaction involving amino acid activation and transfer of the activated amino acid to the cognate tRNA(s) catalyzed by an aminoacyl-tRNA synthetase (aaRS).

Catalytic motifs: highly conserved residues necessary for catalysis (aminoacylation) to occur. These motifs are **HIGH** and **KMSKS** (or close derivatives) for class I aaRSs; and motif 1 (. . . P . . .), motif 2 (. . . FRXE/D . . .) and motif 3 (. . . GXGXGXERFFFF . . .) for class II aaRSs, where F, X, or **bold** characters stand for hydrophobic, non-conserved, or strictly conserved residues, respectively.

Cardiomyopathy: a group of diseases affecting the heart muscle, stemming from multiple causes, for example mitochondrial cardiomyopathy is caused by mitochondrial dysfunction.

Charcot-Marie-Tooth (CMT) disease: an inherited motor and sensory peripheral neuropathy (peripheral nerves in the nervous system are affected); patients present with muscle loss, weakness, and sensory nerve damage in the hands and feet.

Compound heterozygosity: the two alleles of a gene are not identical at the two chromosomal loci.

Early-onset disease: in the case of mitochondrial dysfunction, a disease that strikes immediately or a few hours after birth.

Homozygosity: alleles for a gene are identical on both chromosomes.

HUPRA syndrome: hyperuricemia, pulmonary hypertension, renal failure in infancy, and alkalosis – a severe mitochondrial autosomal multisystem disorder with early-onset presentation. Patients manifest renal failure leading to metabolic alkalosis (high pH in serum), pulmonary hypertension (high blood pressure that affects the arteries in the lungs and the right side of the heart), hypotonia (decrease in muscle tone), and developmental delay.

Hyperuricemia: excess uric acid levels in the blood.

Box 2. The Aminoacyl-tRNA Synthetases: From Housekeeping to Moonlighting Function

aaRSs are ancillary/housekeeping proteins whose main recognized function is to perform aminoacylation of cognate isoaccepting tRNAs. They generally comprise a catalytic domain and a tRNA anticodon-binding domain, but other functional domains are occasionally added. While the 20 aaRSs differ widely in size and oligomeric state (from monomer to tetramer), they have been partitioned into two classes based on the fold of the catalytic domains (Rossmann fold for class I, and an antiparallel β -sheet flanked by α -helices for class II), on the signature **catalytic motifs**, and on the amino acid mode of linkage with either the 2'-hydroxyl or the 3'-hydroxyl of the terminal A of tRNA for class I (ValRS, IleRS, LeuRS, MetRS, CysRS, TyrRS, TrpRS, GluRS, GlnRS, and ArgRS) or class II (SerRS, ThrRS, ProRS, HisRS, AspRS, LysRS, AsnRS, PheRS, AlaRS, and GlyRS) aaRSs, respectively [70,71]. Most class I aaRSs are monomers and class II aaRSs dimers (note that class I TyrRS and TrpRS are dimers and the tRNAs straddle each monomer for recognition). Usually, there is one specific tRNA synthetase for each of the 20 amino acids. However, as in some bacteria (and most archaea), Gln-mt-tRNA^{Gln} is made in human mitochondria by transamidation of Glu-mt-tRNA^{Gln} through the action of an amidotransferase, in agreement with the fact that no gene for mt-GlnRS has been found [72].

In humans, there are two sets of distinct nuclear genes that code for either the cytosolic aaRSs or the mitochondrial aaRSs [16]. The only two exceptions concern GlyRSs and LysRSs, for which cytosolic and mitochondrial forms (differing by the absence or the presence of a mitochondrial targeting sequence, respectively) are encoded by single genes, but are generated either from two translation initiation sites or by alternative mRNA splicing, respectively [73–75]. Interestingly, human cytosolic aaRSs have been recognized to participate in a diversity of alternative (moonlighting) functions beyond translation, connecting them to other cellular activities. Examples include enzymes that operate in pathways linked to, for example, angiogenesis, immune responses, inflammation, tumorigenesis, or neuronal development (reviewed in [43,44]). The functional switch from canonical to non-canonical roles can be achieved by alteration of the polypeptide fold, alteration of surface properties (post-translational modification(s), other), or by atypical cellular organization (different cellular location, relocated in the nucleus or secreted, different macromolecular complex organization, different partnership, etc.) (reviewed in [76,77]). Recently, a first description of an alternative function for a mt-aaRS has been identified: a pro-angiogenic function for rat mt-TrpRS [63]. This suggests that other mt-aaRSs may also play alternative roles in the cell.

Remarkably, the diversity of pathologies is much wider for mutations in mt-tRNAs than in mt-aaRSs. In the CNS-related pathologies, eight mt-aaRSs lead to encephalopathies (mt-ArgRS, mt-AsnRS, mt-CysRS, mt-IleRS, mt-PheRS, mt-ProRS, mt-ThrRS, mt-ValRS), four to **leukodystrophies** (mt-AlaRS, mt-AspRS, mt-GluRS, mt-MetRS), and two to **Perrault syndrome** (mt-HisRS, mt-LeuRS). Mutations also result in isolated pathological conditions such as hearing loss or deafness (mt-MetRS, mt-AsnRS), and intellectual disability (mt-ArgRS, mt-TrpRS). For pathologies not affecting the CNS, three aaRSs provoke **cardiomyopathies** (mt-AlaRS, GlyRS, LysRS), one the **MLASA syndrome** (mt-TyrRS), and one the **HUPRA syndrome** (mt-SerRS). By contrast, mutations in mt-tRNAs are connected with ~50 pathological disorders (and about half are linked to the CNS) displaying a broad clinical spectrum, ranging from mitochondrial myopathy to neurogastrointestinal syndrome, **myoglobinuria**, diabetes mellitus, hypertension, and **Kearns–Sayre syndrome** (see Table S1 in the supplemental material online).

Another outcome is reflected by the contrast between the multiple impacts of mutations in mt-tRNAs versus the singular impact of mutations in mt-aaRSs. Indeed, not only does every mutated mt-tRNA have a broad impact in different major **organ systems**, but each is also linked to more than two diseases [e.g., mutations in mt-tRNA^{Pro} manifest as **MERRF-like disease**, mitochondrial myopathy, or dilated cardiomyopathy] [21]; and, in addition, a mutation in the same tRNA nucleotide can lead to several disorders (e.g., the mutation A5814G in mt-tRNA^{Cys} manifests either as severe encephalopathy or cardiomyopathy) [26,27]. This peculiarity is attributed to the random distribution of heteroplasmic pools of mt-DNA during mitotic segregation [28,29] (Box 1) and, by contrast, is not observed for mt-aaRSs. For 13 of the mt-aaRS enzymes, mutations can lead to a single pathology (e.g., mutations in mt-ProRS lead exclusively to epileptic encephalopathy). There are exceptions where multiple system injuries might be explained by collateral damage, as in the case of mutations in mt-SerRS that are associated with HUPRA syndrome. In this clinical scenario, renal failure (primary injury) might be the cause of **hyperuricemia** and pulmonary hypertension (secondary injuries). When a single mt-aaRS is associated with two unrelated clinical

Isodecoder: tRNA isodecoders share the same anticodon triplet but present sequence differences in other tRNA regions. tRNA isodecoders and tRNA isoacceptors (varying at the anticodon triplet), are charged with the same amino acid. In the human nuclear genome there are more than 270 isodecoder genes (~450 tRNA genes distributed among 49 isoacceptor families).

Kearns–Sayre syndrome: a multisystem disorder mainly affecting the eyes (weakness of the eye muscle and loss of vision, among others). Patients can also present cardiac conduction problems and ataxia.

Late-onset disease: refers to cases where symptoms of a disease strike in childhood or later (early adulthood).

LBSL disease: leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation – a progressive leukodystrophy disorder that affects the white matter of the brain and spinal cord; patients also present elevated lactate levels. Ataxia and spastic paresis are primary clinical symptoms.

Leukodystrophies: a group of disorders affecting white matter in the CNS.

Mitochondrial DNA (mt-DNA): small circular double-stranded DNA; in humans mt-DNA comprises 16 569 bp, coding on both strands for only 37 genes: 13 protein subunits of the respiratory chain complexes, two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). The only non-coding region is the displacement loop (D-loop), also called the control region.

MERRF-like disease: myoclonic epilepsy with ragged-red fibers – a mitochondrial disorder with onset in childhood that affects the CNS and skeletal muscle. The main feature is myoclonus, but patients can also manifest other clinical signs including muscle weakness, ataxia, seizures, dementia, degeneration of the optic nerve, hearing loss, and cardiomyopathy, among others.

Mitochondrial myopathy: mitochondrial disorder manifesting in muscular impairment; primarily muscle weakness.

Mitochondrial unfolded protein response (mt-UPR): a signaling pathway between mitochondria and the nucleus that responds to mitochondrial stress in response to

manifestations (e.g., mt-AlaRS with leukodystrophy or cardiomyopathy, GlyRS with cardiomyopathy or peripheral neuropathy, **Charcot-Marie-Tooth (CMT) disease**, and mt-SerRS with HUPRA or **spastic paresis**), it is always due to distinct sets of mutations [22]. This suggests that, in these cases, organ-specific variations in disease may originate from different aaRS functions associated with different sets of mutated residues [22] (Box 3).

Overall, these findings indicate that, although both mt-aaRSs and mt-tRNAs are involved in aminoacylation, the pathological consequences of mutations in either system probably arise from different molecular mechanisms and, consequently, that our understanding and analysis of the two systems must be dissociated.

Box 3. Mutations in the Context of Protein Architecture May Help To Identify the Molecular Mechanisms of Disease

Structurally, the effects of mutation of a protein residue could be due to its presence at or around any of the binding sites necessary for function (Figure 1) [78–81]. First, mutations in the binding sites for the amino acid, ATP, or the tRNA terminal -CCA end will lead to an absence of catalysis or impaired catalysis (depending on the level of conservation and the structural role). Second, for specificity and tight binding, accessible residues of the aaRSs bind to the cognate tRNAs at multiple locations on their surface (termed the tRNA identity elements), and residues in the anticodon loop play key roles in binding. Mutations at the binding interfaces may alter the dynamics and folding of protein residues, leading to loss of specificity of binding. In addition, aaRSs frequently form oligomers, and mutations may affect the oligomerization process, thereby leading to inactive particles or misfolded or aggregated proteins. Third, mutations at positions far from known key functional regions can still lead to deleterious effects. Indeed, mutation of a buried amino acid may alter communication between domains (e.g., between the binding of a tRNA identity element and the site of catalysis). Similarly, a mutation occurring in a region where no known substrate binds might still impact, through molecular rearrangements and dynamics, on the binding of a substrate or of an alternative molecular partner at a distance. Finally, the state of oligomerization may vary when residues participating in the oligomerization interface are required for alternative function(s). In such a case, a mutation may prevent the adoption of alternative conformations or may prevent an interaction with a new partner, thereby hindering the alternative function.

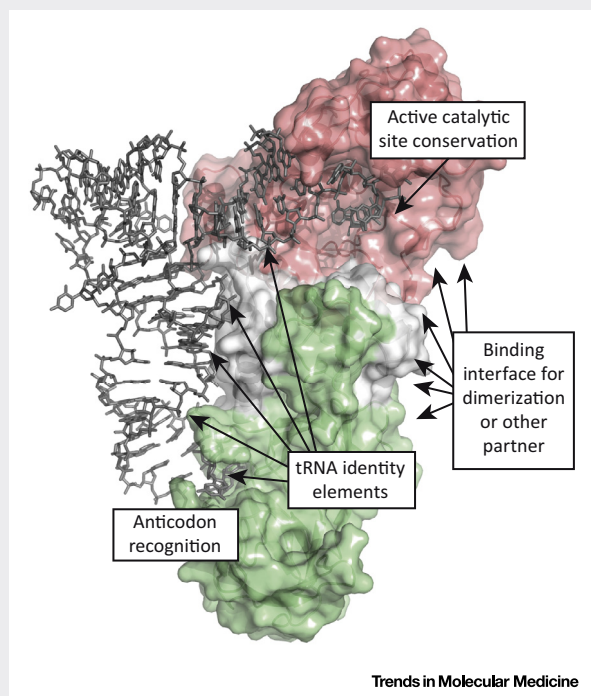


Figure 1. Putative Functional Regions of an aaRS and Its Substrates: The tRNA, the Activated Amino Acid, and Other Possible Protein Partners. The cartoon, generated with Pymol [82], is based on the crystallographic structure of the *E. coli* GlnRS complexed with its cognate tRNA (PDB code 1QTQ [83]).

unfolded or misfolded proteins or to an imbalance in mitochondrial homeostasis.

MLASA syndrome: myopathy, lactic acidosis, and sideroblastic anemia – a mitochondrial autosomal recessive disorder that affects the skeletal muscle and bone marrow.

Myoglobinuria: presence of myoglobin in the urine.

Myoclonic epilepsy: an increase in muscle tone which causes sudden, brief spasm movements that can affect the face or limbs.

Organ system: a group of anatomical structures that together perform specific functions in the body.

Oxidative phosphorylation (OXPHOS): in mitochondria, electrons from electron donors are transferred, in redox reactions, to electron acceptors such as oxygen, which then split, take up protons, and form water. During electron transport, proton pumps in complexes I, III, and IV become activated, leading to the expulsion of protons from the mitochondrial matrix to the intramembrane space. The generated proton gradient combined with electron movement lead to the return of protons to the matrix, thus activating ATP synthase by a proton-motive force. ATP synthase links ADP to inorganic phosphate to generate ATP.

Perrault syndrome: a mitochondrial disorder characterized by hearing loss. Affected females also have ovary abnormalities (abnormal morphology or missing ovaries). Both males and females can also present neurological problems such as ataxia and/or intellectual disability.

Psychomotor regression: deterioration of cognitive abilities and reduction of physical movements.

Respiratory chain complexes: consist of five large multiprotein complexes, located in the inner mitochondrial membrane, that host OXPHOS. The complexes are NADH: ubiquinone oxidoreductase (I), succinate-coenzyme Q reductase (II), coenzyme Q-cytochrome c reductase (III), cytochrome c oxidase (IV), and ATP synthase (V). The sets of proteins involved are of dual genetic origin. A total of 84 subunits and an additional 28 assembly factors are encoded by the nuclear genome while 13 subunits are encoded by the mitochondrial genome. Complex II comprises

Genetics of Disease-associated Mutations in mt-aaRSs

We recently developed a knowledge-based repository coupled with a web server (<http://misynpat.org>) that integrates and links clinical, genetic, and structural data on disease-related mutations of human mt-aaRSs [22]. The MiSynPat repository is one of the sources of the data discussed below and contains an exhaustive list of reported mutations and related references. Presently, 208 different disease-related variations have been identified, either in coding sequences (153) or in intronic regions (55) of mt-aaRS genes.

For a variety of diseases associated with mt-aaRSs mutations, patients are mainly **compound heterozygotes** [22]. **Homozygosity** is sometimes reported (26 of 153 missense or nonsense mutations). Although it seems obvious that mutations in essential enzymes would cause disease, one can also expect that most mutations in such enzymes are lethal. Thus, these observations have suggested that, to be compatible with survival, homozygous mutations should not exert too severe an effect on structure and/or on function [20,30] (Box 1). A unique case is the dimeric mt-TyrRS, for which mutations reported so far are homozygous (with one patient with two different variations affecting the same position, i.e., G191D associated with G191V [31]) (Figures 3 and 4) [22]. The only compound heterozygous patient associates a missense mutation (G191D) with a nonsense mutation (R360X) [32], and only the G191D variation is present in the expressed dimeric protein. This indicates that the pathogenicity of mutated mt-TyrRS exclusively relies on homodimeric mutated forms of the enzyme. Although the relevance to the molecular mechanism is unclear, TyrRS is peculiar because tRNA^{Tyr} binds simultaneously to each of the two monomers of the synthetase (the only other case is TrpRS) [33]. However, the mutated positions are not conserved throughout the global phylogeny and are spread over the 3D structure. Of note, the mt-TyrRS-related MLASA syndrome stands apart from other mt-aaRS-associated diseases because it is the only one known to lead to a musculoskeletal primary defect and no CNS primary injury [34].

For amino acids Gly and Lys, single genes encode both the mitochondrial and the cytosolic aaRSs (Box 2) [16]. Reported mutations in those two genes show either **recessive inheritance** or dominant inheritance, and are thus found in both the cytosolic and the mitochondrial forms of these two enzymes [22]. On the one hand, because the dominant mutations of cytosolic aaRSs (cytosolic TyrRS, AlaRS, HisRS, and MetRS) are linked to peripheral neuropathy CMT disease, it has been suggested that the CMT-related dominant mutations of GlyRS and LysRS (both cytosolic and mitochondrial) probably mainly affect the cytosolic function(s) of these enzymes [35,36]. On the other hand, recessive mutations correlated with cardiomyopathies (for GlyRS and LysRS), or with visual impairment, progressive microcephaly, or hearing impairment (for LysRS), probably mainly impinge on their mitochondrial function(s), but this has not been directly tested. The selective impairment of either cytosolic or mitochondrial forms of these enzymes remains to be carefully investigated experimentally before reaching firm conclusions; indeed, CMT has also been observed to result from mutations in the mitochondrial fusion protein MFN2 [19].

Collectively, these observations emphasize a confounding issue in the field, namely that seemingly comparable mutations in different mt-aaRSs lead to diseases that are extremely different, occurring at different stages in life and in different tissues.

Analysis of Mutated Positions in mt-aaRS Architecture and Potential Functional Implications

Presently, 153 mutations impacting on protein sequences (missense, nonsense, insertion, and deletion) have been reported, and these are spread over the constitutive functional domains of mt-aaRSs (Figure 3). Of note, the tRNA-edge binding domain (found in mt-ArgRS, mt-SerRS, and mt-LeuRS) is the only known functional domain without disease-prone mutations [22], which may

exclusively nucleus-encoded proteins.

Recessive inheritance: conditions where both alleles must be affected to manifest disease.

Selective or evolutionary pressure: molecular features which are essential for cell survival are under selective (or evolutionary) pressure because their disappearance or absence of functionality would lead to either death or lack of progeny. Molecular features that are not under selective pressure can evolve with more freedom, and are said to be neutral at the evolutionary level.

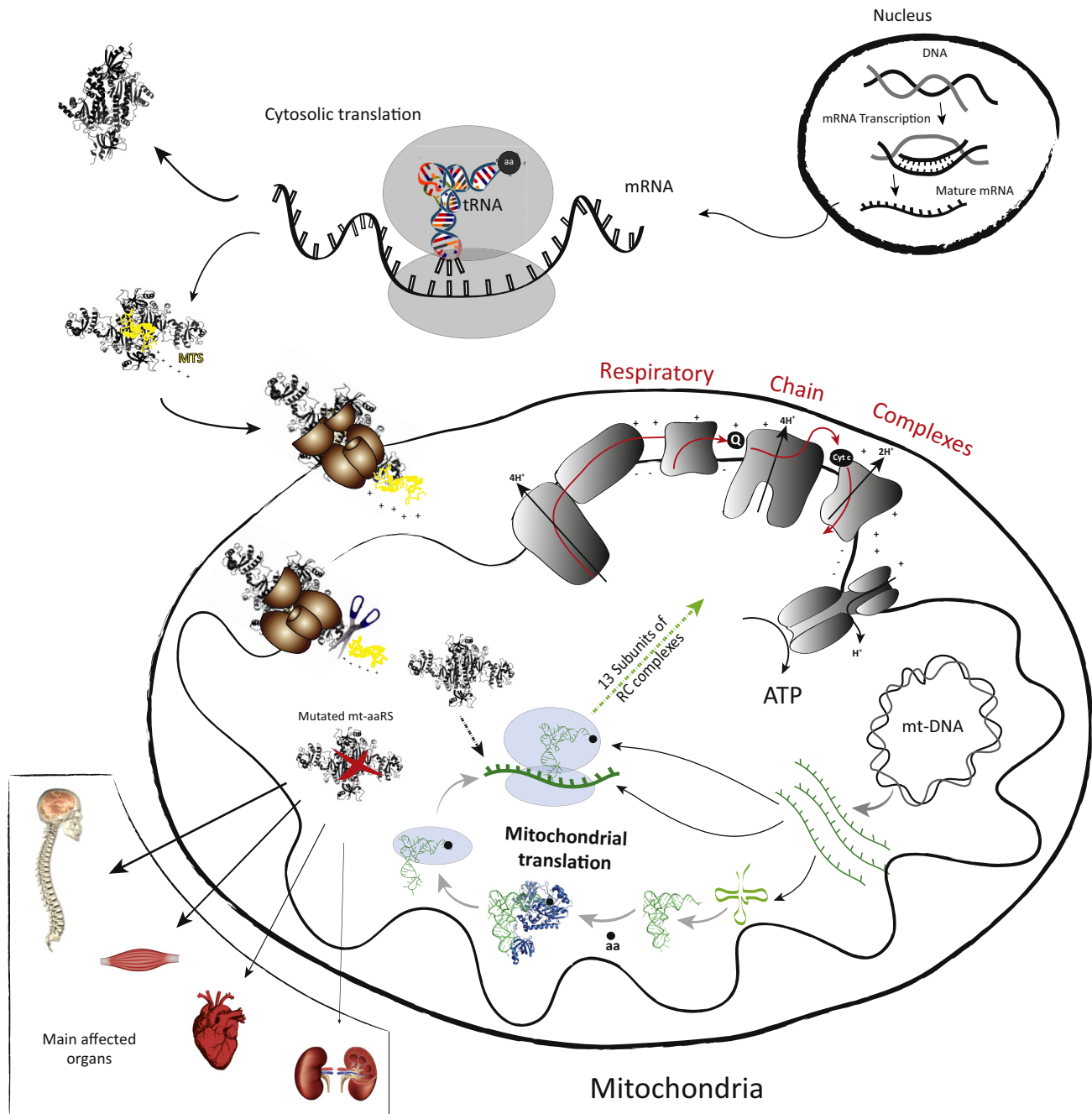
Spastic paresis: affected individuals show an increase in muscle tonus and in tendon reflexes.

Sporadic bilateral optic neuropathy: damage to and/or death of neurons in the optic nerve. The main symptom is loss of vision and damage can be unilateral (affecting only one of the two optic nerves) or bilateral (affecting both optic nerves)

Thylakoids: small structures suspended within the chloroplast that contain sack membranes where chlorophyll is localized and photosynthesis occurs.

Key Figure

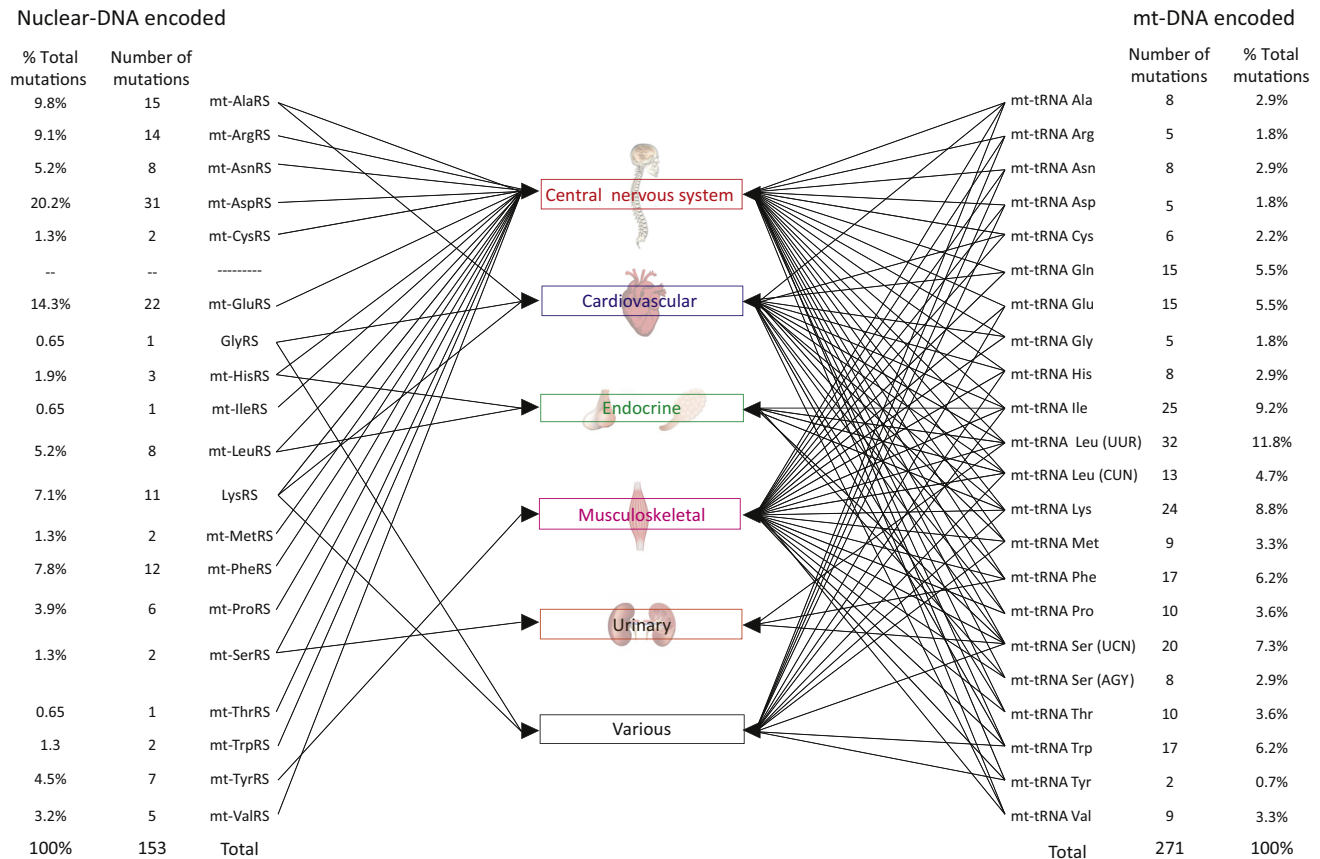
The Canonical Role of Mitochondrial Aminoacyl-tRNA Synthetases (mt-aaRSs) in Mitochondrial Translation and the Tissue-Specific Impact of Disease-Associated Mutations



Trends in Molecular Medicine

Figure 1. Human mt-aaRSs are encoded in the nucleus, synthesized in the cytosol, and delivered and imported into mitochondria via a mitochondrial targeting sequence (MTS) which is cleaved upon entry into mitochondria. mt-aaRSs, key actors in mitochondrial translation, catalyze the specific attachment of each amino acid

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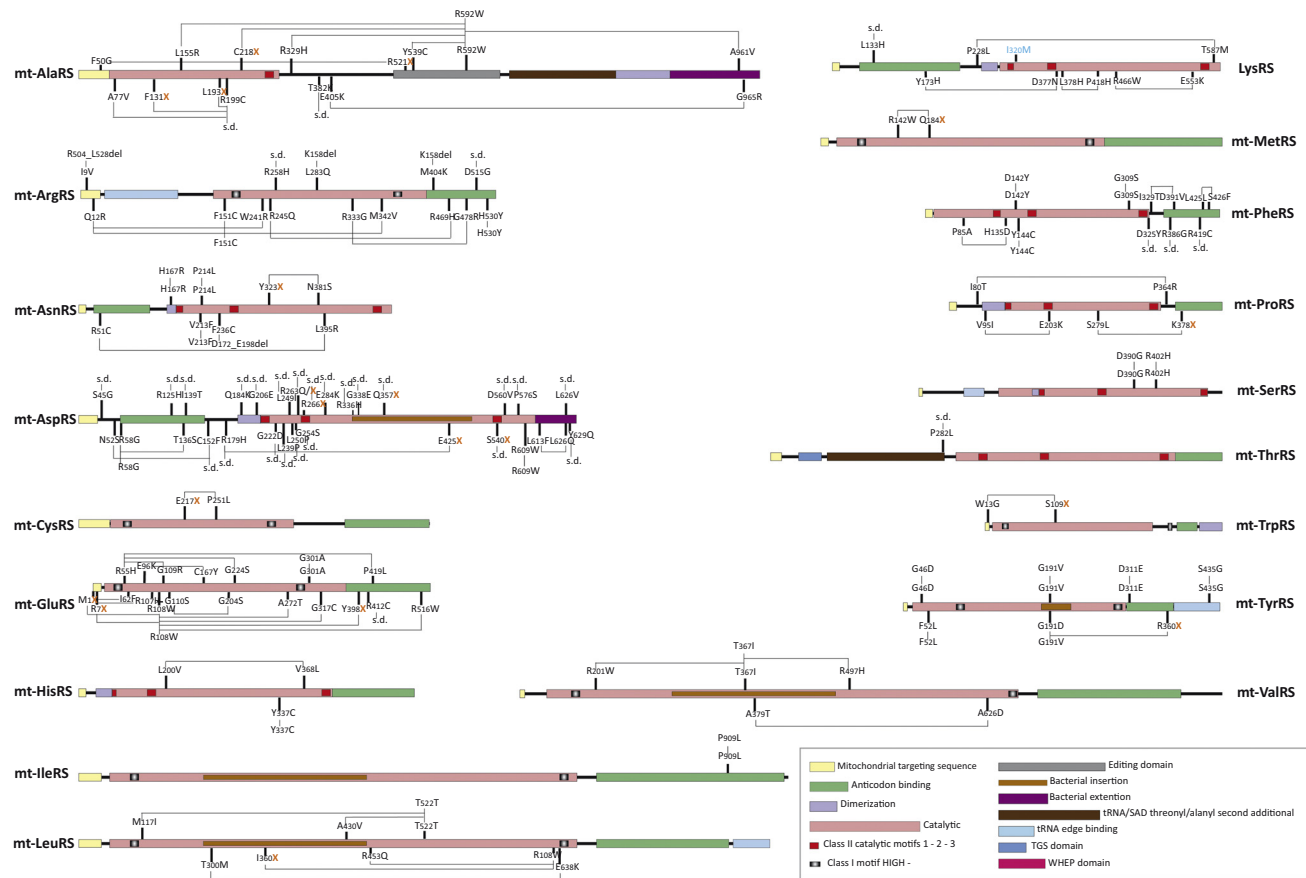
Trends in Molecular Medicine

Figure 2. Mitochondrial Aminoacyl-tRNA Synthetase (mt-aaRS) Versus mt-tRNA Mutations and Affected Organ Systems. The diagram shows the total numbers of known reported mutations and their percentages for mt-aaRSs (left) and mt-tRNAs (right). mt-aaRSs are encoded in the nucleus and mt-tRNAs in the mt-DNA, and thus mutations segregate in patients following Mendelian or maternal inheritance, respectively (Box 1). The primary impacts of the mutations are classified according to five major organ systems: central nervous system (CNS), cardiovascular, endocrine, musculoskeletal, and urinary. A further category named 'various' contains reports of less frequent diseases. Data are extracted from the MiSynPat (<http://misynpat.org> [22]) and MitoMap (www.mitomap.org/MITOMAP [21]) databases for mutations in mt-aaRSs and mt-tRNAs, respectively.

indicate that this domain, in a mitochondrial context, is either non-essential (or neutral) or so essential that its mutations are lethal. Interestingly, a loss of **selective or evolutionary pressure**, reflecting an evolutionary loss of function, has been shown for bacterial insertion domains of mitochondrial AspRSs [37]. Only nonsense mutations have been reported in this domain of human mt-AspRS, and this is interesting because it may be indicative of a lack of specific role for constitutive amino acids, supporting the neutral role of this domain.

We now discuss the effects of mutations on the architecture of mt-aaRSs; this analysis allows the identification of functional domains (Box 3) and thus facilitates the prediction of the potential impact of mutations on these functional domains. Based on an exhaustive list of mutations for these enzymes, in conjunction with 3D models, all gathered in the repository MiSynPat, it is possible to

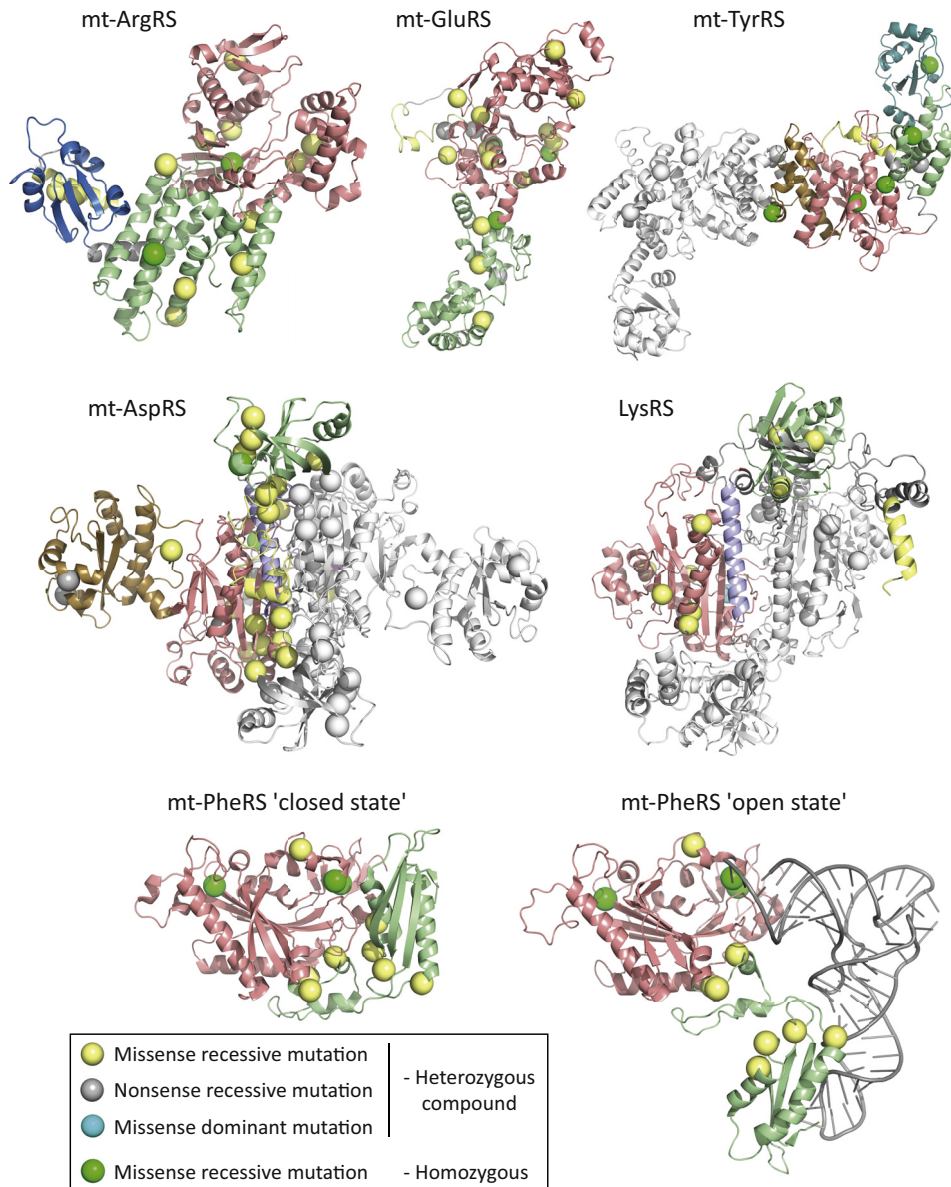
onto the cognate tRNA(s). Mitochondrial translation synthesizes 13 proteins that, together with 84 additional nucleus-encoded proteins, form the respiratory chain complexes. Thus, mt-aaRSs are central to cellular energy production. Mutations in mt-aaRSs have been identified in diverse human diseases, and the main organs affected are the central nervous system, as well as the musculoskeletal, cardiovascular, and urinary systems (indicated in the inset). Fully functional mitochondria require ~1200 additional nucleus-encoded proteins to guarantee mitochondrial genome maintenance and expression.



Trends in Molecular Medicine

Figure 3. Disease-Associated Mutations of Human Mitochondrial Aminoacyl-tRNA Synthetases (mt-aaRSs). The mt-aaRS mutations are shown to scale; known functional domains are color-coded. Allelic compositions, as identified in patients, are linked through black lines for all reported mutations. Amino acid substitutions due to missense mutations are indicated. The orange X indicates a STOP codon created by a nonsense mutation. The mutation in blue (in LysRS) corresponds to a dominant mutation. Data were extracted from MiSynPat (<http://misynpat.org> [22]) which contains all related references. The GlyRS is absent because most reported mutations are dominant and lead to Charcot-Marie-Tooth disease, suggesting an impact on the cytosolic activity of the enzyme rather than on the activity of the mitochondrial enzyme. Abbreviation: s.d., splicing defect or defect involving an intronic region.

reach some conclusions [22]. Protein architectures can be extracted either from crystal structures or from models derived by homology (based on crystallographic structures of homologous proteins). Analysis of these structures has revealed that, for most mt-aaRSs (among those with a significant number of records), mutations occur throughout the known 3D structures, including surfaces that do not interact with tRNAs and, furthermore, the mutations do not localize spatially around functional regions that are necessary for aminoacylation (e.g., mt-ArgRS, mt-GluRS, and mt-TyrRS in Figure 4) [22]. These observations preclude simple mechanistic explanations (Box 3). Occasionally, however, cases do occur where mutations concentrate in identified functional regions. For example, in mt-AspRS, most mutations have been identified at the dimeric interface (mt-AspRS in Figure 4; compare with the visually random distribution of mutations in LysRS, noting that these two enzymes have similar folds and organizations) [22]. Regarding mt-PheRS, the pattern of mutations on the free form of the enzyme provides little information. It is known, however, that the binding of tRNA^{Phe} is accompanied by rearrangement involving a ~160° hinge-type rotation from a ‘closed’ to an ‘open’ state of PheRS, with global repositioning of the anticodon binding domain upon tRNA binding [38]. When displayed in the ‘open’ state, mutations occur in the catalytic core, the anticodon-binding region, and the rearrangement zone, strongly suggesting a direct impact on aminoacylation function (Mt-PheRS in Figure 4).



Trends in Molecular Medicine

Figure 4. Selected Human Mitochondrial Aminoacyl-tRNA Synthetase (mt-aaRS) Mutations in 3D Structural Representations. Coordinates for the 3D models (which include MTSS) of mt-ArgRS, mt-GluRS, mt-TyrRS, mt-AspRS, and LysRS were uploaded from MiSynPat (<http://misynpat.org> [22]). Human mt-PheRS is depicted in a 'closed' state in the absence of cognate tRNA (PDB: 3CMQ [38]) or in the 'open' state when complexed with *Thermus thermophilus* tRNA^{Phe} (in grey) (PDB: 2TUP [84]). For all structures, the mitochondrial targeting sequence (MTS) is in yellow, the catalytic domain in red, the anticodon binding domain in green, and the hinge region in grey, and additional domains are in hues of blue (the color code is the same as in Figure 3). Where appropriate, the second dimer is displayed in light grey. The color codes for missense and nonsense mutations, in homozygous or compound heterozygous contexts, are indicated in the inset. Cartoons were generated with Pymol [82].

Therefore, the distribution of mutated positions on structural models covers almost all regions of the architecture. However, in some instances only, mutations occur at positions that are involved in substrate binding, suggesting a putative impairment in the canonical function of aaRSs [22].

Box 4. Mutations in the Evolutionary Context of Protein Sequences

With the advent of genomics, sequences of homologous proteins can be obtained from a large variety of organisms and organelles. Multiple sequence alignments can help visualize how amino acids are conserved at various positions along the sequence. Sequence alignments are even more powerful when at least one crystal structure is available for one of the sequences. That sequence can then serve as a structural template, and the ensuing structural alignments allow key architectural parameters to be derived for various amino acid positions (e.g., how buried or accessible they are, how close they are to the active site or to some other binding site, whether they occur in a helix or a loop, etc.). The levels of conservation derived from sequence alignments at mutated positions are highly informative for the understanding of the underlying molecular mechanisms. Conservation can be evaluated strictly (always the same type of amino acid) or more broadly, for example whether the variations occur with the maintenance of similar physicochemical properties (charge, size, hydrophobicity).

Conservation can also be analyzed within a single phylum or group of phylae. Residues conserved throughout the whole phylogeny (bacteria, archaea, eukarya, including organelles) are indicative of a **selective pressure** on that position, suggesting either a structural or a functional role related to the central function of the protein (in this case aminoacylation). However, an absence of conservation for an affected position does not call into question the pathogenicity of the mutation. Most importantly, such differentially conserved residues, in other words those that are conserved in only a subgroup or phylum of sequences, reveal loss or gain of selective pressure. Consequently, the impact of mutations at such positions may indicate tissue-specific modulation of translation or a moonlighting (alternative) function (e.g., through the participation in larger molecular complexes). Furthermore, mutation at a non-conserved position (especially with a change in physicochemical properties) may lead to an unstable or misfolded protein *in vivo* (aggregation or degradation) and/or to defective import into mitochondria.

Analysis of the Evolutionary Conservation of Mutated Positions in mt-aaRSs and Potential Functional Implications

The amino acid conservation patterns along the protein sequence can be derived from precise sequence alignments (Box 4). Such patterns convey crucial functional molecular insights on the wild-type protein and potentially further our understanding of the molecular mechanisms of disease (Box 4). An analysis of the amino acid conservation of mutated positions has been recently conducted based on sequence alignments contained and accessible through MiS-ynPat [22]. Overall, among the observed mutations in mt-aaRSs, 67% occur at positions that are not conserved in phylogeny, 13% are conserved based on physicochemical properties, and only 5% are 100% conserved [22]. For some mt-aaRSs, sufficient data are available to conduct more detailed analyses.

For only two aaRSs, the observed mutations occur at highly conserved positions: in mt-PheRS (leading to encephalopathy) and in mt-AlaRS (but only for mutations leading to leukodystrophy) [22]. For mt-PheRS, in agreement with structural observations (see above), experimental *in vitro* aminoacylation measurements reveal a strong impact of these mutations on aminoacylation levels, potentially leading to impaired translation [39]. Indeed, biochemical characterization of recombinant mutant mt-PheRS proteins has shown that mutations can affect synthetase function in different ways, such as by reducing the binding of ATP, phenylalanyl, or tRNA [39], and/or by affecting synthetase folding and stability [39]. No direct experimental data on aminoacylation are currently available for mt-AlaRS, and therefore a quantitative evaluation of the effects of mutations on translation efficiency cannot be presently made.

For three mt-aaRSs, mutations have been reported at conserved positions for at least one allele: mt-ArgRS, associated with encephalopathy, and mt-LeuRS and mt-HisRS, both leading to Perrault syndrome [22]. In these cases, one can envisage that the canonical structures and functions might be affected by these mutations, suggesting that the amount of active mt-aaRS might reside below a minimum biochemical threshold, but this remains to be tested.

For other mt-aaRSs, there is only low or no conservation of mutated positions: mt-GluRS and mt-AspRS (leukodystrophy), mt-ValRS (encephalopathy), mt-TyrRS (MLASA), and mt-AlaRS (with mutations in the latter leading to cardiomyopathy only) [22]. The available aminoacylation

data reveal an absence (e.g., mt-AspRS) or only a weak (e.g., mt-TyrRS) effect of the mutations on the level of aminoacylation [22]. In the case of mt-AspRS, extended investigations on a subset of LBSL-related mutants revealed an absence of a common impact(s) on the molecular, cellular, and/or structural properties of mt-AspRS [17,23,30,40,41]. Consequently, the pathomechanism of LBSL remains unsolved.

Overall, an analysis of the level of conservation leads to the following possibilities. First, there appears to be no linked segregation between conservation of the affected positions and disease (e.g., leukodystrophy is found in two of the classes listed above and encephalopathy in three). Second, there is presumably no correspondence between the level of conservation and the severity of disease (e.g., 'mild' symptoms for mt-AlaRS/leukodystrophy mutated at positions that are highly conserved, vs 'drastic' symptoms for mt-AlaRS/cardiomyopathy mutated at non-conserved positions). Third, the levels of amino acid conservation might reflect different molecular mechanisms, despite similarities in diseases. As a notable example, leukodystrophies linked to mutations in mt-GluRS or mt-AspRS (at non-conserved positions) are probably due to a molecular mechanism that is different from the mechanism underlying leukodystrophy linked to mt-AlaRS mutations (at highly conserved positions) for which aminoacylation deficiencies are strongly suspected. In mt-AspRS, however, it has been shown that, considering exclusively the subphylum of mammals, all reported mutations affect positions that are highly conserved ([30]; preliminary data suggest that this may also be the case for mt-GluRS, but this remains to be validated). This suggests that, for these two enzymes, there may be a selective pressure, possibly restricted to mammals. Of note, numerous functions beyond translation (i.e., 'moonlighting activities') may have emerged from new selective pressures or from the generation of new architectural domains, principally in vertebrates, as has been described for cytosolic aaRSs [42–45] (Box 2). A hypothesis is thus that alternative, albeit uncharacterized functions may also have emerged for these mt-aaRSs.

In summary, the level of amino acid conservation at the mutated positions ranges from low to high for the same aaRS and for similar diseases, suggesting that, although the canonical function of aminoacylation is affected in some cases, other unknown additional functions are also likely to be affected, and this should provide a future area of research.

Towards a Cellular Integration of Mitochondrial Translation Processes

That mutations in mt-aaRSs generally impact on mitochondrial translation is an inescapable conclusion from the preceding sections but, at the same time, it is also an insufficient one, in the sense that we do not know whether, how, why, or when translation is affected. Further, mutations in mt-aaRSs present striking tissue-specificity (see Outstanding Questions and Figure 1) that cannot be rationalized without considering mitochondrial translation as being fully integrated within the cell and actively participating as an environmental sensor [46]. Douglas C. Wallace has evoked the crucial coupling between mitochondria and the nucleus [46]. In his words: 'The nucleus must "know" that mitochondria can generate the required energy before proceeding with DNA replication and transcription'. We suggest that mitochondrial translation plays a pivotal role as an environmental sensor of the state of the cell. We further suggest that the integration of translation occurs through specific modulations and/or connections with other biological processes, which might be tissue-specific or tissue-dependent and which might involve some or all mt-aaRSs. In recent years, several communication and signaling pathways between the nucleus and mitochondria have been uncovered [47]. Thus, it is pertinent to examine the effects aaRSs and their mutations to achieve a better understanding and holistic integration of mitochondrial translation processes within cells. Indeed, mutations in aaRSs may be able to impact upon many or all translational events and signaling pathways, with unsuspected biological consequences. We thus suggest different possibilities for how molecules from the mitochondrial translation machinery might be integrated within the cellular environment.

Integration Through the Unfolded Protein Response

Among the communication pathways between nucleus and mitochondria are the cytoplasmic and **mitochondrial unfolded protein responses** (UPRs) that are intimately linked to a fully-functional translation process. mRNA translation is the result of a wide range of factors and enzymatic activities that must occur to accurately produce correct polypeptides while simultaneously proceeding at the proper speed for native protein folding. The final product, protein folding, is ultimately an error-prone process. In protein synthesis, the types of proteins synthesized vary widely between cell types and environments and, accordingly, so do the levels of nutrients, metabolites, and energy requirements for proper protein folding [48]. Any slowing down or impairment of translation can influence the folding of a nascent protein, leading to the accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum (ER), leading to ER stress and activation of the UPR [48]. As central cofactors, adequate levels of functional aaRSs (and fully functional tRNAs) are essential to achieve smooth translation. For example, a lack of tRNA modifications in the anticodon triplet has been reported to lead to protein misfolding with activation of the UPR signaling pathway in various cell types [49]. Similarly, we can envisage that a decrease in the amount of fully functional aaRSs might trigger the UPR pathway, especially in stringent environments such as those for neuronal cells in the CNS. For example, it is well known that neuronal cells harbor great morphological diversity and extremely high energy demands. Functional mitochondria must be efficiently transported to distal regions of neuronal cells where specific quality-control mechanisms remove dysfunctional mitochondria [50]. Further, as postmitotic cells, neurons are very sensitive to toxic species such as misfolded proteins [51]. Thus, it cannot be excluded that some mutations might lead to either misfolded mt-aaRSs or misfolded products of mitochondrial translation, thereby triggering the mt-UPR pathway. The mt-UPR pathway is linked to the nucleus through the shuttling of transcription factors that can alter nuclear gene expression [52] upon sensing mitochondrial stress [53]. Moreover, the links between mitochondrial protein synthesis and ER occur architecturally through specialized areas – the mitochondria-associated membranes (MAMs). In terms of tissue-specific UPR, one study has shown that conditional knockout mice for heart or skeletal muscle-specific *Dars2* (the gene coding for mt-AspRS) exhibit severe mitochondrial dysfunction and activation of various stress responses in a tissue-dependent manner [54]. However, the activated UPR is only observed in cardiomyocytes, which suggests that this response may be tissue-specific [54]. In error-prone mutant cytosolic AlaRS (with a mutation in the editing domain), protein aggregation also led to death of cerebellar Purkinje cells [55]. Consequently, both sets of observations implicate the mt-UPR and/or misfolded or aggregated proteins in mt-aaRS-associated pathologies.

Integration Through other Communication and Metabolic Pathways

Several examples lend support to the hypothesis that aaRSs are involved in other communication and signaling pathways, and may be used as sensors for the mitochondrial environment. For instance, mt-MetRS has been shown to physically interact with thioredoxin 2, subsequently contributing to mitochondrial redox regulation and detoxification [56]. Furthermore, in cyanobacteria, the ValRS has been demonstrated to be anchored to the membrane of **thylakoids** and is connected to ATP synthase, suggesting that this enzyme may play a role as a functional sensor between elements of gene translation and energy production machineries [57]. Preliminary information seems to indicate that some human mt-aaRSs may also be anchored to the mitochondrial membrane, possibly connecting these two cellular processes; however, this remains to be validated.

Another example includes the synthesis of nonessential amino acids, which are included amongst the numerous essential metabolic pathways hosted by mitochondria. These amino acids are synthesized from precursors generated either by glycolysis (cytosolic) or by the Krebs cycle (mitochondria). For instance, aspartate is the most abundant metabolite in mitochondria

[58] and is tightly connected to the respiratory chain complexes (aspartate synthesis is sensitive to changes in the matrix NADH/NAD redox balance), and is also the precursor of *N*-acetyl-L-aspartate, an essential neuron-specific transmitter [59]. One can envisage that mt-aaRSs could monitor amino acid levels, as reported for the cytosolic LeuRS that was shown to be a leucine sensor in the mTOR (mechanistic target of rapamycin) pathway which regulates both metabolism and protein synthesis [60].

One can also consider that alternative, moonlighting, function(s) may be impacted by disease-associated aaRS mutations. Moonlighting functions have been reported for mitochondrial proteins in the nucleus [61] and for cytosolic aaRSs (Box 2 for examples). Indeed, molecular evolution uses and reuses existing folded proteins for several *ad hoc* purposes. AaRSs are ancient and abundant proteins, and it is possible that moonlighting functions have also arisen for mt-aaRSs. This would correlate with the observation that some mutated positions are exclusively conserved within the mammalian subphylum, and in regions that are not primarily associated with the ancillary functions of aaRSs, meaning that these regions might theoretically display alternative function(s) [22] (Box 3). Of note, mRNAs of mt-aaRSs present various splice variants [62]. Some of the resulting shortened proteins are 'catalytic nulls', implying that they may be repurposed towards an alternative function. Moreover, some disease-related mutations present in genomic DNA may be maintained in the splice variants, and others lost. Therefore, one cannot exclude the possibility that mt-aaRS splice variants carrying mutations might be deleterious, potentially executing impaired alternative functions. Although the splice variant hypothesis cannot constitute a global explanation for each mt-aaRS and for each disease, these truncated/modified proteins might play an important role for specific mt-aaRSs and in specific diseases. Further experimental work is clearly required.

Moonlighting functions, or specialized functions that are necessary only in specific differentiated cells, can be easily contemplated as tissue-specific. The first alternative function of a mt-aaRS was only recently described: rat mt-TrpRS was identified as a novel determinant of angiogenesis in the heart and other tissues by acting as an integrator of pro-angiogenic signaling, directing cell motility and division [63]. Moonlighting activity has also been reported for the human mitochondrial ribosomal protein L12 (MRPL12), for which two mature forms, generated by two-step cleavage during import, have been implicated in translation (canonical role) and in transcriptional regulation (alternative role) [64]. Recently, the coexistence of two forms of human mt-AspRS, matured after importation into mitochondria following the same enzymatic cleavages, has also been demonstrated [65]. It is possible that one of the forms of mt-AspRS harbors a moonlighting activity, and also that processing of mt-aaRSs (presently poorly investigated) represents another means by which to generate and increase the reservoir of alternative functions for mt-aaRSs. However, this hypothesis remains to be tested.

Because disease-related mutations in mt-aaRSs do not lead to multisystemic injuries, and the CNS is the most frequently affected organ system, the preceding observations imply that numerous specific mechanisms may affect, exploit, or modulate the mitochondrial translation apparatus in neuronal cells. Indeed, we believe that future research should focus on identifying the properties of mt-aaRSs that may be specific to neuronal cells in the context of health and disease. By analyzing specific diseases, it may be possible to decipher distinct molecular mechanisms underlying mt-aaRS-related pathologies affecting the CNS. Some examples of neuron-specific molecules or mechanisms have been reported. For instance, one **isodecoder** of cytosolic tRNA^{Arg} has been shown to exist only in neuronal tissues; indeed, mutations in this tRNA^{Arg} can lead to neurodegeneration [66]. In addition, cell type-specific splicing efficiency differences in mt-AspRS mRNA have been observed; correct inclusion of exon 3 in this mRNA occurs less efficiently in neural cells than in other cell types (e.g., glia), as evidenced from *in vitro* experiments using LBSL patient-derived cells [67]. It is therefore essential to reach an

Box 5. Clinician's Corner

The nucleus-encoded mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs) are central components of the mitochondrial translation apparatus; they charge a specific tRNA with the correct amino acid for protein synthesis.

Mutations in mt-aaRSs predominantly affect the CNS, but other organs may be affected.

The term 'mitochondrial disease' covers a large number of different pathologies and disorders. The diversity of onsets, symptoms, organ involvement, and clinical presentations constitutes a challenge for the medical community in terms of diagnosis and even more in the development of therapeutic treatments.

Until now, the clinical management of patients is limited to symptomatic treatments and nutritional supplements. CoQ, vitamins A and E, carnitine, creatine, dichloroacetate, lipoic acid, and L-arginine are some of the supplements that are used routinely in patients with mitochondrial diseases. However, such treatments do not cure the diseases. In recent years numerous therapeutic approaches [including gene therapy using adeno-associated viral (AAVs) to correct the mutation or CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) technology for genome editing] have been developed. Some of those experimental approaches have shown efficacy in animal models. The testing of the efficacy of such treatments in humans is eagerly awaited.

The development and testing of any new therapeutic approach requires an understanding of the molecular mechanisms underlying the disease, and for mt-aaRSs these remain unclear. Therefore, integrative collaborations between clinicians and researchers should be encouraged to (i) assist diagnosis, (ii) counsel patients, and (iii) improve and develop novel and efficient therapeutic approaches.

integrated view of mitochondrial translation in a wide range of cellular environments and also under distinct physiological and pathophysiological conditions (Box 5).

Concluding Remarks

The present discussion indicates that mutations in all mt-aaRSs do not lead to pathologies via a single unique molecular mechanism, even though this might be easy to assume because mt-aaRSs constitute necessary and crucial components of the mitochondrial protein synthesis process. Thus, although many questions are to be pursued (see Outstanding Questions), we suggest that distinct mechanisms are at play in the pathogenesis of mt-aaRS-associated diseases and, furthermore, that these mechanisms may not be necessarily the same for each mt-aaRS. Among such putative mechanisms we have mainly considered that mt-aaRS mutations may generate: (i) dysfunction in the translation machinery, potentially triggering the mt-UPR; (ii) the alteration of alternative mt-aaRS function(s); and (iii) loss of activity as environmental sensors or mediators that integrate cellular pathways. The understanding of complex mitochondrial pathologies requires novel fundamental insights into the mechanisms of mitochondrial translation within cells and on the precise roles that mt-aaRSs play in these integrated molecular networks.

Cells have an absolute need for ATP produced by mitochondria. Unsurprisingly, the cell exerts constant surveillance and quality control of mitochondrial processes, meaning that mitochondria are fully integrated within cellular homeostasis. Mitochondrial translation, which is restricted to the synthesis of 13 proteins in humans, constitutes a point of convergence within the cell, and merges the products of nuclear and mitochondrial genomes. It is thus possible that mitochondrial translation may have been maintained through evolution for networking purposes, guaranteeing crosstalk between the cellular program and cellular energy demands, and enabling the surveillance of metabolic activities and stress. It will therefore be exciting to follow future investigations on the divergent pathways of mt-aaRSs in mitochondrial translation and disease.

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Outstanding Questions

How can mutations in ancillary proteins necessary to perform mitochondrial translation lead to a variety of disorders that impact on a restricted number of tissues/organs and with a great temporal diversity?

Is this marked tissue- and cell-specificity solely due to impaired mitochondrial translation in tissues or cells with high energy demands?

Alternatively, does the tissue-specificity result from non-translation functions played by mt-aaRSs that are only crucial in some tissues or cells? Are both phenomena present for some aaRSs?

Can one systematically assign the observed syndromes to results from direct damage to a single molecular process or to consequences from other linked processes?

Do cells exploit still unknown molecular mechanisms involving nucleus-encoded mt-aaRSs to monitor mitochondrial activity and ATP production?

The mitochondrial UPR connects nuclear and mitochondrial genomic expression. Could some imported and misfolded mutated aaRSs participate in this response, either directly or indirectly, because they slow down translation by impairing aminoacylation?

Alternatively, is mitochondrial translation particularly involved with or dependent upon amino acid metabolic pathways?

We should clarify how mitochondrial translation machineries are idiosyncratically integrated within different cells and tissues. We should disentangle the environmental contributions that either modify or modulate mitochondrial translation from those involved in moonlighting activities.

Neuronal cells present a great diversity of morphologies and an extremely high energy demand. Functional mitochondria must thus be efficiently transported to distal regions of neuronal cells where specific quality-control mechanisms can remove dysfunctional mitochondria. What, and how specific, are the pathways of mitochondrial translation within neuronal cells?

Supplemental Information

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Aminoacyl tRNA synthetases

Met - Leukoencephalopathy
 Ala - Leukoencephalopathy (ovario)
 Glu - Leukoencephalopathy with Thalamus and Brain stem involvement and high Lactate
 Asp - Leukoencephalopathy with Brain stem and Spinal cord involvement and Lactate elevation LBSL
 Cys - Epileptic encephalopathy
 Phe, Asn, Pro - Epileptic encephalopathy (Alpers Encephalopathy)
 Asn - Epileptic encephalopathy (Leigh Syndrome)
 Arg - Epileptic encephalopathy (Pontocerebellar Hypoplasia type 6)
 Thr - Encephalomyopathies
 Val - Epileptic encephalopathy and cardiomyopathy
 Met - Developmental Delay, Poor Growth, and Sensorineural Hearing Loss
 Asn - Nonsyndromic Sensorineural Deafness
 Lys - Nonsyndromic Sensorineural hearing impairment
 Trp - Intellectual Disability
 Phe - Spastic Paraplegia
 Ser - Spastic paresis
 Ile - Visual Impairment and Progressive Microcephaly
 Cataracts (CA), growth hormone deficiency (G), sensory neuropathy (S), sensorineural hearing loss (S), and skeletal dysplasia (S).
 His, Leu - Perrault syndrome

Ala, Gly, Lys - Infantile Mitochondrial Cardiomyopathy

His, Leu - Perrault syndrome

Tyr - Myopathy, lactic acidosis, and sideroblastic anemia-MLASA syndrome

Ser - Hyperuricemia, pulmonary hypertension, renal failure in infancy and alkalosis, HUPRA syndrome

Gly, Leu - Charcot-Marie-Tooth (CMT)

Central Nervous System

Cardiovascular

Endocrine

Musculoskeletal

Urinary

Various

tRNA

Ataxia - Asp, Lys, Phe, Pro, Ser.
 Alzheimer's Disease and Parkinson's Disease - Gln
 Ataxia, Mental deterioration, Deafness - Val, Ser
 Axenfeld-Rieger anomaly - Lys
 Chronic Progressive External Ophthalmoplegia - Phe, Leu, Ile, Ala, Asn, Ser, Glu
 Maternally inherited Deafness or aminoglycoside-induced Deafness - Phe, Ile, Gln, Trp, Ala, Cys, Ser, Lys, Arg, His, Glu, Thr, Pro
 Dementia, Chorea - Trp
 EncephaloCardioMyopathy - Ile
 Encephalomyopathy - Leu, Trp, Asn, Glu, Thr
 Epilepsy - Ile, Phe, Lys, Ser, Asp
 Fatal Infantile Cardiomyopathy Plus a Melas-associated cardiomyopathy - Ile
 KS Kearns-Sayre syndrom - Leu
 Leukoencephalopathy - Lys
 LHON Leber Hereditary Optic Neuropathy - Leu, Gln, Met, Glu, Thr
 LS Leigh Syndrome - Leu, Lys
 MELAS Mitochondrial Encephalomyopathy, Lactic Acidose, Stroke-like episodes - Phe, Val, Leu, Gln; Trp, Lys, His, Glu
 MERRF Myoclonic Epilepsy and Ragged Red muscle Fibers - Leu, Lys, His, Thr, Pro, Phe
 MNGIE Mitochondrial NeuroGastrointestinal Encephalomyopathy - Lys, Val
 Myoclonic Seizures - Ile
 Ophthalmoplegia - Glu
 PEM Progressive EncephaloMyopathy - Leu, Ser, Gly
 PEO Progressive External Ophthalmoplegia - Phe, Ile, Ala, Ser, Lys
 Ptosis, Ophthalmoparesis - Lys, Asn
 Pigmentary Retinopathy - Ile, Glu, Pro
 SensoriNeural Hearing Loss - Phe, Leu, Cys, Ser, Lys,
 Cardiomyopathy - Ile, Leu, Pro
 Hypertension - Leu, Ile, Gln, Met, Ser, Lys, Glu
 Coronary Heart Disease - Ala
 Carotid artery stenosis - Phe
 DM Diabetes Mellitus - Leu, Ile, Glu
 DMDF Diabetes Mellitus, Deafness - Leu, Ser, Lys, Glu
 MTMD Multiple Tumor in Mitochondrial Diabetes - Leu
 Hemiplegia - Val
 Mitochondrial Myopathy - Phe, Leu, Ile, Gln, Met, Trp, Ala, Asn, Cys, Asp, Lys, Gly, Arg, His, Ser
 Chronic Intestinal PseudoObstruction with myopathy - Glu
 Myoglobinuria - Phe, Ile
 Tubulointerstitial Nephritis and Stroke - Phe
 Renal disease - Leu, Ser
 Ineffective Hematopoiesis - Leu
 Progressive Respiratory Failure - Glu
 Cox Deficiency - Met, Glu, Arg
 Lactic Acidose - Met, Glu, Arg
 Metabolic Syndrom - Ile, Leu, Phe
 Exercise Intolerance - Glu, Lys, Met, Ser, Tyr
 Cancer risk - Leu
 Asthenozoospermia - His
 MOF MultiOrgan Failure - Asn, Leu

Objectives of the thesis

The evidence of the involvement of mitochondrial aminoacyl-tRNA synthetases (mt-aaRS) as a cause of mitochondrial disorders has increasingly attracted the interest of the scientific community on these proteins essential for the mitochondrial translation. Our laboratory, as well as others, has made major efforts in the characterization of different functional and structural properties of these mt-aaRS and in trying to understand the physiopathological mechanism(s) involved in the mitochondrial disorders produced as a consequence of their mutations. The objective of my PhD work followed the same order of interest but is aimed at expanding the knowledge towards an unexplored system: the human mitochondrial arginyl-tRNA synthetase. Strikingly, mutations of this enzyme in patients are reported to be the cause of PCH6, a severe neurodevelopmental disease. This work has been organized following three main guidelines:

1. Mitochondrial disorders linked to mutations in mitochondrial synthetases have shown striking tissue specificity towards the central nervous system. However, specific systems diverge towards other organs, producing distinct syndromes or specific affectations. The increasing number of cases reported called for an organization taking into account the clinical parameters related to each case described. This, I did an comprehensive analysis of the clinical data reported in pathologies related to mutations on mt-aaRSs, resulting in a categorization according to the anatomical system they affect. This organization allows us to extract messages and to establish functional research plans, directed mainly towards our system of interest: the mt-ArgRS.

2. The host laboratory, in collaboration with other groups, has been providing important inputs in the characterization and understanding of the mt-AspRS system during the past years. Taking in account all this expertise and making improvements to the existing protocols, I performed studies on my system of interest: the mt-ArgRS. So in this part of the work, I characterized some of the cellular properties of the mt-ArgRS. This, not only allowed to decipher so-far unexplored characteristics to this enzyme, but also when to compare to information previously collected about the mt-AspRS, contributed to reveal that mt-aaRSs have distinct cellular properties.

3. The first two parts were essential for the establishment of this third and final part of the work. Once the clinical analysis of the pathologies was performed; and

some of the intramitochondrial characteristics established, we investigated the impact of selected PCH6-related mutations on i) the newly-established cellular parameters of the mt-ArgRS and ii) on the catalytic function of this protein: the aminoacylation. Additional contributions regarding the mt-AspRS are provided in the Annex of this manuscript.

Integrating different approaches, this thesis aims at contributing to the expansion of the general knowledge of the mt-ArgRS properties in its cellular environment, and beyond that to a better understanding of the mosaicity of effects connected with an *in crescendo* list of mitochondrial diseases correlated with mt-aaRSs. The establishment of different clinical manifestations in the pathologies correlated to mutation on mt-aaRSs, together with the determination of different cellular properties regarding the sub-mitochondrial organization of two mt-aaRSs, strongly suggest new peculiarities, alternate/novel function(s) of mt-aaRSs or their possible involvement into different cellular pathways. Altogether, the outputs of this work should shed light into molecular mechanisms underlying some of the pathologies correlated to the mt-aaRSs.

Results and Discussions

Chapter I **Clinical analyses of pathologies related to mutations in mt-aaRS: spotlight on the central nervous system**

1. Introduction

Mitochondrial disorders are known to produce a large spectrum of clinical manifestations involving different tissues of the human body (Rötig, 2011). Since first identified in 1988 (Holt *et al.*, 1988; Wallace *et al.*, 1988), the concept of mitochondrial diseases has evolved. It includes not only pathologies related with mutations observed in the mitochondrial DNA (mt-DNA) but also genetic mutations in the nuclear DNA (nu-DNA). These latest may alter proteins from the respirator chain complexes, but also proteins involved in any other mitochondrial functions (DiMauro and Schon, 2008). The study of mitochondrial aminoacyl-tRNA synthetases (mt-aaRS), a group of nuclear encoded proteins in charge of the faithful mitochondrial translation, and their relevance as cause of specific neurological disorders has strikingly increased over the last years (Moulinier *et al.*, 2017). Mutations of mt-aaRS rise in autosomal recessive diseases (Konovalova and Tyynismaa, 2013). However, no evidence has revealed the molecular basis of the tissue specificity of these disorders.

Leukoencephalopathy with brain stem, spinal cord involvement and lactate elevation (LBSL) was the first identified mt-aaRS-related disease in 2007 (Scheper *et al.*, 2007), produced by mutations in the *DARS2* gene, that encodes the mitochondrial aspartyl-tRNA synthetase (mt-AspRS). Over the past ten years, all the genes coding for the 19 mt-aaRS have been associated with diseases that exhibit the central nervous system (CNS) as one of the most predominantly affected system (Sissler *et al.*, 2017). The host laboratory has developed a web server dedicated to disease-associated mutations in human mt-aaRS: Misynpat (<http://misynpat.org>; (Moulinier *et al.*, 2017). This database collects the list of publications that contains clinical and genetic information. In addition, this webserver suggest 3D model structures of the mt-aaRSs, and also proposes the analysis of mutations in a context of conservation and evolution of the sequence (Moulinier *et al.*, 2017).

2. Broad range of clinical presentations

The clinical manifestations present with diseases characterized by defined symptoms and/or neuroradiological features (e.g. LBSL), isolated clinical signs (e.g. non syndromic hearing loss), described syndromes (e.g. Perrault syndrome), among others.

Kolanova et al. did the last clinical literature review of the pathologies related to mutations on mt-aaRSs in 2013 (Konovalova and Tyynismaa, 2013). Since then, the number of cases published has increased steadily (Moulinier *et al.*, 2017). The purpose here is to update the clinical scenario and search for common clinical patterns for each mt-aaRS related disorder.

The main objective here is to categorize the mt-aaRSs according to the affected tissue(s) and/or organ system(s). By doing so, we obtained four main groups (**Table 2**) that are further discussed below: i.- mt-aaRS with mutations affecting exclusively the CNS; ii.- mt-aaRS with mutations affecting the CNS AND an other system; iii.- mt-aaRS with mutations affecting the CNS OR an other system and iv.- mt-aaRS with mutations affecting a system other than the CNS.

2.1. mt-aaRS with mutations affecting exclusively the CNS

This part is further divided into two subgroups containing mt-aaRS characterized by epileptic encephalopathies and leukoencephalopathies. In the first subgroup, the common clinical factor is epilepsy. While for the second subgroup, changes in the white matter are the main hallmark in the diagnosis of the disease.

2.1.1. Epileptic Encephalopathy

CARS2

CARS2, encoding the mitochondrial cysteinyl-tRNA synthetase (mt-CysRS), is related to a severe mitochondrial epileptic encephalopathy. Patients harboring mutations in the mt-CysRS manifest severe myoclonus epilepsy; progressive cognitive decline and magnetic resonance imaging (MRI) features show brain atrophy (Coughlin *et al.*, 2015; Hallmann *et al.*, 2014). The age of onset of symptoms was in childhood (9

and 5 years) for the siblings harboring homozygous mutations, whilst the proband with heterozygous mutations presented a neonatal onset (Coughlin *et al.*, 2015; Hallmann *et al.*, 2014). Mitochondrial respiratory chain (MRC) analyses were done in one of the probands and revealed combined deficiency of the activities of complexes I, III and IV in liver and of complexes I and IV in muscle samples. Nevertheless the authors suggested that the lack of statistical significance for these complexes deficiencies is due to a compensatory amplification of mtDNA as a result of mitochondrial dysfunction (Coughlin *et al.*, 2015).

FARS2

Patients with mutations in *FARS2*, the gene encoding for the mitochondrial phenylalanyl-tRNA synthetase (mt-PheRS), present phenotypes that range from spastic paraplegia (Yang *et al.*, 2016) to fatal infantile Alpers encephalopathy (Elo *et al.*, 2012; Walker *et al.*, 2016). Early onset epileptic encephalopathy represents the main disorder reported in the literature (Cho *et al.*, 2017). The MRI in all patients showed progressive brain atrophy. Most of the cases presented with early seizures during the neonatal period, one exception was an infantile onset at 8 years old (Walker *et al.*, 2016). Regarding the type of seizure, myoclonic seizure is the most listed one. Developmental regression was noticed in addition to progressive brain atrophy after seizure onset. Noteworthy, except for two patients, the seizures disappeared after childhood (Cho *et al.*, 2017).

NARS2

NARS2 encodes for the mitochondrial asparaginyl-tRNA synthetase (mt-AsnRS). Mutations in *NARS2* have been correlated with a broad range of clinical phenotypes, varying from non-syndromic hearing loss (NSHL), Leigh syndrome, Alpers' syndrome to intellectual disability with epilepsy and severe myopathy (Mizuguchi *et al.*, 2017). The patients classified with Alpers' syndrome by Safou *et al.* showed MRI with diffuse degeneration of cerebral gray matter, which is characteristic of Alpers' syndrome (Sofou *et al.*, 2015). In 2015, Vanlander *et al.* described two siblings with a phenotype characterized by proximal myopathy, excessive fatigability, and ptosis for one sibling and mild intellectual disability and epilepsy for the other. MRC studies revealed combined deficiencies in complexes IV and I in muscle. Non-syndromic hearing loss was reported solely in one patient with the homozygous

mutation p.V213F (Simon *et al.*, 2015). Curiously, the cases reported with Alpers' syndrome were also homozygous, but with a mutation in the next amino acid (p.P214L) (Sofou *et al.*, 2015). Two brothers reported with Leigh syndrome, characterized by symmetrical lesions in the brain stem and thalamus, had similar clinical features including congenital auditory neuropathy followed by myoclonus, intractable seizures and early death (Simon *et al.*, 2015). We observed that despite the variations in the clinical expression of genetic defects in *NARS2*, a common pattern of developmental delay/regression and epilepsy is present in most of the cases.

PARS2

PARS2, encoding for the mitochondrial prolyl-tRNA synthetase (mt-ProRS), was first described in one patient with fatal Alpers' syndrome (Sofou *et al.*, 2015). The reported case presented with infantile onset focal seizures followed by generalization and signs of psychomotor regression. A brain computed tomography (CT) revealed cerebral cortical atrophy. Biochemical studies of the RC enzyme activities in muscle biopsies revealed decreases in complexes I and IV (Sofou *et al.*, 2015). Other mutations in *PARS2* gene were published in 2017, and the authors described similar neurological features including epilepsy, developmental delay as those previously reported, however fatality was not brought up (Theisen *et al.*, 2017).

RARS2

RARS2, encoding the mitochondrial arginyl-tRNA synthetase (mt-ArgRS), was found to be a disease gene for pontocerebellar hypoplasia type 6 (PCH6) (Edvardson *et al.*, 2007). The characteristic clinical features include severe epileptic encephalopathy with intractable seizures, neonatal lactic acidosis, feeding problems, and deep developmental delay (Cassandrini *et al.*, 2013). MRI revealed severe abnormalities including cerebellar hypoplasia and progressive pontocerebellar atrophy (Lühl *et al.*, 2016). Some patients, however, were presented without the neuroradiologic typical pontocerebellar hypoplasia, thus expanding the phenotypic spectrum of PCH6 (Lühl *et al.*, 2016; Nishri *et al.*, 2016). A classic neuroimaging sign in older patients is progressive cerebral and cerebellar atrophy that derives in striking microcephaly (Namavar *et al.*, 2011). The effects in the activities of the complexes of the respiratory chain are variable, indicating that an absence of impact in MRC activity could not rule out the diagnosis of PCH6 (Zhang *et al.*, 2018).

TARS2

TARS2, encoding the mitochondrial threonyl-tRNA synthetase (mt-ThrRS), was reported in 2014 by Diodato *et al.* in two siblings with fatal mitochondrial encephalomyopathy, presenting axial hypotonia and limb hypertonia, psychomotor delay, and high levels of blood lactate. Both children died a few months after birth. The brain MRI showed lesions involving the corpus callosum and the globi pallidi. Biochemical analysis showed defects of the MRC complex activities in muscle homogenate, and no defect was observed in fibroblasts (Diodato *et al.*, 2014b). We noted that *TARS2* is one of the systems where fewer patients have been reported; one can assume that lethality of mutations in *TARS2* might affect a normal fetal development avoiding a full term pregnancy.

2.1.2. Leukoencephalopathies

DARS2

DARS2, encoding for the mitochondrial aspartyl-tRNA synthetase (mt-AspRS), was the first gene reported to produce a mt-aaRS-related disease (Scheper *et al.*, 2007). Mutations in *DARS2* produce a Leukoencephalopathy with brainstem, spinal cord involvement and lactate elevation (LBSL). Patients diagnosed with LBSL present a large spectrum of phenotypic manifestations including cerebellar ataxia, spasticity, seizures, variable cognitive impairments, and in some cases requirement of wheelchair assistance (van der Knaap and Salomons, 2015). Typical MRI features revealed changes in the cerebral subcortical, periventricular and deep white matter, posterior limbs of internal capsules, centrum semiovale, medulla oblongata, deep cerebellar white matter and variable involvement of the sensory and pyramidal tracts in addition to the brainstem and cerebellar connections (Uluc *et al.*, 2008). Infantile onset was the most common form of presentation, however some patients started their first symptoms in adulthood (van Berge *et al.*, 2014). Severity of LBSL varies from slow and mild neurodegeneration to severe fatal disease; intriguingly three subjects harboring *DARS2* mutations were described asymptomatic (van Berge *et al.*, 2014). However, in some cases of LBSL the symptoms debuted after an infectious or traumatic process. Thus, asymptomatic subjects should be under medical supervision, in the hypothesis that the exposure to a stressful (eventual) process leads to the onset of the disease.

EARS2

Mutations in *EARS2*, encoding the mitochondrial glutamyl-tRNA synthetase (mt-GluRS), have been described in patients with early-onset leukoencephalopathy with thalamus and brainstem involvement and high lactate elevation (LTBL) (Steenweg *et al.*, 2012). LTBL appears during the neonatal period with diverse neurological manifestations including seizures, spasticity, psychomotor regression, and irritability, among others (Talim *et al.*, 2013). The phenotype heterogeneity ranges from fatal outcome concomitant to a severe neurometabolic disorder, with neonatal or very early onset presentation of LTBL (Oliveira *et al.*, 2017). A distinctive brain MRI pattern shows characteristic neuroradiological features with symmetrical abnormalities of cerebral white matter, and symmetrical signal changes in the thalami, midbrain, pons, medulla oblongata and cerebellar white matter (Sahin *et al.*, 2016). High blood lactate and MRC activities defect in complexes I and IV were observed (Talim *et al.*, 2013). In some cases, attenuation of the signs was described. The authors suggested that recovery depends on the severity of brain damage caused by the first episode (Steenweg *et al.*, 2012).

MARS2

Mutations in *MARS2*, encoding the mitochondrial methionyl-tRNA synthetase (mt-MetRS), were first reported to produce the ARSAL syndrome (autosomal recessive spastic ataxia with leukoencephalopathy) (Bayat *et al.*, 2012). Later in 2015, Webb *et al.* reported two additional patients with developmental delay, growth failure, and sensorineural hearing loss, thus, expanding the phenotypic presentation correlated with mutations in *MARS2* (Webb *et al.*, 2015). Enzymatic studies reported deficiencies in complex I in lymphoblastoid cells from patients with ARSAL syndrome and in complex IV in patients with developmental delay (Webb *et al.*, 2015). MRI findings for all patients included cerebellar and cortical atrophy, together with white matter abnormalities (Bayat *et al.*, 2012; Webb *et al.*, 2015).

WARS2

WARS2, encoding the mitochondrial tryptophanyl-tRNA synthetase (mt-TrpRS), was the last enzyme to be correlated with a disorder (Musante *et al.*, 2017). Intellectual disability is a prevalent clinical sign (Burke *et al.*, 2018; Musante *et al.*, 2017; Theisen

et al., 2017). Additional symptoms include athetosis, speech impairment, muscular weakness and ataxia (Musante *et al.*, 2017). MRI studies were done in one patient and revealed abnormalities in the white matter (myelination delayed) and diffused cerebral atrophy (Theisen *et al.*, 2017). Defects in OXPHOS complexes were fluctuating: the activity was relatively unaffected in one patient (Burke *et al.*, 2018), but strongly decreased in complexes I, III and IV found in fibroblast from another proband (Theisen *et al.*, 2017).

2.2. mt-aaRS with mutations affecting the CNS AND an other system

AARS2

AARS2, encoding the mitochondrial alanyl-tRNA synthetases (mt-AlaRS), was found to be mutated in patients with a leukodystrophy characterized by cognitive impairment and motor dysfunction (ataxia, spasticity). The onset of the clinical features appeared in patients during the third decade of life (Götz *et al.*, 2011; Lakshmanan *et al.*, 2017). One important remark is that ovarian failure was described in all known females with leukoencephalopathy caused by *AARS2* mutations (Lakshmanan *et al.*, 2017). Neuroradiological features showed an abnormal diffuse white matter signal affecting the periventricular, tend and corpus callosum with pyramidal tract involvement, typically in the posterior limb of the internal capsule and brainstem (Lakshmanan *et al.*, 2017). Severe defect was observed in the cytochrome c oxidase (Complex IV from the respiratory chain) from muscle homogenate, while activities of respiratory chain complexes were normal in fibroblasts from patients (Dallabona *et al.*, 2014). Mutations in *AARS2* were also correlated with fatal cardiomyopathy (see below). However, the mutated positions in patients with cardiomyopathy are different from those in patients with leukodystrophy (Euro *et al.*, 2015).

HARS2

HARS2, encoding the mitochondrial histidyl-tRNA synthetase (mt-HisRS), was reported as one of the genes producing Perrault syndrome, a rare autosomal recessive disorder characterized by sensorineural hearing loss and ovarian dysgenesis in females (Pierce *et al.*, 2011). Clinical manifestations are slightly variable depending on the age of onset and on the severity. These manifestations include amenorrhea during the third

decade and neurological features including developmental delay or intellectual disability, cerebellar ataxia, and motor and sensory peripheral neuropathy. Solely two patients were reported without neurological signs (Lerat *et al.*, 2016). So far, all males harboring mutations in *HARS2* were fertile (Lieber *et al.*, 2014).

LARS2

Perrault syndrome was also correlated to mutations in *LARS2*, encoding the mitochondrial leucyl-tRNA synthetase (mt-LeuRS)(Pierce *et al.*, 2013). All reported patients display amenorrhea, severe/progressive sensorineural hearing loss with different ages of onset and no concomitant neurological symptoms (Solda *et al.*, 2016). One isolated case of fatal multisystem failure was reported (Riley *et al.*, 2016), expanding the phenotype of *LARS2* mutations.

IARS2

Mutations in *IARS2*, encoding mitochondrial isoleucyl-tRNA synthetase (mt-IleRS), were correlated with a disorder manifesting with cataracts, short-stature secondary to growth hormone deficiency, sensorineural hearing deficit, peripheral sensory neuropathy, and skeletal dysplasia (CAGSSS) (Schwartzentruber *et al.*, 2014). The MRI in one proband showed an atrophied pituitary adenohypophysis and a small neurohypophysis, according with growth hormone deficiency. No cognitive deficit was present (Schwartzentruber *et al.*, 2014). Moreover, compound heterozygous mutations in *IARS2* were identified in a single patient with a form of Leigh syndrome (Schwartzentruber *et al.*, 2014). One last case was described in a child who presented with bilateral hip dislocations, short stature and hypotonia at birth, and developed additional CAGSSS-related symptoms (Moosa *et al.*, 2017).

VARs2

Mutations in *VARs2*, encoding the mitochondrial valyl-tRNA synthetase (mt ValRS), provoke a severe encephalo-cardiomyopathy (Baertling *et al.*, 2017; Bruni *et al.*, 2018; Diodato *et al.*, 2014b). Seventeen patients have been described with a phenotype mainly characterized by severe early onset hypotonia, stridor and respiratory failure (Bruni *et al.*, 2018). Lethality of this disorder was significantly present for the reported patients. Hypertrophic cardiomyopathy is a common additional clinical sign present in this disorder. Involvement of other organs was reported in five patients,

presenting anuric kidney, pancreatitis and hepatosplenomegaly. Nonetheless, the authors presume of the use of some antiepileptic drugs were determinant in affecting those other organs (Bruni *et al.*, 2018). MRI features were non-specific, displaying variable cerebral and cerebellar atrophies, white matter injuries and basal ganglia involvement. None of the biochemical profiles presented a unique pattern, showing defects of complexes IV and I in muscle biopsies, and were almost always normal in patient's fibroblasts (Bruni *et al.*, 2018; Diodato *et al.*, 2014b).

2.3. mt-aaRS with mutations affecting the CNS OR an other system

SARS2

SARS2, encoding the mitochondrial seryl-tRNA synthetase (mt-SerRS), was first reported to be a disease gene for the HUPRA syndrome (hyperuricemia, pulmonary hypertension, renal failure in infancy, and alkalosis) (Belostotsky *et al.*, 2011). Clinical manifestations include early progressive renal failure with hyperuricemia, pulmonary hypertension, failure to thrive, anemia and metabolic hypochloric alkalosis (Rivera *et al.*, 2013). Enzymatic analysis of respiratory chain complexes showed mild deficiencies of complexes I, III and IV. So far, all patients diagnosed with HUPRA syndrome carried autosomal homozygous missense mutations (Belostotsky *et al.*, 2011; Rivera *et al.*, 2013). In 2016, Linnankivi *et al.* described one patient with a homozygous splicing defect in *SARS2*, presenting with progressive spastic paresis instead of HUPRA syndrome, thus expanding the spectrum of phenotypes of mutations in the *SARS2* gene (Linnankivi *et al.*, 2016). MRI features showed atrophy of cerebellar hemispheres and vermis, as well as thalamic signal changes. Kidneys were normal, and there was no hematuria or proteinuria (Linnankivi *et al.*, 2016).

2.4. mt-aaRS with mutations affecting other system than the CNS

AARS2

Severe forms of fatal cardiomyopathy have been associated with mutations in *AARS2* (Götz *et al.*, 2011; Mazurova *et al.*, 2017). The clinical hallmark was a hypertrophic cardiomyopathy and death occurred during the 1st year of life (Mazurova

et al., 2017). No signal of cardiac involvement was observed in foetal ultrasound, except in one case that led to intrauterine death (Mazurova *et al.*, 2017). MRC enzymatic activities reveal combined deficiencies in complexes I and IV (Götz *et al.*, 2011; Mazurova *et al.*, 2017). Interestingly, all the patients with the cardiomyopathy carried the mutation c.1774C>A (p.R592W) at least in one allele (Taylor *et al.*, 2014); some authors hypothesized that the tissue-specific phenotype of the cardiomyopathy could be in relation with variable concentrations of amino acids in different tissues (Götz *et al.*, 2011).

YARS2

Mutations in *YARS2*, encoding the mitochondrial tyrosyl-tRNA synthetase (mt-TyrRS), have been identified as a cause of the tissue specific mitochondrial respiratory chain disorder: Myopathy, Lactic Acidosis, Sideroblastic Anaemia 2 (MLASA2) (Riley *et al.*, 2010). Clinical common features include anemia, lactic acidosis and myopathy (Ardissone *et al.*, 2015). The severity of the clinical course and the age of onset were variable. Indeed, 14/17 of the described patients debuted the clinical symptoms within the first decade of life, only 3/17 patients presented onset in adolescence or adulthood. Sideroblastic anaemia was mostly transfusion dependent (repeated blood transfusions are the only effective therapy). Nevertheless, the three cases with later age of onset manifested spontaneous recovery (Sommerville *et al.*, 2017). A highlight is the absence of neurological manifestations in patients with mutations in *YARS2*. Biochemical measurements in muscle biopsies showed a combination of deficiencies of complexes I, III and IV (Sommerville *et al.*, 2017). mt-TyrRS is the only case for which mutations reported so far are homozygous. Except for one described heterozygous case, in which the G191D mutation is associated with a nonsense mutation, meaning that only the G191D variation is present in the expressed dimeric protein in this patient. Altogether, it indicates that the pathogenicity of mutated mt-TyrRS exclusively relies on homodimeric mutated forms of the enzyme (Sissler *et al.*, 2017).

2.5. GARS and KARS: two systems apart.

GARS and *KARS*, encoding the glycyl-tRNA synthetase (GlyRS) and the lysyl-tRNA synthetase (LysRS), respectively, are the only genes known in Human to code for both the cytosolic and mitochondrial enzymes (Mudge *et al.*, 1998; Shiba *et al.*,

1994; Tolkunova *et al.*, 2000). Mutations in those genes have been reported in both recessive and dominant inheritance with different clinical presentations. Autosomal dominant mutations in *GARS* and *KARS* lead to an affectation of the peripheral nervous system named Charcot–Marie–Tooth disease type 2 (CMT2) (Seburn *et al.*, 2006). However, recessive mutations in these genes have been reported to produce phenotypes similar to those reported by mutations in mt-aARSs (Taylor *et al.*, 2014; Verrigni *et al.*, 2016).

3. LBSL vs PCH6: clinical contrast of disorders affecting the CNS

A comprehensive analysis of the clinical reports and the accumulation of experimental data over the past years led us to identify at least two contrasting situations, exemplified by the two following disorders. LBSL and PCH6 are disorders that affect both the central nervous system. However, clinical manifestations differ in signs and severity. LBSL leads to a neurodegenerative disease, and PCH6 leads to a neurodevelopmental disorder. LBSL patients usually develop movement problems during childhood or adolescence. However, in some cases, the clinical manifestations do not appear until adulthood. The clinical signs mainly presented by individuals with LBSL are spasticity (muscular muscle rigidity) and ataxia (difficulty with coordinating movements). These conditions use to affect more the legs than the arms. In the most severely affected patients the use of the wheelchair assistance is required (van Berge *et al.*, 2014). Regarding PCH type 6, the patients manifests the symptoms early after birth with, in most of the cases, intractable seizures and recurrent apnea (Edvardson *et al.*, 2007). Other neurological signs include generalized hypotonia, microcephaly (unusually small head size caused by impaired growth of some parts of the brain) lethargic, poor sucking and poor feeding requiring nasogastric feeding. The most heavily affected patients live only into infancy or childhood and they never achieved developmental milestones (Joseph *et al.*, 2014).

Mutations within mt-AspRS are correlated with LBSL (Scheper *et al.*, 2007). Investigations performed so far indicate that mutations have idiosyncratic impacts on molecular, cellular, and/or structural properties of the mt-AspRS (Messmer *et al.*, 2011; Sauter *et al.*, 2015; Scheper *et al.*, 2007; van Berge *et al.*, 2013). The absence of clear and common impact(s) on the canonical function of the enzyme strongly suggests a

neuron-specific non-canonical function/property that needs to be unveiled. This hypothesis is further supported by the observations (by others) that activities of respiratory chain complexes are barely or not affected by mutations (Orcesi *et al.*, 2011), and (by the host laboratory) that impacted residues are conserved only within mammals mt-AspRSs indicating a selective pressure restricted to higher eukaryotes, likely related to the emergence of a sub-phylum specific alternate function (Moulinier *et al.*, 2017; Schwenger *et al.*, 2014a). On the other hand, mutations within mt-ArgRS are correlated with PCH6 (Cassandrini *et al.*, 2013). Contrary to the case of mt-AspRS, some evidences led us to think that mutations within the mt-ArgRS would lead to the pathology through impairments of the aminoacylation function. These evidences are: i) the severity of the clinical presentation; ii) the observation of reduced activities of mt-DNA encoded respiratory chain complexes in some muscle biopsies (Lühl *et al.*, 2016), and iii) the observation that at least one of the two mutations (in allelic composition) impact a residue conserved in phylogeny (Moulinier *et al.*, 2017).

The different phenotypic expressions of the two diseases (severe for PCH6 and mild for LBSL), yet both causing central nervous system injuries, call for a better characterization of the cellular properties of the mt-aaRSs. One might suppose that the effect of the mutations produced on the aminoacylation function of the mt-ArgRS will be more drastic than that shown for the mt-AspRS (Scheper *et al.*, 2007). Thus, the next chapter II and III, will be devoted to the establishment of cellular singularities of the mt-ArgRS, and to the study of the impact of the pathologic mutations on the cellular properties and on the aminoacylation function of this enzyme, respectively.

4. Discussion/Conclusion

Two important outputs emerge from this overview of mitochondrial disorders produced by mutations in the mt-aaRSs. The first output is the categorization of the mt-aaRSs into groups according to the clinical phenotypes observed in patients. From this, each message that can be extracted reinforces the variability of the clinical manifestations. Within this variability, we were able to identify two main patterns of presentation in the CNS: epileptic syndromes and leukodystrophic disorders. Another observation is that only few organ systems are affected at the same time as the CNS. It has been suggested that these secondary symptoms are probably the result of a primary defect in the CNS (Sissler *et al.*, 2017). Regarding the correlation between the

mutations and ATP production, none of the subgroups showed an established pattern of affectation of the MRC. Several studies of MRC enzymatic activities in samples issued from affected patients showed variable (or any) effects in the OXPHOS function. However, it should be noted that most of these mitochondrial enzymatic studies have been performed on fibroblasts, which are cells from tissues not affected by diseases linked to mutations on the mt-aaRSs. Tissue-specific variability in the abundance of different components of the mitochondrial translation machinery and tissue-specific differences in the dependence of energy may play a crucial role on the phenotype of these disorders (**Figure 9**). Regarding the age of the manifestation of the clinical signs in the categorized groups, even though the predominance was early or infantile, exceptions occasionally appeared with patients with symptoms that appear later in life. From now on, taking into account this classification, the future cases reported can be assigned to a respective group.

The questions that emerge from here are whether mt-aaRSs belonging to the different subgroups have different cell properties? Or whether it is too early now, and that the emergence of further patients might modify the categorization proposed here?

The second output is the heterogeneity of the genotype-phenotype shown within the four groups associated to mutations in mt-aaRSs. This point is represented by the fact that most of the time the clinical manifestations correlated with mutations in mt-aaRSs rarely occur in the same way, neither at the same age nor with the same severity. There are still many things that need to be done to understand the factors that determine why mutations in the same mt-aaRSs manifest with mild symptoms in one patient and with severe or fatal symptoms in others. Further researches on mt-aaRS are essential to find peculiarities of these family of enzymes that could reveal some clues to the understanding of the molecular mechanism linked to these rare diseases. For this purpose, the next chapter will be devoted to extending the knowledge of the mt-ArgRS through cell models, trying to shed some light on the singularities of this enzyme. In the future, the generation of appropriate animal models and the compilation of clinical information from upcoming patients are a good beginning for the enlightenment of the relationship between genes and clinical presentation of these pathologies.

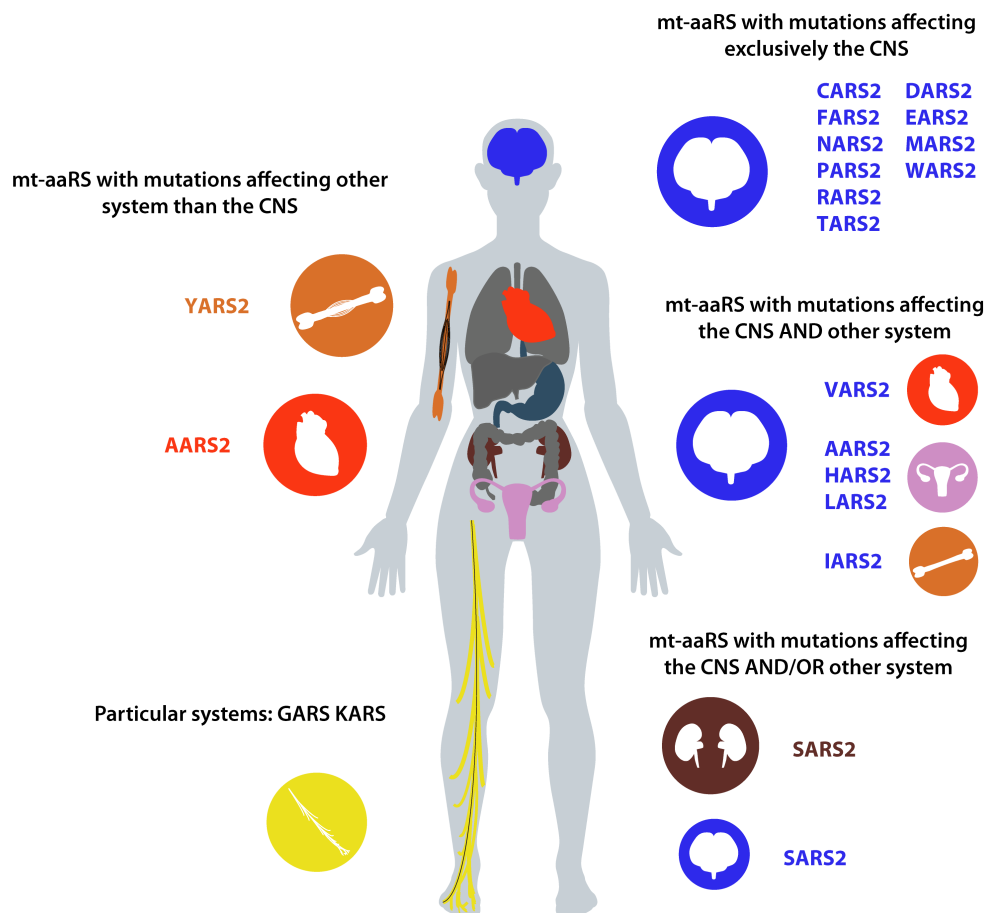


Figure 9. Tissue-specificity of mitochondrial disorders correlated with mutations on human mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs). This representation shows the total set of mt-aaRSs classified in four main groups: mt-aaRSs producing injuries exclusively in the central nervous system (CNS), mt-aaRSs affecting the CNS and another organ, mt-aaRSs affecting alternatively the CNS or other organ, and mt-aaRSs affecting other organ system than the CNS.

GENE	PROTEIN	MAIN PHENOTYPE	MAIN AFFECTED ORGAN	AGE ON ONSET
mt-aaRS with mutations affecting exclusively the CNS				
Encephalopathies				
CARS2	mt-CysRS	Mitochondrial epileptic encephalopathy		Infancy-Childhood
FARS2	mt-PheRS	Alpers encephalopathy		Infancy
NARS2	mt-AsnRS	Alpers' syndrome Leigh syndrome Non-syndromic hearing loss (NSHL),	Brain	Infancy-Childhood
PARS2	mt-ProRS	Alpers' syndrome		Infancy
RARS2	mt-ArgRS	Pontocerebellar hypoplasia type 6 (PCH6)		Infancy
TARS2	mt-ThrRS	Fatal mitochondrial encephalomyopathy		Infancy
Leukoencephalopathies				
DARS2	mt-AspRS	Leukoencephalopathy with brainstem, spinal cord involvement and lactate elevation (LBSL)		Childhood-Adulthood
EARS2	mt-GluRS	Leukoencephalopathy with thalamus and brainstem involvement and high lactate elevation (LTBL)		Infancy
MARS2	mt-MetRS	Autosomal recessive spastic ataxia with leukoencephalopathy (ARSAL syndrome)		Childhood-Adulthood
WARS2	mt-TrpRS	Intellectual disability		Infancy-Childhood
mt-aaRS with mutations affecting the CNS AND other system				
VARS2	mt-ValRS	Fatal mitochondrial encephalocardiomyopathy		Infancy
AARS2	mt-AlaRS	Leukoencephalopathy with ovarian failure	Brain AND other	Childhood-Adulthood
HARS2	mt-HisRS	Perrault syndrome		Childhood-Adulthood
LARS2	mt-LeuRS	Perrault syndrome		Childhood-Adulthood
IARS2	mt-IleRS	CAGSSS Leigh syndrome		Infancy-Childhood
mt-aaRS with mutations affecting the CNS OR other system				
SARS2	mt-SerRS	Hyperuricemia, Pulmonary hypertension, Renal failure in infancy, and Alkalosis (HUPRA)	Kidney	Infancy
mt-aaRS with mutations affecting other system than the CNS				
AARS2	mt-AlaRS	Hypertrophic cardiomyopathy	Heart	Infancy
YARS2	mt-TyrRS	Myopathy, Lactic Acidosis, Sideroblastic Anaemia 2 (MLASA2)	Muscle/Bone narrow	Childhood-Adulthood

Table 2. Classification of the pathologies produced by mutations on human mitochondrial aminoacyl-tRNA synthetases.

Chapter II Deciphering (some) cellular properties of the mt-ArgRS

1. Introduction

The import of nuclear-encoded proteins is required for the correct functioning of the mitochondria. Once inside the mitochondria, the proteins follow the fluid-mosaic model proposed by Singer and Nicolson in 1972 (Singer and Nicolson). According to this model the proteins can be located either on the outer membrane, inner membrane, intermembrane space or on the matrix. The proteins found in the membrane can be embedded into the membrane (integral proteins) or loosely attached to the membrane (peripheral proteins). The import process is achieved for many of the mitochondrial proteins thanks to the presence of an N-terminal sequence named mitochondrial targeting sequence (MTS). The maturation process of the MTS is poorly defined, but is essential among others, for the proper folding and functioning of the imported protein into the mitochondria (e.g. (Chacinska *et al.*, 2009)). Indeed, as recalled in the introduction, the relevance of the process of mitochondrial precursors by a sorting signal in the form of an N-terminal extension to direct the precursor across the outer and inner membranes into the mitochondria has been extensively described (reviewed in e.g. (Mossmann *et al.*, 2012)).

In addition to this essential role for the mitochondria biogenesis, it has been shown at several instances that the N-terminal sequence of a protein may have a significant role on its expression on bacterial strains. For instance, the host laboratory has established the impact of selected N-termini on the expression, the solubility, the stability and the activity of the recombinant human mt-AspRS, expressed in a bacterial strain (Gaudry *et al.*, 2012). As another example, it has been shown in the case of the mt-LeuRS that the N-terminal of the protein has an important influence on aminoacylation and that its activity is lower when a part of the sorting mitochondrial signal remains upstream of its sequence (Yao *et al.*, 2003).

Previous studies from the host laboratory have revealed specific cellular characteristics/peculiarities for the human mt-AspRS, such as: the coexistence of two forms of the enzyme inside the mitochondria, produced by alternative cleavages of the MTS after importation (Carapito *et al.*, 2017), and the establishment of the double localization of the mt-AspRS inside mitochondria, present both in the membrane fraction and in the soluble fraction. Considering these above-recalled previous works, the aim of the present chapter is to establish

some of the cellular characteristics of the mt-ArgRS, making use of the tools and the experimental strategies previously developed for the mt-AspRS. We thus have 1) determined the *in cellulo* N-termini of the mt-ArgRS and searched for the most soluble recombinant version of the protein for optimal expression in a bacterial strain, 2) established the intra-mitochondrial location of the mt-ArgRS, and 3) identified its mode of membrane anchoring. In what follows, the results are compared and discussed with those previously obtained for the mt-AspRS.

2. N-Termini determination of the human mt-ArgRS

Mitochondrial targeting sequence of mt-ArgRS is predicted *in silico* to be cleaved between residues Val₁₆ and Leu₁₇ by the webserver MitoProt (Claros and Vincens, 1996). However, Agnes Gaudry (Assistant Engineer in the host laboratory) carried out a series of experiments of *in vitro* expression of the mt-ArgRS using a recombinant protein starting at this N-terminal Leu₁₇, without any success. Although sometimes the use of *in silico* prediction is useful, it appeared not to be effective in the present case.

The identification of the cleavage site on the N-terminal MTS of the mt-ArgRS remains a challenging task because of the absence of consensus sequences for cleavage sites and the possibility of multiple proteolytic steps for a single precursor protein (Carapito *et al.*, 2017). Protein sequencing by Edman degradation is a first strategy to identify the N-terminus of a protein (Edman, 1949). This technique, however, requires important quantities of purified protein and may not work if the N-terminus has been chemically modified (Edman, 1949). Facing these difficulties, several strategies have been developed to determine the N-termini of mitochondrial proteins. Within these strategies, one can mention an adapted version of the classical shotgun proteomics. The classical LC-MS/MS analysis identifies fully-tryptic peptides, properly cleaved on both ends by trypsin. In the present case of matured mitochondrial protein, the N-terminal peptide may be cleaved only on C-terminal end by trypsin (semi-tryptic peptides). The identification of such semi-tryptic peptides thus required the elaboration of a dedicated database that contains all theoretically calculated possible N-terminal peptides. Theoretical masses of processed N-terminal peptides were then compared to the experimentally detected peptides by mass spectrometry (Carapito *et al.*, 2017). However, when the maturation of the precursor protein occurs after a lysine or an arginine, the N-terminal peptide will be detected as a fully-tryptic peptide. In this situation, one cannot distinguish whether this peptide is an internal peptide (properly cleaved on both ends by trypsin) or a newly generated mature N-terminus (cleaved only on C-terminal end by trypsin) (Carapito *et al.*, 2017). For these ambiguous peptides, the Doublet N-terminal Oriented Proteomics (dN-TOP) approach is an

accurate strategy (Vaca Jacome *et al.*, 2015). Briefly, the dN-TOP technique is based on the double labeling of free protein's N-termini using simultaneous chemical labeling with light and heavy trimethoxyphenyl phosphonium (TMPP). It enables the characterization, in a single experiment, of both the free protein N-termini and all other internal peptides (Vaca Jacome *et al.*, 2015). Thus, in collaboration with Christine Carapito (LSMBO/IPCC, Strasbourg), we used the dN-TOP technique to identify N-Termini MTS of mt-ArgRS. This technique was previously applied in the laboratory and allowed to determine the presence of two forms of the mt-AspRS within the mitochondria, produced by alternative cuts of MTS after importation (Carapito *et al.*, 2017).

2.1. Chemical N-Terminal labelling prior to mass spectrometry analysis reveal four possible processed forms of mt-ArgRS

The preparation of sample provided to Carapito's team for the dN-TOP method was as follows. Mitochondria (~500 mg) were enriched from HEK293T cells (~ 8.6×10^8 cells from ten cell dishes of 150 mm) transfected with the pCI plasmid expressing the WT mt-ArgRS protein with a C-terminal His₆-Tag. Over expression of the mt-ArgRS was performed so that to increase its abundance over the other mitochondrial proteins. Then, protein enrichment was done with resin Ni-NTA Agarose in a NaH₂PO₄/Na₂HPO₄ pH 7.5 buffer. The dN-TOP strategy was applied on this enriched sample of mt-ArgRS, following the published experimental procedure (Vaca Jacome *et al.*, 2015). Briefly, the samples were separated on a SDS-PAGE, the bands corresponding to the expected size were cut off and the contained proteins were in-gel digested with trypsin. The resulting peptides were then subjected to MS analysis. Validation of detected TMPP peptides requires very strict criteria to be met: the light and heavy labeled forms of the potential N-terminal peptide must be identified using database searches, both peptides must to be perfectly coeluted and the peptide sequence must be unique in the searched database (**Figure 10**, adapted from (Vaca Jacome *et al.*, 2015)). An important advantage of this method is that internal peptides are not removed, thus, contributing to the identification of proteins (Vaca Jacome *et al.*, 2015). This strategy allowed the identification of four N-termini for the mt-ArgRS at positions Asn₁₈, Ser₂₇, Leu₇₂, Ile₁₂₇ (**Figure 11**).

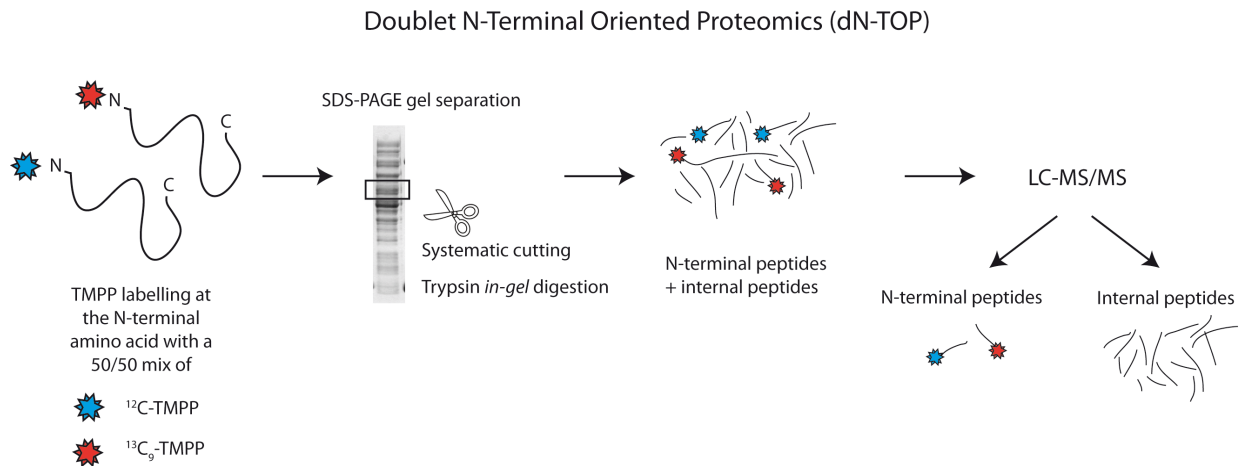


Figure 10. Schematic representation of the principle of the Doublet N-terminal Oriented Proteomics (dN-TOP) strategy. TMPP stands for trimethoxyphenyl phosphonium (Figure adapted from (Vaca Jacome *et al.*, 2015))

Several studies have been devoted to the understanding of the complex process carried out for the import and cleavage of proteins into the mitochondria (Chacinska *et al.*, 2009; Mossmann *et al.*, 2012; Neupert, 1997). Among the most described peptidases in charged of the maturation of the MTS are: the mitochondrial processing peptidase (MPP), mitochondrial intermediate peptidase (MIP), the inner membrane peptidase (IMP). For the MPP the cleavage site motif has been analysed in several precursor proteins of different species and was previously grouped into four different classes according to the position of an arginine relative to the mature N-terminus: \mathbf{R}_{-2} motif ($\mathbf{R}_{-2}\mathbf{X}\downarrow\mathbf{X}$), \mathbf{R}_{-3} motif ($\mathbf{R}_{-3}\mathbf{X}(\mathbf{Y}/\mathbf{F}/\mathbf{L})\downarrow(\mathbf{S}/\mathbf{A}/\mathbf{X})$), \mathbf{R}_{-10} motif ($\mathbf{R}_{-10}\mathbf{X}\downarrow(\mathbf{F}/\mathbf{L}/\mathbf{I})\mathbf{XX}(\mathbf{T}/\mathbf{S}/\mathbf{G})\mathbf{XXXX}\downarrow\mathbf{X}$) and the R-none motifs ($\mathbf{XXX}\downarrow\mathbf{X}(\mathbf{S}/\mathbf{X})$). IMP is constituted by two catalytic subunits Imp1 and Imp2, and non-overlapping substrate specificity has been showed. Whereas Imp1 recognizes hydrophobic residues at position -3 and an asparagine residue at position -1 relative to the cleavage site, the only known substrate of Imp2 has a serine (-3) and alanine (-1) residue at the respective positions. MIP is a soluble monomeric metalloprotease in the mitochondrial matrix. In contrast to IMP, which can cleave presequences independent of MPP, MIP only recognizes and cleaves its substrate after initial processing by MPP. The recent identification of further substrates in yeast allowed the analysis of the cleavage site in more detail and confirmed predominantly arginine in -10 , phenylalanine and leucine in position -8 , and the abundance of serine and threonine which are distributed across positions -5 , -6 and -7 of the presequence (Gordon *et al.*, 2001; Mossmann *et al.*, 2012; Neupert, 1997). However, it has been reported situations with the absence of motifs for the N-Termini cleavage (Gavel and von Heijne, 1990). This last R-none motif has been described in a small group of mitochondrial proteins without assignment of other peptidase, and their processing remaining unexplained (Gakh *et al.*, 2002a).

When analyzing the mt-ArgRS sequence (**Figure 11.B**), we found that only N₁₈ fits with a MPP R₋₃ motif (**R₁₅V₁₆L₁₇↓N₁₈**). For the position S27, if we consider that mutational analysis of the asparagine in position -1 showed that Imp1 tolerates a broad variety of structurally distinct residues at these positions (Chen *et al.*, 1999), we can suppose a cleavage by the Imp 1 corresponding to (**L₂₄I₂₅T₂₆↓S₂₇**). For the other two positions, we can hypothesized that they result from the relatively high degree of amino acid sequence degeneracy at MPP cleavage sites (Gakh *et al.*, 2002a), thus limiting the level of confidence for the prediction.

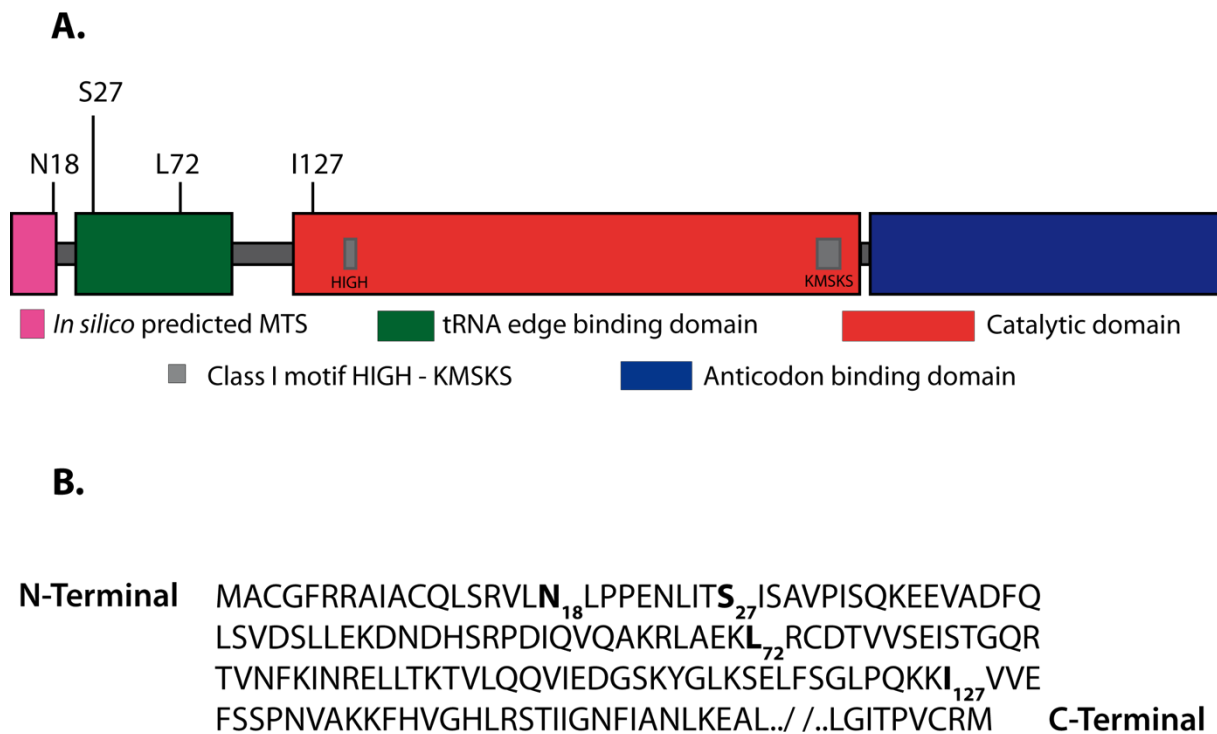


Figure 11. N-Terminal variants of the mt-ArgRS. **A)** Schematic representation of the human mt-ArgRS with the positions of the four possible N-Terminal as identified by dN-TOP experiments. **B)** N-terminal sequence of the human mt-ArgRS where the four possible N-terminal residues are highlighted in bold characters.

The cleavage of the MTS of mitochondrial proteins was described for some of the mt-aaRSs. The dN-TOP approach was applied on total purified mitochondria analyzing in a systematic way all other members of the mt-aaRSs family were generated (Carapito *et al.*, 2017). The results obtained from the dN-TOP method for another mt-aaRS, the alanyl-tRNA synthetase, were also successfully exploited for the production of a recombinant soluble version of this protein (Hilander *et al.*, 2018). Thus, supporting the accuracy of the dN-TOP results determining the mt-aaRSs.

In the case of the mt-AspRS, this same dN-TOP approach identified three chemically modified peptides, revealing three possible N-terminal residues (Leu₃₃, Ile₄₁, Pro₄₂). It has been proposed that the forms starting at Leu₃₃ and Ile₄₁ could be produced either in response to a R-

₁₀ motif (**R**₃₁**S**₃₂↓**L**₃₃**L****Q****S**₃₇**S****Q****R****R**₄₁↓**I**₄₂), cleaved by MPP and MIP between residues Ser₃₂ and Leu₃₃, and residues Arg₄₀ and Ile₄₁, respectively; or in response to two R-2 motifs (**R**₃₁**S**₃₂↓**L**₃₃ and **R**₄₀**R**₄₁↓**I**₄₂) independently cleaved by MPP at the same two positions. For the mt-AspRS Pro₄₂ form, the hypothesis was a subsequent cleavage of the protein starting at Ile₄₁ by the Aminopeptidase P (APP), for which a mitochondrial isoform (APP3 or XPNPEP3) has been described (Carapito *et al.*, 2017). These results were consistent with previous *in vitro* import experiments that revealed different processed forms of mt-AspRS after importation into the mitochondria (Messmer *et al.*, 2011). Corroborating this proposal of the co-existence of two forms of mt-AspRS, we frequently observed the presence of two specific bands with different sizes in western blots on mitochondrial samples. Unlike the mt-AspRS, only one band is observed by western blot for the *in cellulo* detection of the mt-ArgRS.

Concerning the results for N-termini of the mt-ArgRS, even if they represent preliminary data, we decided to continue the experiments and start the expression and solubility test with the four N-Termini possibilities. One could consider the repetition of the dN-TOP method to attempt to increase the accuracy of the preliminary results.

2.2. Cloning, expression and solubility test of different N-termini mt-ArgRS variants

A second important objective is to produce the recombinant mt-ArgRS in bacterial strain so that to investigate its enzymatic properties by *in vitro* aminoacylation assays and eventually to test for the impact of pathology-related mutation on this property (see Chapter 3). Thus, four forms of the mt-ArgRS starting at each of the four potential N-termini identified above were cloned and expressed in *E. coli*. To do this, 5'-primers were designed so that to initiate the protein sequence at each of the identified N-termini (Asn₁₈, Ser₂₇, Leu₇₂ and Ile₁₂₇) and to introduce a start codon coding for methionine indispensable to translation initiation. In addition, 3'-primers were designed so that to introduce a His₁₀-Tag at the C-terminus of the mt-ArgRS. The resulting constructions, amplified from the template sequence (see below) were cloned into a pDEST14 plasmid. To facilitate the nomenclature, resulting recombinant proteins (with N-terminal Met and C-terminal His₁₀-tag) are named mt-ArgRS-N₁₈, mt-ArgRS-S₂₇, mt-ArgRS-L₇₂ and mt-ArgRS-I₁₂₇ (for sake of simplicity, the three-letter code of amino acids has been replaced by the one-letter code).

The template used to produce these constructions was an optimized sequence of the human mitochondrial *RARS2* gene for expression in *E. coli* strain designed by Agnes Gaudry and purchased in Integrated DNA Technologies-IDT®. In this optimization, the codons of the

human sequence of the mt-ArgRS were replaced by synonymous codons with more frequent usage and reading in bacterial strain. To test the impact of these different N-Termini on the expression and solubility of mt-ArgRS, BL21 (DE3) *E. coli* strain were transformed with each of the constructions as described in section **2.6. Material and Methods**. The site Mitoprot predicted Leu₁₇ as the potential N-termini for mt-ArgRs (Claros, 1995). Thus, this construction (mt-ArgRS-L₁₇) was also used in the expression/solubility tests. The IPTG was used to induce protein expression taking advantage of the *lac* operon present in the pDEST plasmid. In a first series of experiment, two conditions were compared: control (no IPTG) versus induction with IPTG. Cell extracts containing the expressed protein variants were disrupted through sonication and then centrifuged so that to separate soluble from insoluble proteins. Total extract (disrupted cells before centrifugation) and soluble extract (supernatant after centrifugation) were loaded and separated in a SDS-PAGE gel. Protein bands of the mt-ArgRS were detected by western blot using anti His-Tag antibody. The size of the bands corresponded each time to the theoretical molecular weights of the different variants: mt-ArgRS-L₁₇ – 65.5kDa, mt-ArgRS-N₁₈ – 65.14kDa, mt-ArgRS-S₂₇ – 64.15kDa, mt-ArgRS-L₇₂ – 59.13kDa, mt-ArgRS-I₁₂₇ – 52.95kDa.

Expression and solubility were estimated taking into account the intensity of the bands by western blot detection. mt-ArgRS-L₁₇ and mt-ArgRS-L₇₂ both showed the best expression and solubility. A subtle difference was shown between the amounts of protein in the two conditions (with and without IPTG), with a tiny increase of the intensity of the band detection in the fraction with IPTG (**Figure 12**). This indicates that the presence of the *lac* operon in the pDEST14 plasmid positively increased the protein expression. Regarding mt-ArgRS-N₁₈ and mt-ArgRS-S₂₇, the amounts of protein produced were remarkably low when compared to mt-ArgRS-L₁₇ and mt-ArgRS-L₇₂. For mt-ArgRS-N₁₈ and mt-ArgRS-S₂₇, a slight increase in quantity was shown when IPTG was used. Concerning mt-ArgRS-I₁₂₇, protein was detected in the same way in the total extract with and without IPTG. However, no bands were detected in the soluble extract, indicating that this variant is lost during the centrifugation step. This could be due to instability of the protein, or to its degradation.

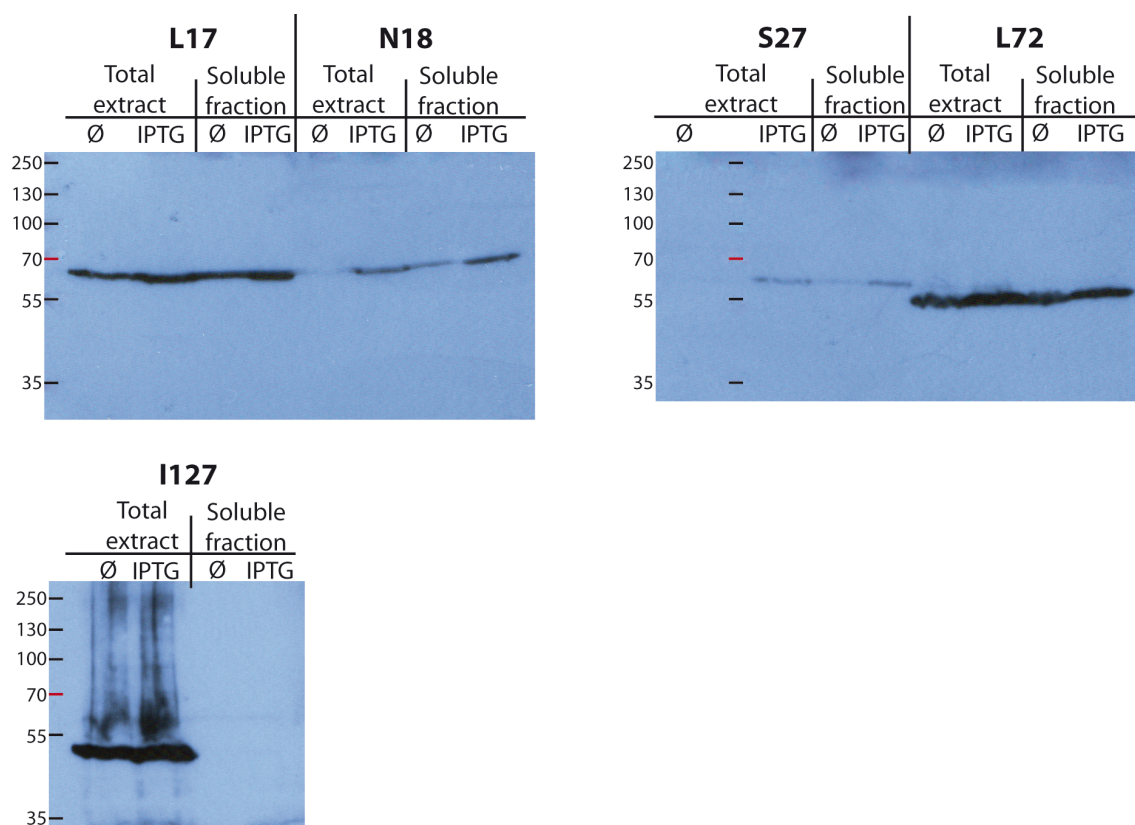


Figure 12. Influence of N-terminal sequence on expression and solubility of recombinant mt-AspRS variants. mt-ArgRS-L₁₇, mt-ArgRS-N₁₈, mt-ArgRS-S₂₇, mt-ArgRS-L₇₂ and mt-ArgRS-I₁₂₇ starting at residues L17, N18, S27, L72 and I127, respectively, were expressed in BL21 DE3. Cell extracts and soluble fractions were loaded and separated onto a 10% SDS-PAGE gel and the mt-ArgRS was detected by Western Blot analysis using anti-His antibody. The expected size of the mt-ArgRS variants is L17~65.5kDa, N18~65.14kDa, S27~64.15kDa, L72~59.13kDa, I127~52.95kDa. Ø: no IPTG was used, IPTG: induction was done with IPTG 250 µM

Based on these results, the variant mt-ArgRS-L₇₂ was chosen for the investigation of the impact of individual mutations on the aminoacylation activity of the mt-ArgRS (see Chapter III), because of its good expression and solubility.

3. Intramitochondrial localization of the human mt-ArgRS

Earlier studies from the laboratory have suggested that mt-aaRSs may have different sub-mitochondrial localizations, some of them getting located in both soluble (matrix or intramembrane space) and membrane associated, and others being exclusively found in one of the two mitochondrial fractions. Nevertheless, those were preliminary results that needed to be validated. Later on, a former PhD student, Loukmane Karim, improved and established a mitochondrial fractionation protocol, validating the data concerning the double localization of the mt-AspRS. He clearly showed that the mt-AspRS is located both in the soluble and the membrane fractions of the mitochondria. In the present work, the objective was focused on the mt-ArgRS. The same mitochondrial fractionation approach was applied, however the results

obtained were different. The article #2 include the results and discussions of this study, as well as the determination of the mode of anchorage of those proteins to the mitochondrial membrane.

Additionally to the results presented in the article, further data concerning the location of mt-ArgRS and mt-AspRS in other cell lines are included and discussed below.

3.1. The mt-ArgRS is located exclusively in the membrane fraction of the mitochondria

Mitochondria from Human Embryonic Kidney cells (HEK293T) were enriched and fractionated into soluble fraction (S in **Figure 13**) containing molecules from the matrix and intermembrane space, and membrane fraction (M in **Figure 13**) containing molecules from the outer and the inner membranes. Different proteins of known intramitochondrial location were previously used by Loukmane Karim to assess the quality of the fractionation process (see details in article #2, Figure 1B). Prohibitin and SOD2 were systematically detected and analyzed in all experiments as controls of the soluble and membrane fractions, respectively.

A typical experiment focused on the mt-ArgRS is shown in **Figure 13**. It reveals the presence of the human mt-ArgRS in solely the membrane fraction. This observation was constant in each mitochondrial fractionation experiment performed with HEK293T cell line. For this experiment, the detection by western blot of mt-AspRS was also routinely performed, and the identification of this protein was each time in both fractions of the mitochondria (soluble and membrane) according to the previous data highlighted by Loukmane Karim.

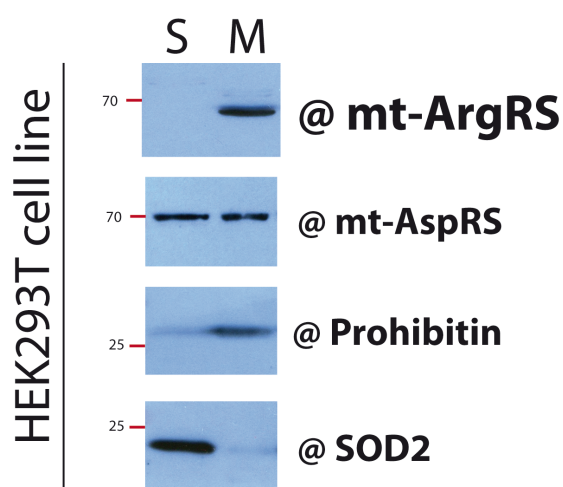


Figure 13. Intramitochondrial localization of mt-ArgRS *in cellulo*. Mitochondria were purified from HEK293T cell line and fractionated into soluble (matrix and intermembrane space) and membrane (outer and inner membranes) fractions. Endogenous mt-ArgRS, mt-AspRS, prohibitin and SOD were detected with specific antibody. S, soluble fraction. M, membrane fraction.

Regarding mt-ArgRS and its exclusive presence in mitochondrial membrane, no trans-membrane domain was predicted based on the sequence of the protein using TMHMM Server, <http://www.cbs.dtu.dk/services/TMHMM-2.0/>. Other aaRSs have been reported to relocate into

a membrane under certain conditions. This relocation occurs in some cases to regulate the translation in a positive or negative way, or to allow the protein to perform another function alternative to translation. Early on, the MetRS was found to be associated with detergent-insoluble components of the rough endoplasmic reticulum in cultured vertebrate cells (Dang *et al.*, 1983). In this case, the mode of membrane association remained unknown, as well as the functional significance of the ER-association for the aaRS. It has been suggested, however, that this localization brought the aaRSs close to ribosomes, which may provide a high local concentration of aaRS for maximal efficiency of protein biosynthesis (Dang *et al.*, 1983). The human cytosolic LysRS, naturally anchored to the cytosolic MSC, was shown to be relocated to the plasma membrane upon laminin-dependent phosphorylation at residue Thr52. Once in the plasma membrane, the LysRS associates with and stabilizes other membrane proteins such as p67LR and integrin $\alpha 6$, resulting in an increased cellular migration and invasion and in a possible implication in the tumorigenesis (Kim *et al.*, 2012). The authors suggest that the recruitment of a key translational component to regulate cell migration is justified by the fact that it may reduce the level of operational translation machinery (Young *et al.*, 2016). A similar postulate of negatively controlled protein synthesis has been proposed for the *E. coli* LeuRS. The enzyme is naturally soluble in the cytoplasm, but sequestered to the cytoplasmic membrane during metabolic stress or growth restriction caused by e.g. low levels of leucine (Williamson, 1993). Conversely, in human, the translocation of LeuRS into the lysosome membrane is induced by leucine addition. In this situation, LeuRS acts as an intracellular leucine sensor and activates the mTOR-signaling pathway, which regulates translation, cell size and autophagy (Choi *et al.*, 2017; Han *et al.*, 2012).

Strikingly, four aaRSs were shown to be anchored to the thylakoid membrane through an additional protein domain named CAAD (for Cyanobacterial Aminoacyl-tRNA synthetases Appended Domain) (Olmedo-Verd *et al.*, 2011). The membrane-anchored ValRS was further shown to directly interact with the ATP-synthase, connecting elements from gene translation and energy production machineries (Santamaria-Gomez *et al.*, 2016). This is the only description, so far, of an aaRS constitutively anchored to the membrane, and not responding to a stimuli, as it is the case for the human mt-ArgRS. This form of constitutive presence in the mitochondrial membrane displayed by the mt-ValRS and the mt-ArgRS, lets us speculate that probably the mt-ArgRS may in some way be involved in some alternative function of the mitochondria as well, other than translation.

3.2. Sub-mitochondrial localization of mt-aaRSs in other cell models

After having validated that mt-ArgRS and mt-AspRS are present at different intramitochondrial locations using HEK293T as a cellular model, we extended the study to other cell lines.

In collaboration with Dr. Etienne LEFAI (CarMeN Laboratory, Lyon-France), mitochondrial fractionation protocol was performed on mitochondria extracted from human myotubes, from HepG2 and from Huh7 cell lines. Human myotubes were isolated from muscle biopsies of patients without comorbidities. Lefai's team performed the isolation and differentiation of the human primary muscle cells following a protocol published by Chanon et al. (Chanon *et al.*, 2017). HepG2 and Huh7 are both immortalized cell lines from hepatocellular carcinoma. Mitochondrial activities were shown to vary in the different tissues. For instance, there is a high-energy demand in terms of ATP in muscles and the metabolic pathways as The Krebs cycle, and fatty acid oxidation are extremely important in the liver (Barclay, 2017; Rui, 2014). It is then of particular interest to investigate the intra-mitochondrial locations of the mt-AspRS and the mt-ArgRS in those tissues. The results, represented in **figure 14**, show that the location pattern of both enzymes is the same as the one established in HEK293 cells: the mt-ArgRS is exclusively located in the membrane fraction, and the mt-AspRS is double localized both in the soluble and the membrane fraction.

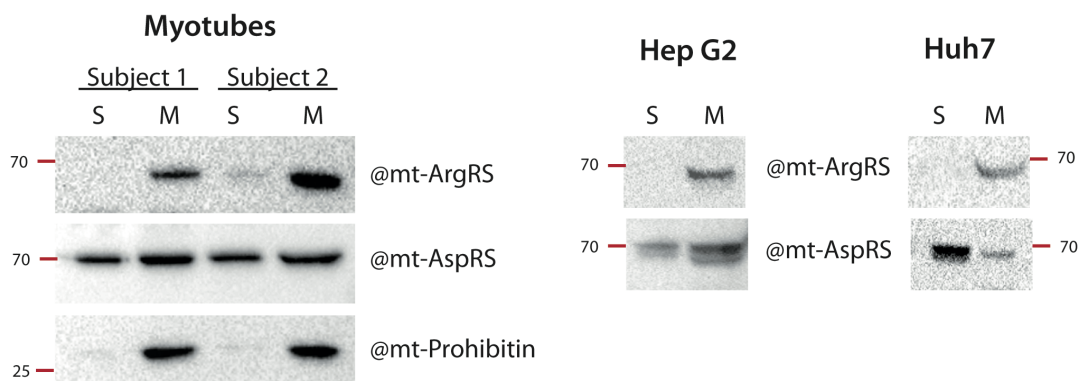


Figure 14. Intramitochondrial localization of mt-ArgRS in selected human cells. Mitochondria were purified from myotubes, Hep G2 and Huh7 cells and fractionated into soluble (matrix and intermembrane space) and membrane (outer and inner membranes) fractions. Endogenous mt-ArgRS, mt-AspRS, prohibitin and SOD2 were detected with specific antibodies. S, soluble fraction. M, membrane fraction.

Since the pathologies correlated with mutations in mt-ArgRS and mt-AspRS lead to disorders in the central nervous system, the next step was to investigate the location of these enzymes in a cellular model that simulate more closely the neurophysiologic environment. In order to do this, human neuroblastome cell line, SH-SY5Y, were used in two stages,

undifferentiated and differentiated. First, fractionation of enriched mitochondria from undifferentiated SH-SY5Y was performed. At this stage, the profile was again: in the membrane fraction for the mt-ArgRS and in both the membrane and soluble fraction for the mt-AspRS (**Figure 15**). Then, Florian Pierre (Engineer in the host laboratory) initiated in the laboratory the *in vivo* differentiation of the SH-SY5Y cells to neurons. This process of differentiation requires optimizations that are still being carried out. It consists of sequential changes of culture medium during defined timelines. These changes include different concentrations of main components in the serum as: the quantity of proteins containing in the serum, the introduction of hormonal and neurotrophic factors, among others (Shipley *et al.*, 2016). This allows the selection of the phenotype of neuronal cells against epithelial cells, resulting in homogeneous populations of neuronal cultures. To validate the neuronal differentiation, an antibody against the microtubule associated protein 2 (Map2 antibody, Sigma-Aldrich®) was used. The preliminary results so far obtained for differentiated SH-SY5Y maintain the same profile, exclusive localization of the mt-ArgRS in the membrane fraction of the mitochondria and in the membrane and soluble fraction for the mt-AspRS (**Figure 15**).

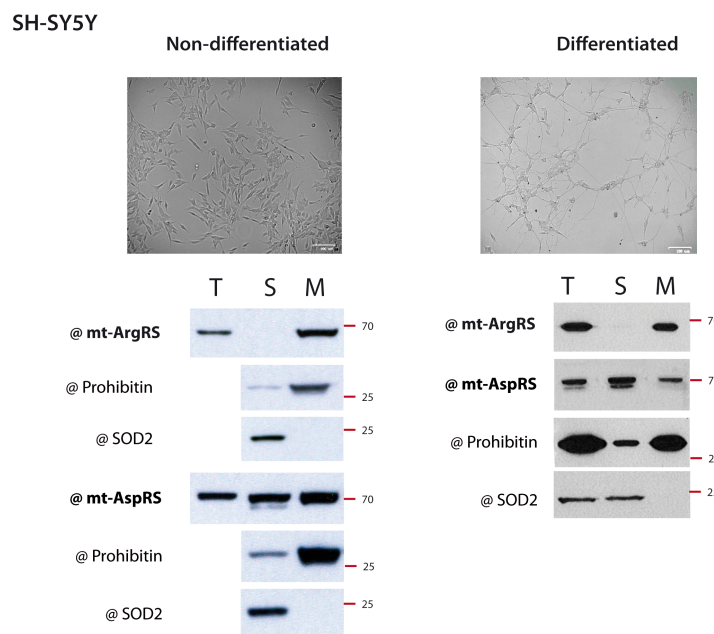


Figure 15. Intramitochondrial localization of mt-ArgRS in undifferentiated and differentiated SH-SY5Y. Mitochondria were purified from SH-SY5Y in two stages: undifferentiated and differentiated into neurons (as shown in the insets). They were fractionated into soluble (matrix and intermembrane space) and membrane (outer and inner membranes) fractions. Endogenous mt-ArgRS, mt-AspRS, prohibitin and SOD2 were detected with specific antibodies. S, soluble fraction. M, membrane fraction.

The different pattern of location of mt-ArgRS and mt-AspRS maintained in both undifferentiated and differentiated conditions. Thus, suggesting that this cellular peculiarity is more ubiquitous than associated with a specific cell type.

4. Diverse types of mt-aaRS interactions with the mitochondrial membrane

Distinct modes of membrane anchorage exist and they are schematically recalled in **figure 16**. A protein can either be ‘integral’ and permanently attached to the membrane *via* a transmembrane domain or hydrophobic region(s); or be ‘peripheral’ and temporarily adhered to the membrane through either electrostatic or ionic interactions (*via* another protein or *via* membrane lipids), disulfide bond interactions, or a covalently-bound lipid anchor.

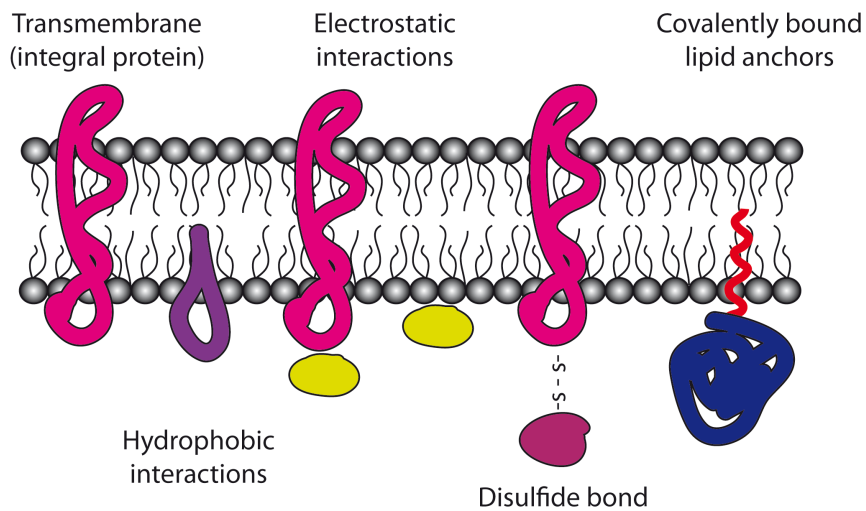


Figure 16 (adapted from Figure 2A of the article #2). Schematic representation of the different modes of protein interaction with membranes.

A selection of disrupting chemical agents reported to produce type-specific release of membrane-bound proteins was used at the moment of sonication of the enriched mitochondria, prior the fractionation process. Those were 8M Urea (Singh and Panda, 2005), 0.5M KCl (Arnoult *et al.*, 2009; Rajala *et al.*, 2014), 0.1M Na₂CO₃ pH11 (Fujiki *et al.*, 1982; Herlan *et al.*, 2003; Kim *et al.*, 2015), 0.4M DTT (Winger *et al.*, 2007), 1M NH₂OH pH 7 or 1M NH₂OH pH 11 (Cockle *et al.*; E.N. *et al.*, 1985; Okubo *et al.*, 1991) Previous work by Loukmane Karim established that human mt-AspRS is a peripheral proteins interacting with membrane *via* electrostatic interactions.

The fact that the mt-ArgRS is located exclusively in the membrane fraction made essential the characterization of the type of anchoring to the membrane. The same protocol was performed to study the mt-ArgRS. As shown in **figure 17.A**, solely urea allowed the completely release of mt-ArgRS from the mitochondria membrane. SOD2 and Prohibitin are systematically detected/analyzed as control experiments from the matrix and the membrane fractions, respectively. While SOD2 remains soluble whatever is the applied chemicals, prohibitin was detected in the soluble fractions upon urea, carbonate pH 11 and hydroxylamine pH 11

treatments. Carbonate treatment (pH 11) was reported to lead to the release of some integral proteins with mode of anchorage weaker than complete transmembrane domain (Fujiki *et al.*, 1982; Herlan *et al.*, 2003; Kim *et al.*, 2015).

We further studied the impact of urea over the membrane location of the mt-ArgRS to determine which concentration of urea was able to release the protein from the membrane fraction (Figure XX.B). In order to do this, different concentrations of urea were used: 1M, 2M, 4M and 8M. The detection of the mt-AspRS was performed to compare the effect of urea on these two enzymes. We can appreciate in **figure 17.B** that the responses of mt-ArgRS and mt-AspRS to urea were different. For the mt-ArgRS, 8M of urea were required to completely release the protein from the membrane fraction, as it has been reported for the solubilization of integral proteins (Devraj *et al.*, 2009). In contrast, lower urea concentrations produce an effect on mt-AspRS location.

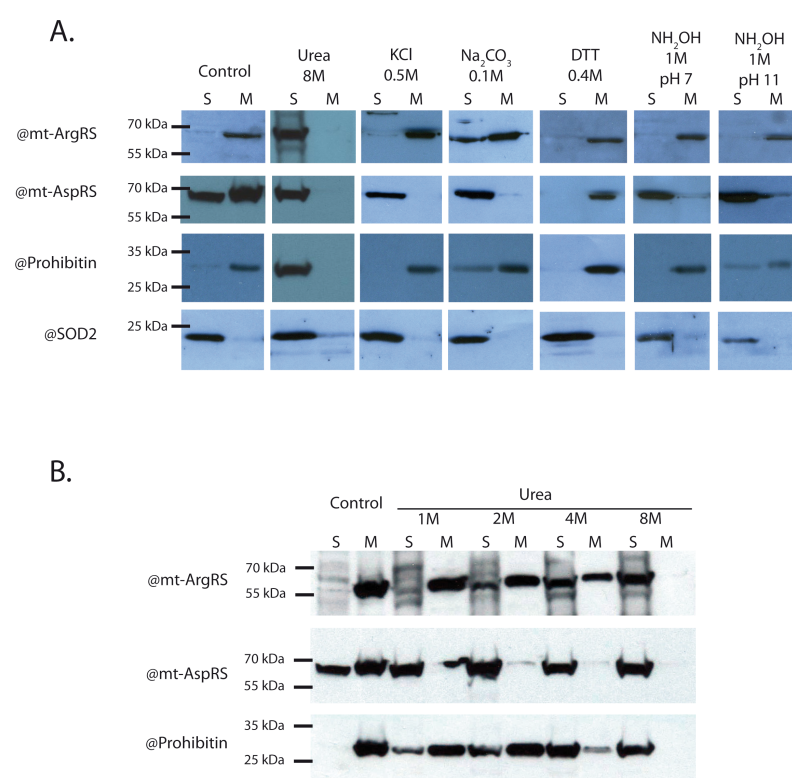


Figure 17. Diverse interaction modes of mt-ArgRS and mt-AspRS with the mitochondrial membrane. The sensitivity of mt-ArgRS and mt-AspRS membrane anchoring to chemical disrupting agents (A.) and to urea concentration (B.) was deciphered by investigating the release of proteins from the membrane to the soluble fractions. For each experiment, mitochondria were enriched from HEK293T cells. Chemical disrupting agents, used at indicated concentrations, were applied during the sonication of the enriched mitochondria, prior to the fractionation. mt-ArgRS, mt-AspRS, prohibitin and SOD2 were detected by Western blot analysis with specific antibodies. S, soluble fraction. M, membrane fraction.

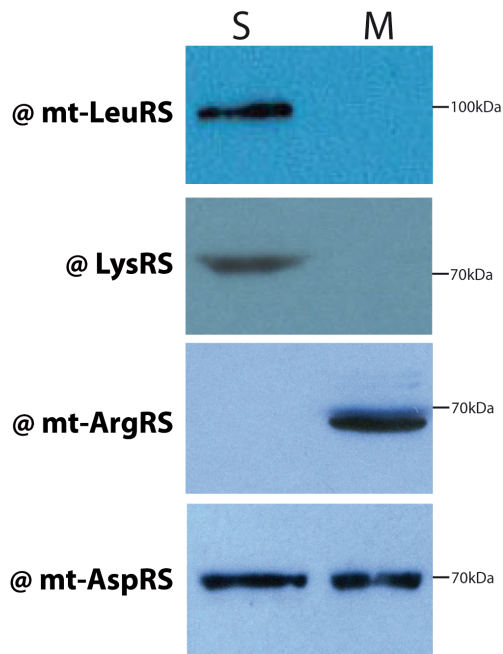
Altogether, our results showed that membrane-anchored fractions of mt-AspRS and mt-ArgRS respond to different chemistry, indicating that the mode of anchorage is distinct: salt sensitive for mt-AspRS, indicative of an electrostatic-mode of anchorage; urea-sensitive for mt-ArgRS, indicative of an hydrophobic mode of anchorage for mt-ArgRS. These results, as well as the methodology applied for the study of the type of interaction between the aaRSs and the mitochondrial membrane are also published and discussed in article #2.

5. Conclusion and perspectives

Mitochondrial aaRSs are synthesized in the cytosol, and subsequently translocated across the mitochondrial membranes. For most of the proteins addressed to the mitochondria, this is performed thanks to the presence of a N-terminal MTS, which has to be cleaved upon entry into the organelle for the release of a properly folded and functional protein. The results we obtained in the present work allow us to enlarge the view of the mt-aaRSs in different topics: the study of the N-Termini for the mt-ArgRS, the search of the best recombinant and soluble form for the bacterial expression of the mt-ArgRS, the intramitochondrial localization of the mt-ArgRS and its mode of anchorage to the mitochondrial membrane.

Regarding the study of the N-termini, the objective of finding a recombinant and soluble version of the mt-ArgRS was attained. When comparing the expression and the solubility of the four tested recombinant variants of the mt-ArgRS, mt-Arg-L₇₂ appeared to be the best one. It will allow the investigation of the impact of selected PCH6-mutations on the aminoacylation function of the mt-ArgRS (See Chapter III).

Former PhD students from the host laboratory initiated the investigation of the intra-mitochondrial localization of some mt-aaRSs. Loukmane Karim demonstrated that the mt-AspRS is dual localized, present both in the soluble and in the membrane fractions. He further showed that the mt-AspRS is anchored to the membrane via electrostatic types of interactions. Here we extend this analyses and I could demonstrate that the mt-ArgRS its exclusively present in the mitochondrial membrane. Chemical treatments further revealed that the enzyme is anchored to the membrane *via* hydrophobic interactions. Altogether, these results demonstrated that the two mt-aaRSs compared here have unique and distinct characteristics regarding their intra-mitochondrial localizations. This encourages us for the characterization of the additional mt-aaRSs. These experiments are currently under way, performed by Florian Pierre and master students hosted by our laboratory. The analysis of the sub-mitochondrial localization of the mt-LeuRS and the LysRS after fractionation of the mitochondria revealed an additional situation since these two enzymes are exclusively detected in the soluble fraction of the mitochondria (**Figure 18**).



HEK293T cell line

Figure 18. Intramitochondrial localizations of selected mt-aaRSs in human cell lines. Mitochondria were purified from HEK293T cell line and fractionated into soluble (matrix and intermembrane space) and membrane (outer and inner membranes) fractions. Endogenous mt-LeuRS – 102 kDa, LysRS – 74 kDa, mt-ArgRS – 66 kDa and mt-AspRS – 73 kDa were detected by Western blot analysis with specific antibodies. S, soluble fraction. M, membrane fraction.

Previous investigations show that some of the key actors for mitochondrial translation machinery are located at the vicinity of the inner membrane, likely to allow for the direct incorporation of hydrophobic proteins into the membrane (reviewed in e.g. Ott and Herrmann BBA 2010 (Ott and Herrmann, 2010)). This is for instance the case for the mito-ribosome, tethered to the matrix side of the mitochondrial inner membrane (Liu and Spremulli, 2000) *via* a protuberant domain (the mitochondrial ribosomal protein L45, MRPL45; (Greber *et al.*, 2015)) and for the mitochondrial elongation factor Tu, associated with the inner mitochondrial membrane, independently of the mito-ribosome, *via* a combination of ionic and hydrophobic (Bogenhagen *et al.*, 2014; Suzuki *et al.*, 2007). One could imagine that the direct or indirect association of the mt-aaRSs to the mitochondrial membrane should be required to perform translation. However, the data revealed by this work puts aside this idea. The analysis of these four mt-aaRSs shows that the situation is more complex than it appears. The establishment of variable sub-mitochondrial locations for these four mt-aaRSs strongly suggests the existence of additional enzyme properties that will require further investigations to be identified. Additionally, much remains to be done to clarify how is the interaction/assembly of all the components in the mitochondrial translation. *In organello* translation and co-immunoprecipitation may shed light on the possible functional associations among the components of the mitochondrial translation, especially within the set of mt-aaRSs and the rest of the members of the mitochondrial translation machinery.

The knowledge of the intramitochondrial organization clearly provides an invaluable knowledge about these enzymes. However, much remains to be done to be able to understand

the relationship between the synthetases and the pathologies produced. Therefore, the next chapter will be focused to the study of the impact of pathological mutations on i) the *in cellulo* intramitochondrial localization for the mt-AspRS and mt-ArgRS and ii) the *in vitro* analysis of enzymatic function of the mt-ArgRS.

Article # 2

**Three human aminoacyl-tRNA synthetases
have distinct sub-mitochondrial localizations
that are unaffected by disease-associated
mutations**

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Human mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs) are key enzymes in the mitochondrial protein translation system and catalyze the charging of amino acids on their cognate tRNAs. Mutations in their nuclear genes are associated with pathologies having a broad spectrum of clinical phenotypes, but with no clear molecular mechanism(s). For example, mutations in the nuclear genes encoding mt-AspRS and mt-ArgRS are correlated with the moderate neurodegenerative disorder leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) and with the severe neurodevelopmental disorder pontocerebellar hypoplasia type 6 (PCH6), respectively. Previous studies have shown no or only minor impacts of these mutations on the canonical properties of these enzymes, indicating that the role of the mt-aaRSs in protein synthesis is mostly not affected by these mutations, but their effects on the mitochondrial localizations of aaRSs remain unclear. Here, we demonstrate that three human aaRSs, mt-AspRS, mt-ArgRS, and LysRS, each have a specific sub-mitochondrial distribution, with mt-ArgRS being exclusively localized in the membrane, LysRS exclusively in the soluble fraction, and mt-AspRS being present in both. Chemical treatments revealed that mt-AspRS is anchored in the mitochondrial membrane through electrostatic interactions, whereas mt-ArgRS uses hydrophobic interactions. We also report that novel mutations in mt-AspRS and mt-ArgRS genes from individuals with LBSL and PCH6, respectively, had

no significant impact on the mitochondrial localizations of mt-AspRS and mt-ArgRS. The variable sub-mitochondrial localizations for these three mt-aaRSs strongly suggest the existence of additional enzyme properties, requiring further investigation to unravel the mechanisms underlying the two neurodegenerative disorders.

Mitochondria are double-membrane organelles with essential activities in cellular energy production as well as in a number of pathways linked to cellular life, disease, aging, and death. They possess their own genome and an independent translation machinery devoted in human to the synthesis of 13 proteins. The latter are hydrophobic subunits of the respiratory chain complexes embedded in the mitochondrial inner membrane (1). The human mitochondrial translation machinery is of dual genetic origin where RNA constituents are encoded by the mitochondrial genome (mt-DNA) and the protein constituents are encoded by the nuclear genome. Previous investigations show that some of the key actors for mitochondrial translation machinery are located at the vicinity of the inner membrane, likely to allow for the direct incorporation of hydrophobic proteins into the membrane (reviewed in Ref. 2). This is for instance the case for the mitoribosome, tethered to the matrix side of the mitochondrial inner membrane (3) via a protruberant domain (the mitochondrial ribosomal protein L45, MRPL45) (4, 5) and for the mitochondrial elongation factor Tu, associated with the inner mitochondrial membrane, independently of the mitoribosome, via a combination of ionic and hydrophobic interactions (6).

Among the proteins involved in mt-DNA expression the aminoacyl-tRNA synthetases (aaRSs)⁵ play a crucial role in mitochondrial protein translation by charging tRNAs with cognate amino acids. In human there is a specific set of aaRSs for mitochondrial localization (mt-aaRSs) distinct from the one for cytosolic localization (with the exceptions of LysRSs and

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This article contains Table S1 and Figs. S1 and S2.

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⁵ The abbreviations used are: mt-aaRS, mitochondrial aminoacyl-tRNA synthetase; LBSL, leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation; HEK, human embryonic kidney; mt-ArgRS, mitochondrial arginyl-tRNA synthetase; SOD2, superoxide dismutase 2; VDAC1, voltage-dependent anion selective channel protein 1; PCH6, pontocerebellar hypoplasia type 6; PEI, polyethylenimine; BHK, baby hamster kidney.

GlyRSs, where cytosolic and mitochondrial versions are encoded by single genes (7)). As for all proteins participating in the mitochondrial translation, the mt-aARSs are encoded by the nuclear genome and are synthesized within the cytosol, addressed to, and imported into, the mitochondria thanks to mitochondrial targeting sequences (MTS). MTS are cleaved upon entry into the mitochondria (8). Although the macromolecules involved in mammalian mitochondrial translation have been under investigation for many years, there is an increasing interest for the investigation of the human mt-aARSs since the discovery of a large and growing number of mutations in the encoding genes that are linked to a variety of pathologies (reviewed in Refs. 9–11). Despite being ubiquitously expressed and having a common role in the mitochondrial translation process, mt-aARSs are impacted in various ways. Their mutations cause an unexpected variety of phenotypic expressions, including mainly neurological disorders but also non-neurological issues. Today, the number of reported cases is steadily growing (12), but the way mutations affect mt-aARSs in their structure and/or function remains to be elucidated. The fact that comparable mutations in mt-aARSs lead to diverse diseases, with different ages of onset, and within different tissues represents a confounding issue.

The most prominent case of disease-related mt-aARS gene concerns *DARS2*, the gene coding for the mitochondrial aspartyl-tRNA synthetase (mt-AspRS). Presently, more than 60 different clinically relevant mutations have been identified and associated with leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) (12, 13). LBSL is a progressive neurodegenerative disorder that affects the brain white matter, and leads mostly to abnormal muscle stiffness and difficulties with coordinating movements. Most affected patients eventually require wheelchair assistance (14). Mutations within *RARS2*, the gene coding for the mitochondrial arginyl-tRNA synthetase (mt-ArgRS), are correlated with pontocerebellar hypoplasia type 6 (PCH6) (15). Symptoms such as severe impairment of brain development, hypotonia, lethargy, poor sucking and/or recurrent apnea appear on the first days after birth. For infants surviving beyond the newborn period, the growth of the head is arrested and progressive microcephaly is observed (16). Up to date, only 22 cases of PCH6 are reported and most of the patients are presently deceased. Investigations performed so far showed no or variable impacts on the canonical properties of the enzymes, indicating that the housekeeping role of the mt-aARS in the protein synthesis is not the general target of the mutations (17–19).

Here, we investigate the cellular properties of human mt-AspRS and mt-ArgRS, and establish that the two enzymes have different mitochondrial localizations, despite their involvement in the same mitochondrial translation process. In addition, we report new LBSL and PCH6 patients, compound heterozygous for two mutations in *DARS2* and *RARS2*, respectively. We investigate the impact of a series of disease-associated mutations, affecting the mt-AspRS and mt-ArgRS, on newly established cellular properties. Combined with previous work, the present results open new perspectives, which may shed new light on the links between mutations and the related diseases.

Results

Mitochondrial aminoacyl-tRNA synthetases have different intra-mitochondrial localizations

To establish the sub-mitochondrial localization of mt-AspRS, mt-ArgRS, and LysRS, mitochondria from human embryonic kidney cells (HEK293T) were enriched and fractionated into a soluble fraction (**S** in Fig. 1A) containing molecules from the matrix and intermembrane space, and membrane fraction (**M** in Fig. 1A) containing molecules from the outer and the inner membranes. Western blots using antibodies against proteins of known sub-mitochondrial localization were used to assess the quality of the fractionation process. The superoxide dismutase 2 (SOD2) (20) is a marker for soluble matrix proteins. The voltage-dependent anion selective channel protein 1 (VDAC1) (21, 22) and prohibitin (23) are integral proteins, anchored to the outer and inner membranes, respectively. The mammalian mt ribosome (3), cytochrome *c* (Cyt *c*) (24), creatine kinase (CKMT1A) (25), and heat shock protein 60 (Hsp60) (26, 27) are reported to be dual localized in the soluble and membranes fractions as peripheral proteins. All marker proteins were detected in agreement with the literature (Fig. 1B), attesting for the quality of the fractionation protocol and the absence of cross-contamination.

The intra-mitochondrial localizations of mt-AspRS, mt-ArgRS, and LysRS were established using specific antibodies. This experiment reveals that the mt-AspRS is distributed in both the soluble and membrane fractions, whereas the mt-ArgRS is exclusively found in the membrane fraction and the LysRS is exclusively found in the soluble fraction (Fig. 1B). Identical results, showing different intra-mitochondrial localizations for two of the mt-aARSs, is observed using anti-FLAG antibodies on BHK21 cells expressing FLAG-tagged mt-AspRS or on HEK293T cells expressing FLAG-tagged mt-ArgRS (Fig. 1C).

Mitochondrial aspartyl-tRNA synthetase and arginyl-tRNA synthetase have different modes of membrane anchoring

A protein can either be “integral” and permanently attached to the membrane via a transmembrane domain or hydrophobic region(s); or be “peripheral” and loosely adhered to the membrane through either electrostatic or ionic interactions (via another protein or membrane lipids), disulfide bond interactions, or a covalently bound lipid anchor. A series of disrupting chemical agents have been reported to produce type-specific release of membrane-bound proteins. Examples of chemicals and their specificity of action are provided in Fig. 2A. A selection of these disrupting chemical agents (8 M urea (28), 0.5 M KCl (26, 29), 0.1 M Na₂CO₃ pH 11 (30–32), 0.4 M DTT (33), 1 M NH₂OH, pH 7, or 1 M NH₂OH, pH 11) (34–36) was applied on isolated and enriched mitochondria, prior to the fractionation process. As shown in Fig. 2B, all conditions except 0.4 M DTT led to the release of mt-AspRS from the membrane fraction. Of note, in the latter experiment, the portion of mt-AspRS in the soluble fraction is either relocated within the membrane fraction, or lost during the experimental treatment. Conversely, solely urea and carbonate treatments allowed either the total or the partial release of mt-ArgRS, respectively, which remained

Intra-mitochondrial distributions of mt-AspRS and mt-ArgRS

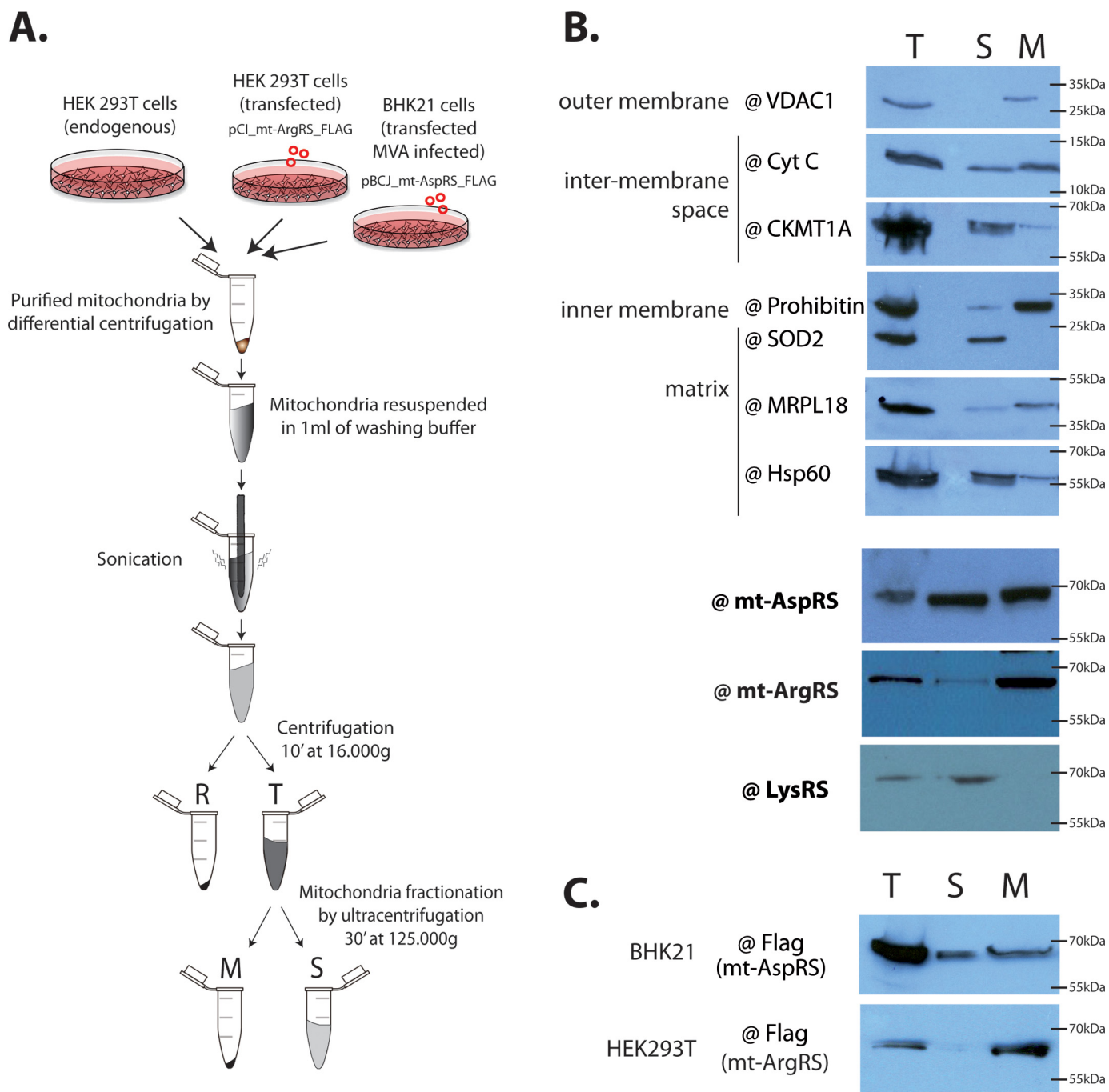


Figure 1. Sub-mitochondrial localizations of mt-AspRS, mt-ArgRS, and LysRS. *A*, flow chart of the experimental procedure. Cells are either unmodified HEK293T (for results shown in *B*), or transfected and infected BHK21 for FLAG-tagged mt-AspRS (following the protocol published in Ref. 19) and transfected HEK293T for FLAG-tagged mt-ArgRS (for results shown in *C*). *B*, the purity of the soluble and membrane fractions was assessed by Western blotting detection of mitochondrial marker proteins of known sub-mitochondrial localization: outer membrane (VDAC1), inter-membranes space (Cyt *c* and CKMT1A), inner membrane (prohibitin), and matrix (SOD2, MRPL18 and Hsp60). Mt-AspRS, mt-ArgRS, and LysRS were detected using specific antibodies. *C*, sub-mitochondrial localizations of FLAG-tagged mt-AspRS or mt-ArgRS detected by Western blotting using an antibody against the FLAG tag. **R**, residual fraction; **T**, total mitochondria; **S**, soluble fraction; **M**, membrane fraction.

otherwise anchored to the mitochondrial membrane. SOD2 and prohibitin are detected/analyzed as control experiments from the matrix and the membrane fractions, respectively. Although SOD2 remains soluble whatever is the applied chemical, prohibitin was detected in the soluble fractions upon urea, carbonate, pH 11, and hydroxylamine, pH 11, treatments. Carbonate treatment, pH 11, was reported to lead to the release of some integral proteins with mode of anchoring

weaker than the complete transmembrane domain (30–32). Altogether, these results showed that membrane-anchored fractions of mt-AspRS and mt-ArgRS respond to different chemistry, indicating that the mode of anchoring is distinct: salt sensitive for mt-AspRS, indicative of an electrostatic mode of anchoring; salt-resistant but urea-sensitive for mt-ArgRS, indicative of a hydrophobic mode of anchoring for mt-ArgRS.

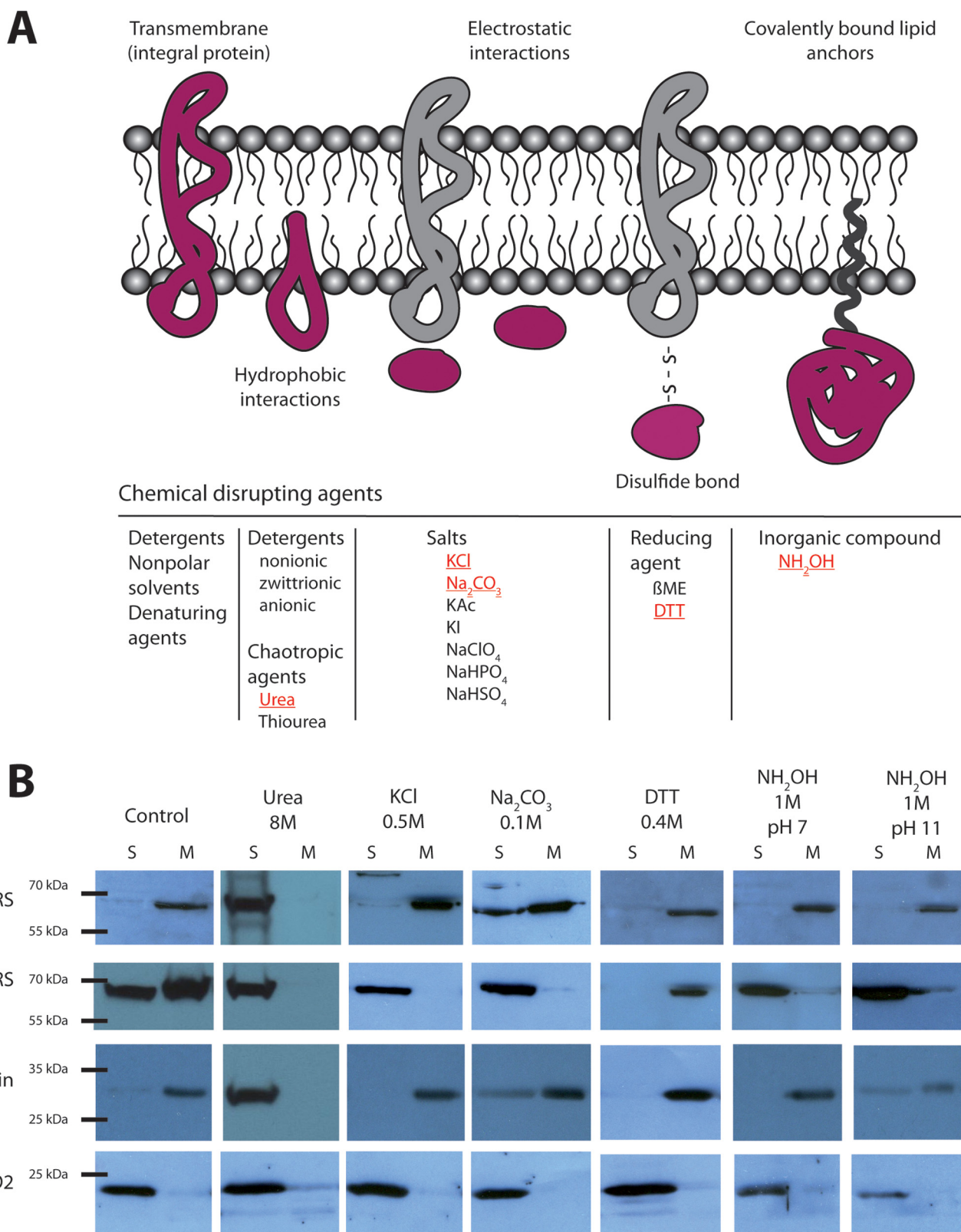


Figure 2. Different modes of membrane anchoring of mt-AspRS and mt-ArgRS. A, schematic representation of the different modes of protein membrane anchoring or binding. Some chemicals reported to be used for the release of anchored protein are indicated on the scheme, those indicated in red have been applied in the present study (for references, see text). B, Western blot analysis (using antibodies targeted against mt-ArgRS, mt-AspRS, prohibitin, or SOD2) of the soluble and membranes fractions after treatment as indicated on the top.

Intra-mitochondrial distributions of mt-AspRS and mt-ArgRS

Impact of disease-associated mutations on mt-AspRS and mt-ArgRS intra-mitochondrial distributions

The impact of LBSL-related mutations on the solubility of the human mt-AspRS was investigated in a previous study (19). To do so, WT and mutant versions of the mt-AspRS were expressed in modified vaccinia Ankara-infected BHK 21 cells (37). Mt-AspRS being a dimeric enzyme (7) and, as demonstrated here, being dual localized, the choice was made to analyze further mutant mt-AspRSs in the same heterologous expression system. A possible impact of disease-related mutations on the mt-AspRS dual localization will thus be more easily detectable because none of the used antibodies (against either the C-terminal FLAG tag or a peptide specific to the human mt-AspRS) will detect the endogenous hamster mt-AspRS. Mt-ArgRS being a monomeric enzyme and located exclusively in the membrane fraction, any impact on the localization, if observed, would necessarily arise from the disease-related mutation. The choice was thus to express WT and mutant versions of the mt-ArgRS in HEK293T cells (which contains endogenous WT mt-ArgRS). Experiments shown in Fig. 1 confirm that the distribution of both mt-AspRS and mt-ArgRS is conserved in the two types of cultured cells, indicating that the two model systems are appropriate for further investigations.

The effects of eight LBSL-related and 12 PCH6-related mutations, identified in patients in *DARS2* and *RARS2*, respectively, were investigated regarding their possible impacts on the sub-mitochondrial localization of the corresponding enzyme. WT and mutants mt-AspRS and mt-ArgRS were individually expressed in cellular models and their distributions between soluble (S), membranes (M), and residual (R) fractions were determined by Western blotting. All experiments were repeated three times and the percentage of each fraction was calculated, assuming that $S + M + R = 100\%$ of the total expressed protein. Representative Western blots are given in Fig. 3A and histograms corresponding to the relative distribution of mt-AspRS variants are given in Fig. S1. None of the PCH6-related mutations alter the expression (Fig. S2) and the membrane-anchored localization (Fig. 3A) of the mt-ArgRS. A statistically significant (p values < 0.05) reduction in the soluble fraction was observed for Q184K⁶ and R263Q mutants of mt-AspRS (Fig. 3A, Fig. S1). Even with variations, no significant effect was noticed in the membranes fractions of mt-AspRS mutants. The residual fraction of the Q184K mutant was significantly increased compared with WT mt-AspRS (p value < 0.05), consistent with the lower solubility already observed for this variant (19).

Fibroblasts derived either from the LBSL patient (p.R76SfsX5/p.G338E), from the PCH6 patient (p.T100Cfs*5/p.A10V) (see “Experimental Procedures” for cases reports), or from a healthy control were fractionated following the same procedure. Of note, due to the lower abundance of mitochondria in skin fibroblasts as compared with immortalized HEK or BHK cells, a

number 10-fold greater of cells was necessary ($\sim 2 \times 10^7$ fibroblast cells as compared with $\sim 2 \times 10^6$ required for HEK or BHK cells per experiment). No difference of growth between healthy and LBSL- or PCH6-derived fibroblasts was noticed. Detections of either the endogenous mt-AspRS or the endogenous mt-ArgRS within the two patient-derived cell lines show distributions strictly comparable with the ones within fibroblasts from healthy control (Fig. 3B).

Discussion

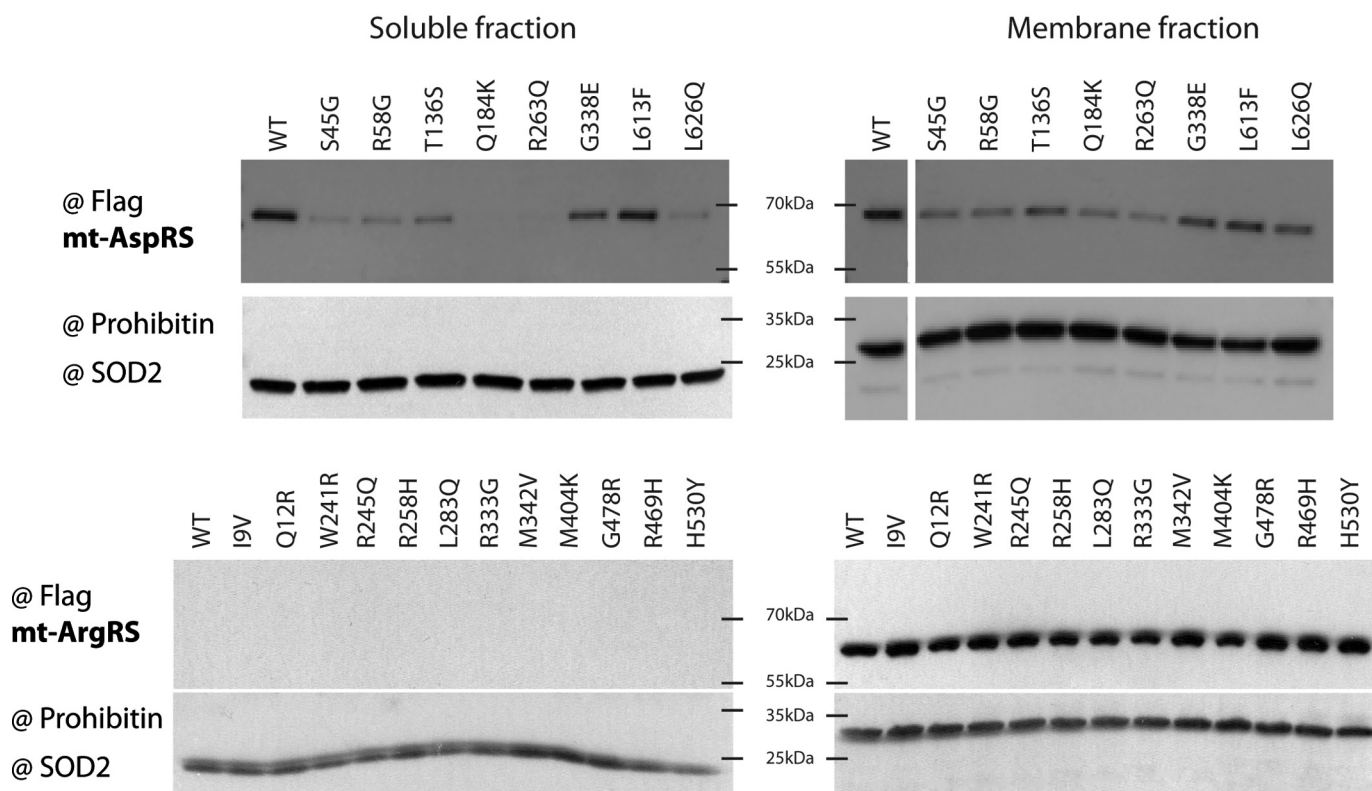
aaRSs are housekeeping enzymes that catalyze the aminoacylation of cognate isoaccepting tRNAs, at least as the main recognized function. The human cytosolic aaRSs have been extensively investigated and the organization of nine of them within a macromolecular complex was established (e.g. Refs. 38 and 39). Alternative cellular and/or extracellular activities linked to metabolism, angiogenesis, immune response, inflammation, tumorigenesis, or neuronal development, among other functions, have also been reported (38, 40–42). In contrast, the knowledge regarding cellular organization of mt-aaRSs and possible alternative function(s) remains scarce. It is only recently that a pro-angiogenic function of the rat mt-TrpRS (43) and a cysteine polysulfidation activity of the mice and the human mt-CysRSs (44) have been identified.

In the present study, we establish the sub-mitochondrial localization of three mt-aaRSs and demonstrate that regardless of their common role in the aminoacylation of cognate tRNAs, they have different distributions. The applied fractionation protocol separates soluble proteins from the intermembrane space or the matrix (soluble fraction) from those anchored to the inner or the outer membranes (membrane fraction). We show that the mt-ArgRS is exclusively localized in the membrane, the LysRS is exclusively in the soluble fraction, and the mt-AspRS is dual localized, being present in both soluble and membrane fractions. Multiple localizations have been observed at several instances for mitochondrial proteins. Few reports have in addition established a link between multiple localizations and multiple functions or modulated activities of the corresponding protein (e.g. Refs. 29, 45, and 46). Previously, we showed that the human mt-AspRS is processed after mitochondrial importation into three different products of maturation from which two co-exist in the mitochondria (8). This observation open new perspectives in the biological understanding of the mt-AspRS and is considered in line with the discovery of two mature forms of the mt-ribosomal protein of the large subunit MRPL12, generated by a similar multiple steps cleavage process during import and demonstrated to display distinct functions (47). The question now is whether there is a correlation between the two mature forms of mt-AspRS, the two sub-mitochondrial localizations described in the present study, and the possibility of this enzyme to have distinct functions or modulated activities.

We further demonstrate that the mode of membrane-anchoring is different between the membrane fractions of the mt-AspRS and the mt-ArgRS: salt-sensitive for the mt-AspRS, indicating an electrostatic mode of membrane anchoring, and urea-sensitive for the mt-ArgRS, indicating a hydrophobic mode of membrane anchoring. None of the two proteins has

⁶ Rigorously, amino acid conversion of a given mutation should be preceded by the “p” letter to indicate that the protein level is considered and the amino acid change should be indicated using the three-letter nomenclature. For sake of simplicity, the “p” is omitted and the one-letter code is used so that the mutation p.Gln184Lys is named here Q184K.

A. Cellular models



B. Fibroblasts from patients

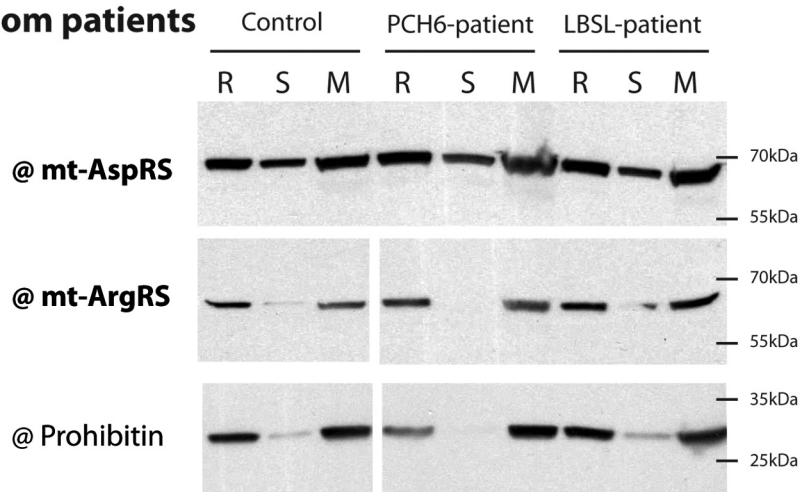


Figure 3. Impact of disease-associated mutations on the intra-mitochondrial localization. A, cellular models. Shown are representative Western blots of three independent experiments detecting WT and mutant mt-AspRS (top) and mt-ArgRS (bottom) using anti-FLAG antibody. SOD2 and prohibitin were detected to attest for the quality of the fractionation and used as loading controls for the soluble and membrane fractions, respectively. B, fibroblasts from patients. Shown are representative Western blots detecting WT and mutant mt-AspRS, mt-ArgRS, and prohibitin using specific antibodies. Control skin fibroblast is from a healthy control person. PCH6-patient and LBSL-patient correspond to skin fibroblasts from patient 2 (p.T100Cfs*5/p.A10V) and patient 1 (p.R76SfsX5/p.G338E), respectively. R, residual fraction; S, soluble fraction; M, membrane fraction.

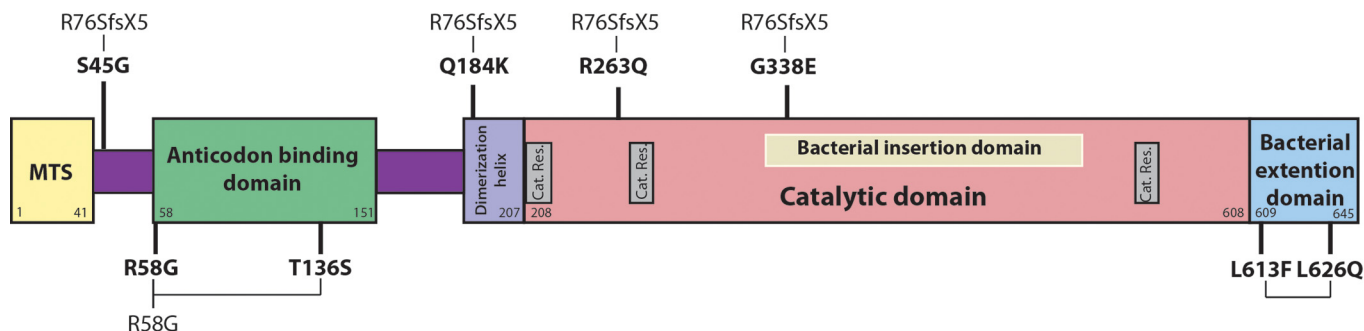
a sequence-based predictable transmembrane domain (tested in the TMHMM Server, <http://www.cbs.dtu.dk/services/TMHMM-2.0> (48), not shown).⁷ There are several examples where an aaRS is relocated into a membrane upon stimuli. This

is either to positively or negatively regulate the translation or to allow the occurrence of an alternate function of the aaRS by removing it from translation. For instance, the human cytosolic LysRS is naturally anchored to the cytosolic multi-synthetase complex but relocated to the plasma membrane upon laminin-dependent phosphorylation. Once at the plasma membrane, the LysRS associates with membrane proteins p67LR and the

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Intra-mitochondrial distributions of mt-AspRS and mt-ArgRS

A. mt-AspRS



B. mt-ArgRS

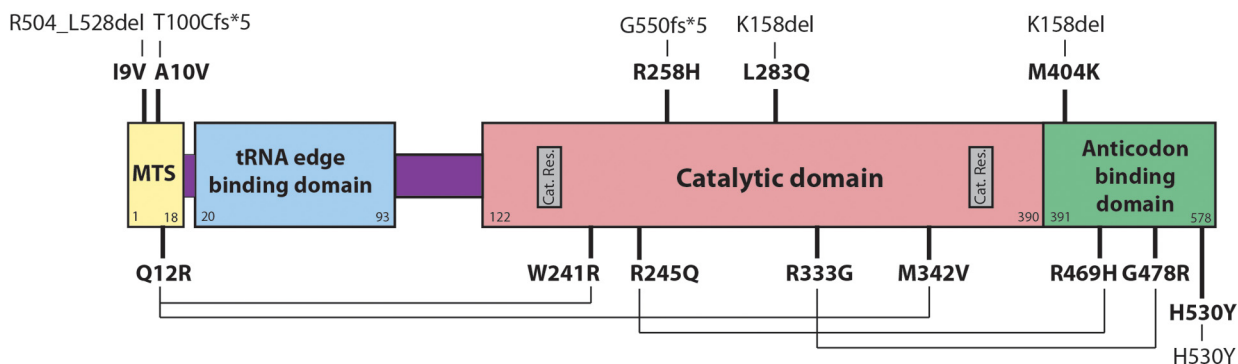


Figure 4. Investigated disease-associated mutations of human mt-AspRS (A) and mt-ArgRS (B). The mt-aars are shown to scale; known functional domains are named and colored. *Cat. Res.* stands for “catalytic residues.” Allelic compositions, as identified in patients, are linked through *black lines*. R76SfsX5 is a truncated variant of mt-AspRS due to the c.228–20_21delTTinsC mutation. T100Cfs*5 and G550fs*5 are truncated variants of mt-ArgRS due to the c.298G_1→A and the c.1651–2A→G mutations, respectively. Mutations investigated in the present study are highlighted in *bold characters*. Rigorously, amino acid conversion of a given mutation should be preceded by the “p” letter to indicate that the protein level is considered. For sake of simplicity, the “p” is omitted. All data are extracted from Ref. 12, which contains all related references. Figures were adapted from Ref. 11.

integrin $\alpha 6$, increasing cellular migration and invasion (49). The authors suggest that the recruitment of a key translational component to regulate cell migration may reduce the level of operational translation machinery (50). As another example, the human cytosolic LeuRS is translocated to the lysosome membrane upon leucine addition. In this situation, LeuRS acts as an intracellular leucine sensor and activates the mammalian target of rapamycin-signaling pathway, regulating translation, cell size, and autophagy (51, 52). Finally, in cyanobacteria, four aars are anchored to the thylakoid membranes thanks to an additional protein domain named CAAD (for cyanobacterial aminoacyl-tRNA synthetases appended domain) (53). The membrane-anchored ValRS was shown to directly interact with the ATP-synthase, linking elements from gene translation and energy production machineries (54). In this case, the aars is constitutively anchored to the membrane (and not relocated upon stimuli), a situation similar to what we observed for the human mt-ArgRS.

Over the past 10 years, disease-related mutations affecting *DARS2* and *RARS2* have been reported in patients with LBSL or PCH6 syndromes, respectively (12). Schematic representations of the modular organizations of mt-AspRS and mt-ArgRS are given in Fig. 4. Previous studies, on the same subset of mt-AspRS mutants, revealed that only some had lower aminoacy-

lation activities (mutants L626Q and R263Q) (17, 55). Additional investigations showed that the analyzed mutations have no impact on the mt-AspRS architecture (19), but distinct and variable impacts on mt-AspRS expression (T136S, Q184K, L626Q) (17), dimerization (Q184K) (17), translocation from the cytosol to the mitochondria (S45G) (56), or on *in cellulo* solubility (Q184K, L626Q) (19). Strikingly, mutations R58G and L613F have no noticeable impact on any of these parameters. Regarding PCH6-related mutations of mt-ArgRS, limited investigations have been performed so far. A striking reduction in the amount of the mt-tRNA^{Arg} was found in patient’s fibroblasts (with the combination of the K291R mutation and the IVS2 + 5 (A→G) mutation, which causes exon 2 skipping) with, however, the observation that the residual mt-tRNA^{Arg} transcript was almost fully acylated, suggesting that the uncharged mt-tRNA^{Arg} become unstable (15). Lower mt-ArgRS expression and activity have been observed in cultured skin fibroblasts from two patients (with p.R245Q/p.R469H or p.W241R/p.Q12R combinations of mutations). A drastically less significant impact has, however, been reported in a third patient (p.I9V/p.R504_L528del) (18). Altogether, the fact that disease-related mutations impact variably the investigated properties of the proteins is perplexing and points to the fact that the housekeeping role of mt-aars in protein synthesis is

not the general target of the mutations, and that different mechanisms are at play in the pathogenesis of mt-aaRS-associated diseases.

In the present study, we have investigated the impact of LBSL- and PCH6-causing mutations on mt-AspRS dual localization and on mt-ArgRS membrane localization, respectively. With the exception of Q184K, none of the investigated mutations significantly impairs the intra-mitochondrial distribution of the corresponding mt-aaRS, neither in cells expressing mutant proteins nor in skin fibroblast cells derived from patients. The Q184K mutation affects the matrix localization of mt-AspRS, consistent with the lower solubility and higher propensity to aggregate, previously determined for this mutant *in vitro* by biophysical approaches (19). It is worth mentioning that the two patient-derived skin fibroblasts are heterozygous compounds and contain a missense mutation and a splicing defect. The splicing defect in the case of a *DARS2*-related LBSL patient was reported to be leaky and to allow the production of a significant amount of WT protein (13, 14). The same authors have further demonstrated the cell-type dependence of splicing of mt-AspRS mRNA and that the mutations have a larger effect on exon 3 exclusion in neuronal cell lines than in non-neural cell lines (57). This may explain the lack of visible impact on skin fibroblast cells expressing both a missense mutation and a splicing defect.

The case of mutant S45G is puzzling. Indeed, it was previously shown that this mutation impairs the import of the protein into isolated mitochondria purified from HEK293T cells, but does not affect the targeting or the processing (cleavage) as demonstrated by *in cellulo* and *in vitro* approaches, respectively (56). In the present study, the S45G mutant is expressed *in cellulo* and shown to be soluble and dually localized in mitochondria similarly to the WT protein. It is thus hypothesized that the S45G mutation, situated near one of the mt-AspRS processing sites (8), may alter the efficiency of the import but not the import itself. This process might then be more sensitive in isolated mitochondria than in a cellular context. Corroborating this hypothesis, the targeting and import of a same protein was demonstrated to be different from one cell or tissue to the other one (58). Also, mutation in the huntingtin protein was shown to affect the mitochondrial import into neurons but not into other cells, leading to premature neuronal death in patients with Huntington disease (59).

Outlook

We have established the different intra-mitochondrial localizations for the human mt-aaRSs in kidney and skin fibroblasts cells. This distribution should be further investigated in other tissues, *e.g.* in neuronal cells and/or under distinct physiological or pathophysiological contexts. It is worth mentioning that both LBSL and PCH6 are neuronal affections. Similarly, the absence of impact of disease-associated mutations on mt-AspRS or mt-ArgRS intra-mitochondrial localizations does not exclude a possible impact in other cellular contexts. This is consistent with the observation of the “lack of clear biochemical phenotypes (OXPHOS or mitochondrial protein synthesis defects) in skin fibroblasts and myoblasts from most of mutant

mt-aaRSs patients,” previously described (60). Similar investigations should now be performed on neuronal cellular models.

The discovery of different localizations and diverse modes of membrane anchoring for two mt-aaRS should be considered in line with the observation that the two related diseases have distinct onsets and degrees of severity. The observation of contrasting scenarios further points to the fact that different mechanisms are likely at play in the pathogenesis of mt-aaRS-associated diseases and suggests distinct intra-mitochondrial roles of the two mt-aaRSs, which will need to be unveiled.

Experimental procedures

Informed consent for diagnostic and research studies was obtained for all subjects in accordance with the Declaration of Helsinki protocols and approved by local Institutional Review Boards in Paris.

Cells, biochemical, and chemicals

HEK293T were from Invitrogen. Skin fibroblasts (patient-derived and control) were from the Imagine Institute (Paris). Baby hamster kidney cells strain 21 (BHK21) (ATCC number CRL-12072) and modified vaccinia Ankara strain (MVA-EM24) were gifts from Robert Drillien (IGBMC, Strasbourg). Polyclonal anti-human mt-AspRS was produced in rabbit by the service of antibodies production at the IGBMC (Illkirch) and raised against the peptide ⁴⁸⁶LFLPKEENPREL⁴⁹⁷. Antibodies against human mt-ArgRS, human LysRS, human superoxide dismutase (SOD2), prohibitin, voltage-dependent anion-selective channel (VDAC1), and cytochrome *c* (Cyt *c*) were purchased from Abcam®. Antibodies against mt creatine kinase (CKMT1A), heat shock protein 60 (Hsp60), and anti-FLAG® were purchased, respectively, from ProteinTech, Bethyl Lab, and Sigma. Mt-ribosomal protein L18 (MRPL18) antibody was a gift from Dr. Entelis (GMGM, Strasbourg). Horseradish peroxidase-conjugated goat anti-rabbit and sheep anti-mouse secondary antibodies were from Bio-Rad and GE Healthcare, respectively. Chemiluminescent detection kit was from Pierce (Thermo Scientific), Mini-Protean® TGX Precast polyacrylamide gels and the Trans-Blot Turbo system were from Bio-Rad. Arrest™ protease inhibitor mixture and polyethylenimine (PEI, linear 25 kDa) were purchased from GBiosciences and Polysciences, respectively. Tryptose phosphate broth (TPB) was from Sigma. Trypsin, penicillin/streptomycin, phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium, and Glasgow's minimum essential medium (GMEM) were purchased from Gibco. Fetal bovine serum (FBS) was from Eurobio.

Cases reports

Patient 1—Patient 1, a girl, was born to healthy unrelated parents after a term pregnancy and normal delivery. She did well during her first years of life. At six years, she mentioned short, recurrent and increasingly frequent episodes of tingling sensation of the inferior limbs, originally ascribed to Lyme disease. She subsequently presented recurrent attacks of unilateral lower limb weakness, frequent falls, and gait ataxia and limb intention tremor. Left hemiparesis with pyramidal syndrome, brisk deep tendon reflexes, cerebellar ataxia and *pes cavus* were

Intra-mitochondrial distributions of mt-AspRS and mt-ArgRS

noted but she could attend school normally. Brain MRI revealed bilateral hyper intensity of pyramidal track, pons, cerebellar peduncles and corpus callosum. Her metabolic work up including plasma lactate and pyruvate was normal. Next-generation sequencing revealed compound heterozygosity for two *DARS2* variants, a missense variant (c.1013G>A; p.G338E) and a previously reported nonsense truncating variant (c.228–21_228–20delinsC; p.R76SfsX5) (13).

Patient 2—Patient 2, a girl, was born to second-cousin Turkish parents after a term pregnancy and normal delivery. At day 4, she presented bouts of myoclonic jerks unresponsive to valproate and carbamazepine. She developed myoclonic encephalopathy with trunk hypotonia and inability to follow with eyes at aged 2 months. Brain MRI showed severe sus- and sub-tentorial brain atrophy with pericerebral effusion, with no basal ganglia involvement but NMR spectroscopy evidence of an important lactate peak (cerebrospinal fluid lactate: 4.2 mmol/liter, normal < 2.1). Next-generation sequencing revealed compound heterozygosity for two *RARS2* variants, a missense variant (c.29C>T; p.A10V) and a splicing variant (c.298G-1→A) causing a frameshift and a premature termination codon (p.T100Cfs*5).

Plasmid constructions for protein expression in mammalian cells

For expression in cultured cells, all sequences were cloned with a downstream FLAG (DYKDDDDK) epitope tag coding sequence, so that all proteins are FLAG-tagged at their C termini. The gene for WT mt-AspRS and those carrying mutations c.172C→G (p.R58G), c.406A→T (p.T136S), c.550C→A (p.Q184K), c.788G→A (p.R263Q), c.1837C→T (p.L613F), and c.1876T→A (p.L626Q) were constructed and cloned as previously described (19). The ones carrying the c.133A>G (p.S45G) and c.1013G>A (p.G338E) mutations were generated by directed mutagenesis on a derivative of pBCJ739.14 (37). The gene for WT mt-ArgRS was cloned into the NdeI and XhoI sites of the pCI vector (Promega) with standard molecular biology procedures. Mutations c.25A→G (p.I9V), c.35A→G (p.Q12R), c.721T→A (p.W241R), c.734G→A (p.R245Q), c.773G→A (p.R258H), c.848T→A (p.L283Q), c.997C→G (p.R333G), c.1024A→G (p.M342V), c.1211T→A (p.M404K), c.1406G→A (p.R469H), c.1432G→A (p.G478R), and c.1588C→T (p.H530Y) were introduced by site-directed mutagenesis using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The primers used for mutagenesis are listed in Table S1.

Cell culture and transfection

BHK21 cells were cultured in Glasgow's minimum essential medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 5% tryptose phosphate broth in 5% CO₂ at 37 °C. Transfection of BHK21 cells was performed as described (19). Briefly, cells were washed with PBS, infected with modified vaccinia Ankara virus expressing isopropyl 1-thio-β-D-galactopyranoside-inducible T7 polymerase, and subsequently transfected with plasmid expressing either WT or mutant AspRSs under the dependence of T7 promoter using PEI. Plasmid-PEI complexes were prepared in a ratio of 1:4.5 (w/w) and incubated for 15 min at 20 °C prior to the transfection

(5 and 15 μg of DNA per plate of 60 and 150 cm², respectively). Protein expression was induced by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside.

HEK293T cells and skin fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% penicillin/streptomycin in 5% CO₂ at 37 °C. HEK293T cells were transfected with PEI (with same procedure and plasmid/PEI ratio as mentioned above) at 50% confluence with constructs expressing mt-ArgRS-FLAG (WT and mutants). The transfected cells were incubated at 37 °C for 3 days and then analyzed by Western blotting.

Mitochondrial enrichment

Cells were collected, washed with PBS, and resuspended in an isotonic buffer (220 mM mannitol, 70 mM sucrose, 1 mM MgCl₂, 1 mM EDTA, 10 mM HEPES/KOH, pH 7.4) containing a protease inhibitor mixture. Cells were then disrupted mechanically using 2-mm diameter ceramic beads in a FastPrep-24TM 5G machine (MP Biomedicals). Intact cells, nuclei, and debris (pellet) were discarded after 10 min of centrifugation at 400 × g (4 °C). Supernatant was centrifuged 10 min at 12,000 × g (4 °C) to collect fraction enriched in mitochondria (~40 mg were obtained per 150-cm² confluent plate).

Mitochondrial fractionation

Flow charts of experimental procedures to fractionate mitochondria are given in Fig. 1A. Mitochondria (~30 mg) were resuspended in 1 ml of washing buffer (10 mM K₂HPO₄/KH₂PO₄, pH 7.5, 300 mM mannitol, 1 mM EDTA, containing a protease inhibitor mixture), and sonicated 6 × 10 s on ice. Sonicated mitochondria were centrifuged during 10 min at 16,000 × g (4 °C). The resulted pellet (named residual fraction **R**) corresponds to unbroken mitochondria and aggregates, if any. The supernatant (**T**, for total mitochondria) was further ultracentrifuged 30 min at 125,000 × g (4 °C) to separate soluble (**S**) from membranes (**M**) fractions.

Chemical treatment of mitochondria

Enriched mitochondria (~30 mg/experiment) were sonicated 6 × 10 s on ice in the presence of different chemical agents (1–8 M urea, 0.1 M Na₂CO₃, pH 11, 0.5 M KCl, 0.4 M DTT, or 1 M NH₂OH, at pH 7 and 11) in 1 ml of 10 mM K₂HPO₄/KH₂PO₄, pH 7.5, 300 mM mannitol, 1 mM EDTA, and containing a protease inhibitor mixture for 10 min at 20 °C. After sonication, mitochondria underwent the fractionation protocol as described above.

Western blotting

Soluble (**S**) fractions were concentrated up to a volume of 80 μl using Amicon → Ultra-0.5 centrifugal filters (10K, Millipore). Residual (**R**) and membrane (**M**) fractions were solubilized in 80 μl of washing buffer. The protein concentration of each fraction was quantified using Bradford assay. All fractions were supplemented with 20 μl of Laemmli dissociating buffer, heated at 95 °C for 10 min. Twenty μl of each fraction (containing ~20 μg of proteins for total, ~140 μg for the soluble fraction, and ~65 μg for the membrane fraction) were loaded on a 10% SDS-PAGE. Proteins were blotted on a polyvinylidene

difluoride membrane and detected with specific antibodies. Chemiluminescent detection was carried out using the Pierce Detection Kit according to the manufacturer's instructions.

Autoradiographs were digitized using Epson Perfection 3490 Photo. For comparison between mt-aaRS WT and mutants, the relative amount of proteins was estimated from band intensities using ImageJ software (61), and corrected based on SOD2 and prohibitin intensities as loading controls. Soluble membranes and residual fractions obtained from each set of experiments have been quantified out of a same autoradiograph, assuming that all mt-aaRS protein has been recovered during treatment and percentage of each fraction in individual experiments has been calculated ($R + S + M = 100\%$) so that the relative distribution of proteins in each fraction for each condition is independent of the expression rate. Mean values and S.D. were calculated out of three independent replicates. Values were then normalized for comparison purposes to the corresponding WT fraction, artificially set to a value of 1. Due to the low abundance of mt-AspRS mRNA when compared with other mt-aaRS mRNAs in skin cells and when compared with other cells (<http://biogps.org>),⁷ the detection of the corresponding protein by Western blotting experiments was harder to reproduce in the three fibroblast cell lines.

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Chapter III Analysis of the impact of pathologies-related mutations on mitochondrial aminoacyl-tRNA synthetases properties

1. Introduction

In Chapter I, an exhaustive analysis of the disorders produced by mutations in the nuclear genes encoding for the mt-aaRSs was done. The ideas that emanated from this analysis were useful for the organization of the clinical manifestations. Since it was clearly demonstrated that PCH6 and LBSL present different clinical manifestations (even if they both affect CNS), distinct cellular properties were anticipated for the two mt-aaRSs.

Chapter II follows then from the need to characterize the cellular peculiarities of these enzymes. Important new data allowed us to establish knowledge regarding the mt-ArgRS and compare them with previous results, identifying that the mt-aaRSs have distinct intra-mitochondrial localizations.

In this third chapter, we explore if this "different localization" parameter could be impacted by the pathologies-related mutations and could possibly explain the pathologies. The study of the impact of the mutations on the mt-aaRSs was divided into two parts: First, we evaluate *in cellulo* the impact of diseases-associated mutations on the intra-mitochondrial locations of mt-AspRS and mt-ArgRS. The results from this work are part of article #2. In addition to this and considering the previously mentioned arguments (e.g. mutations at conserved positions for at least one allele, severity of the clinical manifestations; see chapter I), we had initially anticipated the possibility of an impact of PCH6-related mutations on the aminoacylation property of the mt-ArgRS. We thus performed *in vitro* analysis of the aminoacylation activity of the mt-ArgRS. The enzyme activity of mutated enzymes was established and compared to the wild-type form of the mt-ArgRS.

2. Impact of pathologies-related mutations on mt-aaRSs localizations *in cellulo*

The discovery of different intra-mitochondrial localizations for distinct aaRSs not only suggested the existence of additional enzyme properties, but also raises the possibility that this cellular property might be affected by the pathologies-related mutations. We thus investigated the impact of selected mutations reported to be correlated to LBSL and PCH6 pathologies on the mt-aaRSs intra-mitochondrial localizations.

Regarding mutations linked to LBSL, a former PhD student, Loukmane Karim, started the research work on mt-AspRS and established that *in cellulo* the double localization of this enzyme was not affected by the LBSL-associated mutations. Additionally, experiments analyzing the impact of mutations on the solubility of the mt-AspRS were performed, showing that for two missense mutations the proteins presented lowest solubility and highest propensity to aggregate *in cellulo* (Sauter *et al.*, 2015).

Twelve PCH6-related missense mutations were studied *in cellulo*: c.25A>G (p.I9V), c.35A>G (p.Q12R), c.721T>A (p.W241R), c.734G>A (p.R245Q), c.773G>A (p.R258H), c.848T>A (p.L283Q), c.997C>G (p.R333G), c.1024A>G (p.M342V), c.1211T>A (p.M404K), c.1406G>A (p.R469H), c.1432G>A (p.G478R), c.1588C>T (p.H530Y). The localizations of these mutations throughout the functional domains of the mt-ArgRS are represented in **Figure 19** (highlighted with blue dots). As seen in figure 16, amino acid substitutions either extend, shorten or delete side chain. We observe that all the mutated amino acids include all chemical natures, however, so far none of the incorporated amino acid has an electrically charged (negative) side chains. Thus, the diversity of reported substitution, affecting all functional domains, renders prediction(s) about a common mechanism difficult.

Moreover, two new mutations, one linked to LBSL and the other to PCH6, were described. This two new reported cases are included in the article #2. LBSL patient was a girl who presented the clinical symptoms onset at six years, presenting different affections in the inferior limbs. Neuroimaging studies showed various alterations in the pyramidal track, pons, cerebellar peduncles and corpus callosum. Next generation sequencing revealed compound heterozygosity for two *DARS2* variants, a missense variant (c.1013G>A; p.G338E) and a previously reported nonsense truncating variant (c.228-21_228-20delinsC; p.R76SfsX5). PCH6 patient was also a girl whose onset was in the neonatal period presenting myoclonic jerks. Neuroimaging studies revealed localized brain atrophy. Next generation sequencing

revealed compound heterozygosity for two *RARS2* variants, a missense variant (c.29C>T; p.A10V) and a splicing variant (c.298G-1>A) causing a frameshift and a premature termination codon (p.T100Cfs*5).

For either the PCH6 or LBSL patient, it should be noted that the clinical manifestations are similar to those previously described in previous reports. In these new cases we found the typical lower limb involvement in LBSL and the seizures in PCH6. As for the results of the imaging studies we also find neuroimaging standards described in chapter I for LBSL. However, although brain atrophy was described for PCH6, we did not obtain precise data on whether there is marked pontocerebellar hypoplasia.

The results obtained from this *in cellulo* analysis show that regardless of the mutation studied, or regardless of the cellular system used (HEK293T or human fibroblasts), the exclusive presence of mt-ArgRS in the mitochondrial membrane remains unchanged (See **Figure 20**). To go a little further in this study, we also explore whether the mutations studied have any effect on the total expression of the protein *in cellulo*. Here also, regardless of the mutation studied, the expression of the protein was unchanged when compared to the wild type mt-ArgRS. The image corresponding to this is represented in the supplementary information of article #2. Discarding the fact that the location of synthetase is part of the pathogenesis of the disease prompts us to continue exploring the properties of the enzyme that could explain the disease.

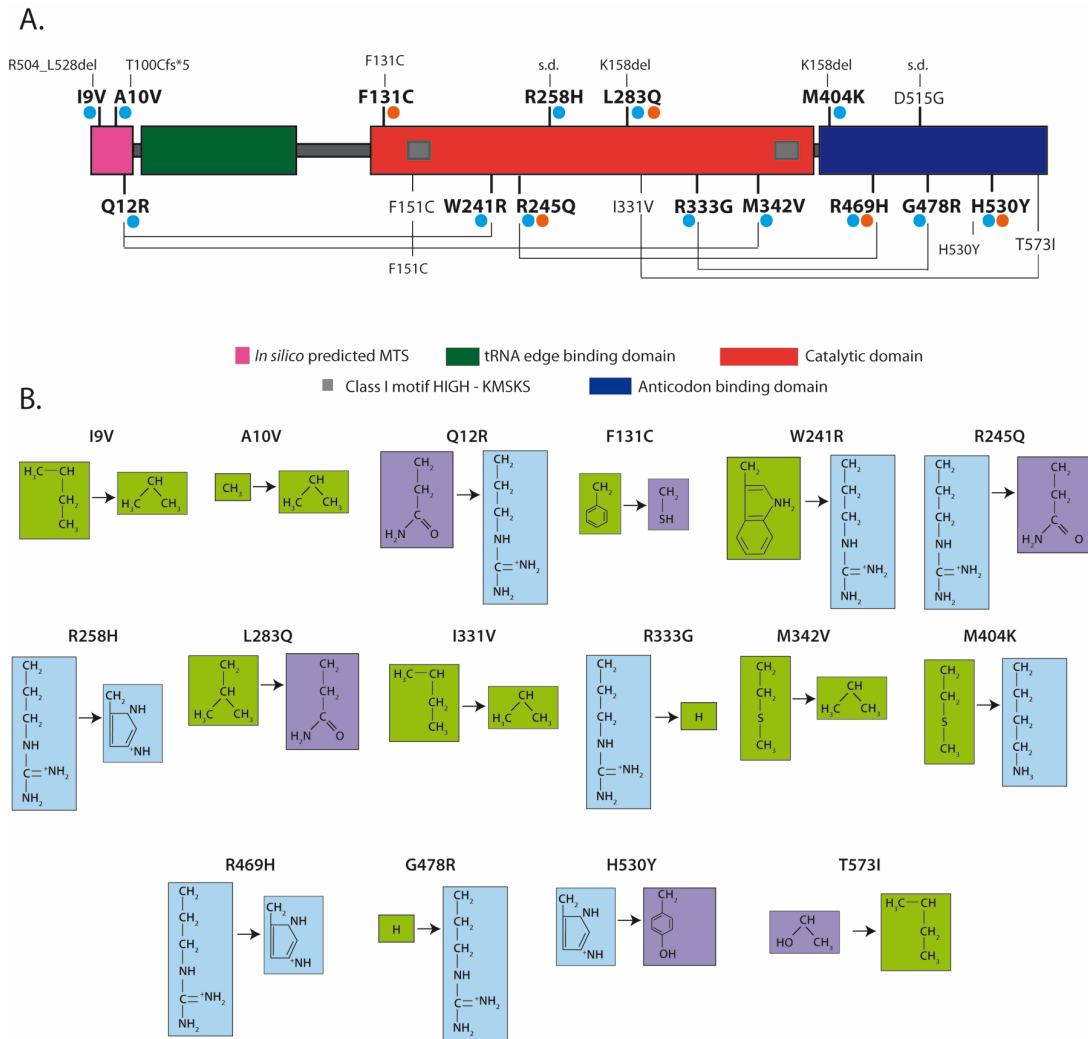


Figure 19. Locations and chemical nature of the amino acid substitutions of PCH6-associated mutations within functional domains of mt-ArgRS. (A) Schematic representation of the modular organization of the human mt-AspRS. Allelic compositions, as identified in patients, are linked through black lines. Mutations investigated in the present study, are highlighted in bold characters. Those investigated for their impact on membrane localization of the mt-ArgRS are indicated by the blue dots, and those for their impact on *in vitro* aminoacylation property of the enzyme by orange dots. Rigorously, amino acid conversion of a given mutation should be preceded by the “p.” letter to indicate that the protein level is considered. For sake of simplicity, the “p.” is omitted. All data are extracted from <http://misynpat.org> (Moulinier *et al.*, 2017), which contains all related references. (B) Representation of amino acid substitutions by their chemical nature. A code color was used to illustrate corresponding chemical nature: blue for non-polar side chains, purple for polar side chains, blue for electrically (basic) charged.

Since mt-ArgRS is a monomeric enzyme and is located exclusively in the membrane fraction, any impact on location, if it exists, would necessarily arise from the disease-related mutation. It is worth mentioning that the fact of using HEK293T transfected, the results would be the result of the participation of the endogenous mt-ArgRS and the mutated transfected version of the protein. While an anti-ArgRS antibody will detect both enzymes, the anti-flag-antibody will reveal solely the exogenous mt-ArgRS. Experiments studying the location of

mt-AspRS and mt-ArgRS were also performed in fibroblasts from these patients harboring these new mutations (**Figure 20.B** taken from figure 4 of article #2). Either in HEK293T cells or in human fibroblasts, the locations of mt-ArgRS and mt-AspRS were not affected independently of the studied mutations. The experimental process performed, as well as the discussion of the results obtained from this study, were included and published in article #2.

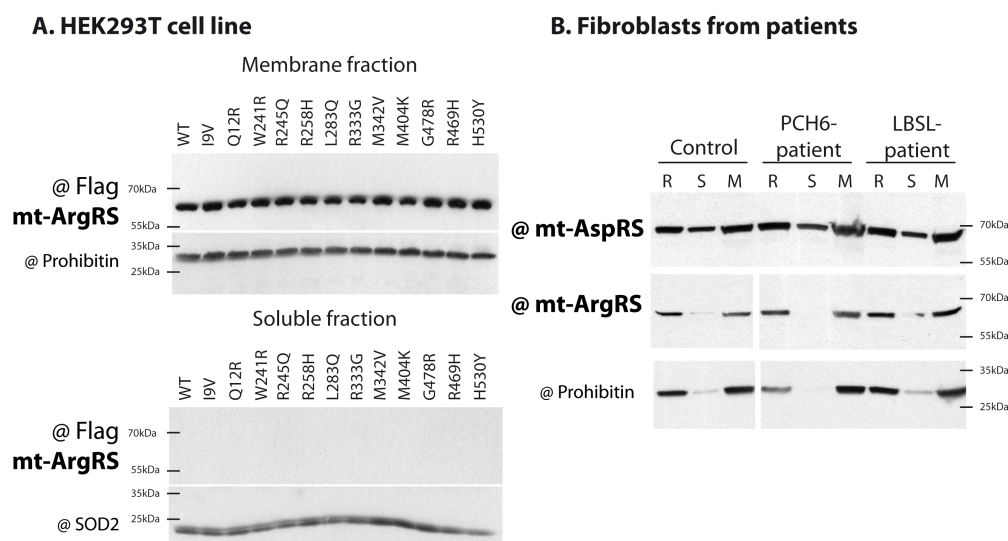


Figure 20 (taken from figure 3 of article #2) Impact of PCH6-related mutations on the exclusively membrane location of the mt-ArgRS *in cellulo*. A) HEK293T cells were transfected with different construction of mt-ArgRS (WT and mutants). Mitochondria were enriched and fractionated into soluble (containing matrix and intermembranes space proteins) and membrane (containing inner and outer membrane proteins) fractions. Representative western blot of WT and mutants mt-ArgRS detected with anti-Flag antibody. Detection of both SOD2 and Prohibitin were performed as loading control of fractions. B) Fibroblasts from patients. Representative western blots detecting WT and mutants mt-AspRS and mt-ArgRS, and prohibitin as a control, using specific antibodies. "Control" skin fibroblast is from a healthy control person. "PCH6-patient" and "LBSL-patient" correspond to skin fibroblasts from patient PCH6 (p.T100Cfs*5/p.A10V) and patient LBSL (p.R76SfsX5/p.G338E), respectively. R: Residual; S: soluble fraction; M: membrane fraction.

3. *In vitro* analysis of the impact of pathology-related mutations on mt-ArgRS canonical function

In the first chapter, the description of the disease caused by the mutations in *RARS2*, encoding for the mt-ArgRS, was made. The severity of the pathology was established when the clinical manifestations of the disease were compared to those produced by other mt-aaRSs. An example of this is that in some cases the symptoms in some patients are limited to weakness in the lower limbs, as has been reported for some LBSL patients. However, regarding PCH6 patients, the motor and cognitive functions of the patients are so affected that most of the time

they are unable to carry out their daily activities on their own (Zhang *et al.*, 2018). Due to the severity of the clinical manifestations of PCH6, and to the high level of conservation of the mutated amino acid (at least in one of the two mutated alleles, see mysinpat.org) one could assume that the aminoacylation function of the mt-ArgRS could be affected as a result of the mutations. In this part of the work, the objective was to evaluate the impact of five reported mutations on the canonical function of the mt-ArgRS.

The chosen mutations to be studied were: p.Phe131Cys, p.Arg245Gln, p.Leu283Gln, p.Arg469His and p.His530Tyr. To facilitate the nomenclature, the “p.” will be omitted and the one-letter code will substituted the three-letter code to name the mutations, thus, the mutation p.Phe131Cys will be named here F131C. The selected studied mutations are distributed in the catalytic domain of the enzyme and in the anticodon-binding domain (see orange dots in **Figure 19** and **Figure 21** (mutations on the structure 3D)).

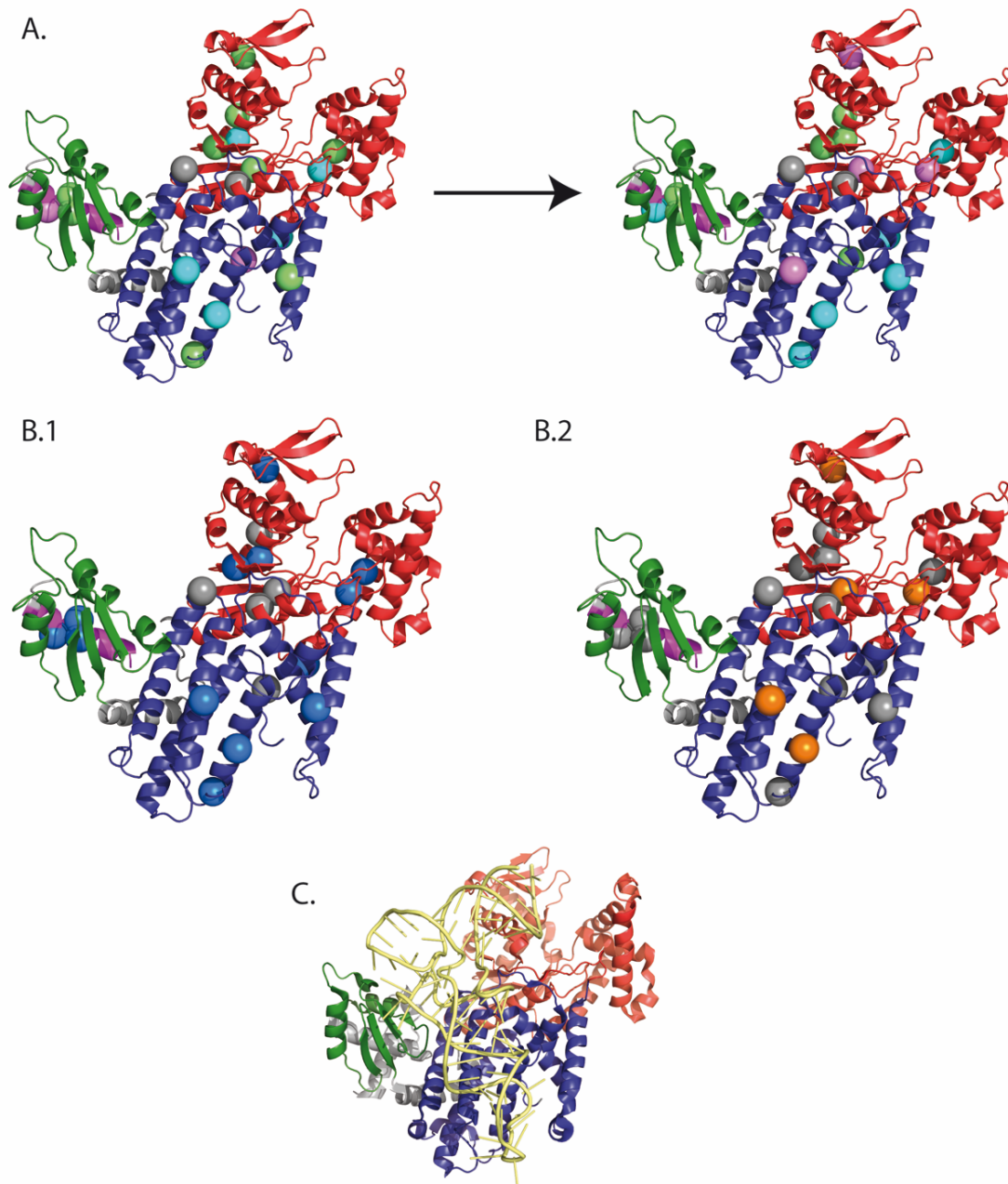


Figure 21. Human mitochondrial aminoacyl tRNA synthetases (mt-tRNA) mutations in 3D structural representation. Coordinates for 3D model (including MTS) of mt-ArgRS were uploaded from MiSynPat (<http://misynpat.org> (Moulinier *et al.*, 2017)). **A)** Representation of the reported mutated positions highlighted by the chemical nature, using the code colour showed in **figure 16**); left, chemical nature of the amino acid in the WT mt-ArgRS before the mutation; right, chemical nature of the new amino acid in the mutated mt-ArgRS. **B.1)** Representation in blue spheres of the selected studied mutations *in cellulo*. **B.2)** Representation in orange spheres of the selected studied mutations *in vitro*. **C)** Stereo view of the crystal structure of the yeast mt-AgRS (PDB: 1F7V) with its tRNA substrate in yellow (Delagoutte *et al.*, 2000). For all structures, the MTS is in pink, the tRNA edge-binding domain in green, the catalytic domain in red, and the anticodon-binding domain in blue.

These five mutations were chosen to represent different situations found in patients reported with PCH6. We thus considered i) mutations in a heterozygous state (R245Q and R469H are

present in a same patient, and L283Q is combined with a truncated protein); ii) mutations in an homozygous state (F131C in the catalytic domain and H530Y in the anticodon-binding domain); and iii) the conservation of the mutated residue within the sequence alignment (the arginine in position 469 affected by the R469H mutation is 100% conserved; see misynpat.org (Moulinier *et al.*, 2017)). As already mentioned above mt-ArgRS is a monomeric enzyme, in this case heterozygous composite patients present a mixture of two mutated proteins where each one carries a different mutation. It should be mentioned that we are not in a position to estimate whether these two populations are present in equivalent amounts or not. This situation is totally different from what exists for mt-AspRS, which is a homodimeric protein. Indeed, a LBSL patient *a priori* possesses a mixture of homo- and hetero-dimers.

3.1. Cloning, expression and purification of the mt-ArgRS WT and mutants

The mutations were introduced by directed mutagenesis in the pDEST plasmid containing the sequence coding for the mt-ArgRS-L₇₂ recombinant protein with a His₁₀-Tag in the C-terminal, following the protocol described in the section 3.1.2. In this experiment, the form mt-ArgRS-L₇₂ is representative of the wild type (WT) mt-ArgRS (see Chapter II). Expression and purification were performed simultaneously for the WT enzyme and a single mutant at one time following the same experimental conditions (in order to obtain two sets of proteins prepared under strictly comparable conditions). **Figure 22** represents an example of the protein purification. While the western blot seems rather encouraging, the coomassie blue staining shows that the protein remains highly contaminated after concentration. Given that the yield of protein expression is not highly effective (since 3 L of culture are necessary to obtain ~10 to 20 mg of crude proteins extract) and the level of contamination is identical for both versions of the protein (WT or mutant), we agreed to perform this first series of experiments in the conditions described as follows. However, we are aware that these represent only preliminary data, and the conditions of purifications should be optimized to improve the quality and the purity of the protein.

Expression of recombinant proteins was induced with IPTG, under the control of the *lac* operon and the T7 promoter. The purification protocol is based on previous expertise in the laboratory with purification of other mt-aaRSs by affinity chromatography (see section 5 in Materials and Methods). Briefly, cell extracts were sonicated and ultracentrifuged. The resulting supernatant was loaded onto a HIS-Select[®] HF Nickel Affinity Gel resin column previously equilibrated in a phosphate buffer. After extensive wash of the column, the proteins

were eluted with a linear gradient containing imidazole. All steps were monitored using the *BioLogic DuoFlow System* from BIO-RAD. Fractions containing the protein were pooled and concentrated. All the constructions were expressed and soluble. As already mentioned, an optimal level of purity of the enzyme was not reached; this was evidenced by multiple unspecific bands in the coomassie gel. To try to improve the purity of protein samples, some modifications were made to the buffers, like increasing the NaCl concentration from 150mM to 450mM. This attempt resulted, however, to the aggregation of the protein. Several aliquots were taken during the different purification steps, and loaded onto a 10% SDS-gel. Protein bands were detected by Western blot analysis using anti-His antibody (**Figure 22**). In figure 19 we can appreciate a typical result of the version mt-ArgRS-L₇₂ versus the F131C mutant. For each experiments, the proteins samples were analyzed on two gels: one to perform western blot analysis and the second one for blue coomassie staining.

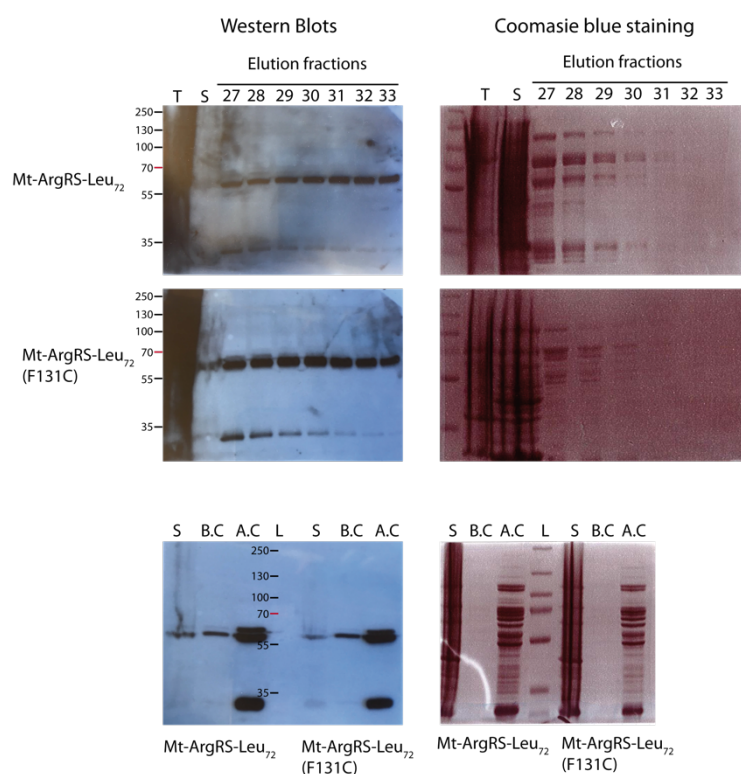


Figure 22. Expression, purification and concentration of the Mt-ArgRS-Leu₇₂ and the variant Mt-ArgRS-Leu₇₂(F131C). *E. coli* BL21 cells were transformed with the plasmid pDEST carrying the mt-ArgRS coding sequence starting at amino acid Leucine 72. Samples were taken after sonication (T-Total), ultracentrifugation (S-Soluble), and different elution fractions, and were loaded onto a 10% SDS-PAGE gel (in duplicate). One of the gels was used to western blot detection using an anti-His-tag (left column gels), and the other gel was used to a coomassie blue staining (right column gels). Samples obtained before and after concentration (B.C and A.C, respectively) of the eluted protein were also loaded onto a 10% SDS-PAGE gel.

3.2. Aminoacylation assays

The purified WT mt-ArgRS and five missense variants were used to perform *in vitro* aminoacylation in order to determine their ability to aminoacylate tRNA^{Arg}. Protein concentration was estimated using NanoDropTM spectrophotometer considering the theoretical extinction coefficient of mt-ArgRS ($\epsilon = 59120 \text{ M}^{-1}\text{cm}^{-1}$).

In vitro arginylation was performed in the presence of an established concentration of isotopic mixture of Arginine, total tRNA from *E. coli* and mt-ArgRS. At the end of the reaction, the detection of the radioactivity in counts per minute (cpm) allowed the quantification of the tRNA^{Arg} (in pMoles) aminoacylated with radioactive arginine. Total tRNA from *E. coli* was habitually purified from bacterial cultures in the laboratory by Agnès Gaudry. From the quantification of the total tRNA we estimated the concentration of the tRNA^{Arg} considering that this tRNA represents 1/20 of the total tRNA population. Routinely 40 pmoles of tRNA^{Arg} from *E. coli* were used for each aminoacylation reaction, however this concentration was modified in some assays to observe the possible changes in the reaction in the presence of more (or less) substrate. Different concentrations of enzyme (50 nM, 100 nM, 200 nM, 250 nM, 400 nM, 800 nM and/or 1500 nM) were also tested. The aminoacylation reaction mixture was equilibrated to 37°C before the addition of the enzyme to a final volume of 50 µl. At different times (5 min, 10 min, 15 min and 30 min), 10 µl of the aminoacylation reaction were spotted onto Whatman papers previously equilibrated with 5% trichloroacetic acid (TCA). The papers were immediately placed in cold 5% TCA to precipitate tRNA and terminate the reaction. The papers were then washed and dried. The dried papers were placed in counting vessels and incorporation of radioactive arginine was measured by liquid scintillation counting using a scintillation counter.

Due to the particularities of the human mt-ArgRS (not known before the present study) as for example the attachment to the mitochondrial membrane by hydrophobic interaction, experiments previously performed in the laboratory attempting to purify the human mt-ArgRS never yielded promising results. Thus, the results of experiments in which *in vitro* assays were tested with the human mt-ArgRS never showed a catalytic activity. One of the slightest but seemingly favorable changes for enzyme purification was to change the component of the buffer from potassium to NaH₂PO₄/Na₂HPO₄ pH 7.5. Until now it was a determining factor that showed us the most encouraging results.

Since no pre-existing functional enzyme was available for positive controls, it was absolutely necessary to verify whether the experimental conditions were optimal to the functionality of our human mt-ArgRS-L₇₂ in aminoacylation assay. To establish the experimental conditions, one of the most important factors to consider for the *in vitro* aminoacylation assays is the ATP/MgCl₂ ratio (Sohm *et al.*, 2003). Previous *in vitro* aminoacylation assay of mt-aaRSs commonly used a buffer with a ratio ATP/MgCl₂ ~0.20 (Bonfond *et al.*, 2005a; Sohm *et al.*, 2003). However this ratio did not work for the mt-ArgRS

requiring hardly optimization by several assays by Agnes Gaudry, until reach an optimal ratio of 1 (8 mM ATP/ 8 mM MgCl₂). Thus, the first step was to test the WT mt-ArgRS-L72. Even though the step of protein purification was improved in our experiments, we are aware that the low level of purity of the protein represents a major limitation to our work. Thus, unusually large amount of enzyme was needed to perform the aminoacylation assays. Nevertheless, the fact that the experiments were performed in the same established conditions allow comparing the enzymatic activity obtained between the WT mt-ArgRS and the mutants. Here 10 µl of the purified WT mt-ArgRS-L72 were used to perform the reaction. The results of the assay are shown in **Figure 23**, revealing that the mt-ArgRS-L72 aminoacylates its cognate tRNA from *E. coli*. This result showing the aminoacylation even up to ~50 pMoles of tRNA^{Arg} indicates that probably the initial calculated tRNA^{Arg} concentration was underestimated. While trying to improve the aminoacylation conditions, some others tRNAs (*in vitro* tRNA^{Arg} transcript and yeast tRNA^{Arg} commercially purchased) were used. None of them were efficiently aminoacylated by the mt-ArgRS, under our experimental conditions. Of note, an enzymatic activity was efficiently measured solely with the total tRNA from *E. coli*. The experimental conditions were, however, not optimal to reach a plateau aminoacylation in 30 min of reaction. These results further suggest that the quality and/or the quantity of the enzyme was not sufficient.

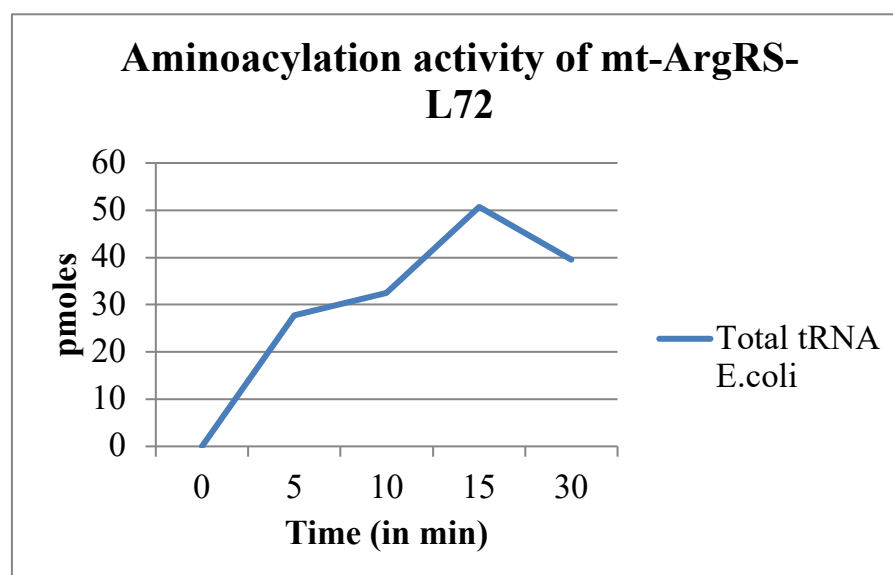


Figure 23. Aminoacylation activity of the human mt-ArgRS-L72. Aminoacylation assay was performed using 100 nM of protein and 40 pmol tRNA^{Arg} from *E. coli* in a buffer pH 7.5 at 37°C. Aliquots of 10 µl were taken at 0, 5, 10, 15 and 30 min of reaction.

4. Impact of PCH6-mutations on aminoacylation activity

To test the impact of PCH6-related mutations on the aminoacylation function of the mt-ArgRS, aminoacylation reactions were performed using each time the "WT" mt-ArgRS (L72) and one mutant in the same experiment (**Figure 24**). Aminoacylation assays were performed at least in two individual experiments (n=2) using protein samples obtained from a single purification process. Enzymatic activities were calculated in UE/mg, representing the pMoles of amino acid incorporated per mg of protein per minute. The means of the mt-ArgR-L72 enzymatic activities were normalized and arbitrarily set to the value of 1, in order to compare and establish a relation between the enzymatic activities of the WT vs. the mutants. The results are given in **table 3**.

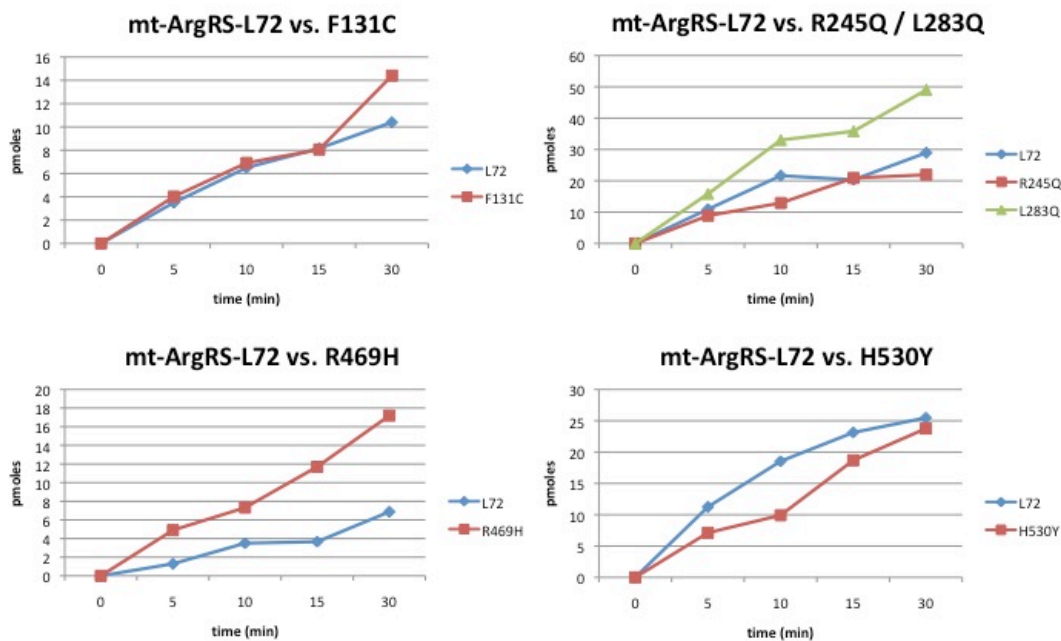


Figure 24. Arginine charging of mt-tRNA^{Arg} by the wild type human mt-ArgRS and F131C, R245Q, L283Q, R469H and H530Y mutants in *in vitro* aminoacylation assay. Aminoacylation assays were performed using ~100nM of protein, and total tRNA from *E. coli* (in a quantity that correspond approximately to 40 pmol of tRNA^{Arg}) at 37°C. Aliquots of 10 µl were taken at 0, 5, 10, 15 and 30 min.

	Relative enzymatic activity
mt-ArgRS-L72 (WT)	1
mt-ArgRS-L72/F131C	1.1
mt-ArgRS-L72/R245Q	0.4
mt-ArgRS-L72/L283Q	0.15
mt-ArgRS-L72/R469H	3

Table 3. Relative enzymatic activity of the mt-ArgRS-L72 compared with five PCH6-related mutations.

Table 3 shows that mt-ArgRS-L72/F131C has an enzymatic activity very similar to the one of mt-ArgRS-L72(WT). mt-ArgRS-L72/R245Q, mt-ArgRS-L72/L283Q and mt-ArgRS-L72/R469H have activities 2.5 fold decreased, ~6 fold decreased and 3 fold increased, respectively. These results were extremely striking. We previously proposed that the severity of the symptoms caused by PCH6 could be linked to drastic effects on the aminoacylation function of the mutated enzyme. Surprisingly, in all the studied mutations, the catalytic function of the enzyme was maintained, very mildly affected or even increased. Indeed, one of the cases to highlight is the mutation of arginine 469, which is 100% (strictly) conserved in the alignment of the sequences (Moulinier *et al.*, 2017). It is widely believed that mutation of strictly conserved residues would be detrimental to the architecture or the activity. We show here that the relative enzymatic activity of the mutation R469H is reproducibly increased 3 fold when compared with the WT enzyme. R469H is present in patients in a heterozygous state, associated with mutation R245Q. Strikingly, the R245Q mutation leads to a decrease of 2.5 fold of the aminoacylation activity, suggesting a compensatory effect that would lead to normal activity. This is however speculative. Actually, our *in vitro* aminoacylation assays do not reproduce the genetic situation of the patients, where the activity of the synthetase is determined by a combination of two different mutant alleles, with possibly two distinct levels of expression. Thus, we are not able to determine whether a compensatory effect by one mutation could rescue (or impair in cases of increased impacts) the enzymatic activity of the protein. Nonetheless, the experiments performed here reveal that none of the tested PCH6-related mutations has a drastic impact on the arginylation property of the mt-ArgRS, which leaves unsolved the pathogenic mechanism of these mutations.

Earlier aminoacylation studies for the impact of disease-related mutations on other mt-aaRSs had been performed, and variable effects had been shown. The available aminoacylation data reveal diverse (e.g., mt-AspRS (Scheper *et al.*, 2007), mt-PheRS (Walker *et al.*, 2016)) or only a weak (e.g., mt-TyrRS (Riley *et al.*, 2013), mt-LeuRS (Riley *et al.*, 2016)) effects of the disease associated mutations on the level of aminoacylation. The host laboratory has carried several investigations in order to decipher the relationship between LBSL mutations on the enzyme activity of the mt-AspRS. A series of mutations have been tested and showed variable impacts on the aminoacylation property of the human mt-AspRS, ranking from no impact, to a decrease of ~80 fold (Scheper *et al.*, 2007; van Berge *et al.*, 2013). Most importantly, the authors have shown situations where non-deleterious mutations are present in a same patient. This investigation has been extended to other functional and structural parameters and revealed that the analyzed mutations have no impact on the mt-AspRS architecture (Sauter *et al.*, 2015), but distinct and variable impacts on mt-AspRS expression (van Berge *et al.*, 2013), dimerization (van Berge *et al.*, 2013), translocation from the cytosol to the mitochondria (Messmer *et al.*, 2011), *in cellulo* solubility (Sauter *et al.*, 2015), or on intramitochondrial localization (Gonzalez-Serrano *et al.*, 2018). Strikingly, two of the investigated mutations have no noticeable impact on any of these parameters. Altogether, the fact that disease-related mutations impact variably the investigated properties of the mt-AspRS is perplexing and points to the fact that the housekeeping role of the enzyme in protein synthesis is not the general target of the mutations.

For mt-PheRS, experimental *in vitro* aminoacylation measurements revealed an impact of the p.I329T replacement on aminoacylation levels (~4-fold decrease in the catalytic efficiency of amino acid activation) (Elo *et al.*, 2012). Biochemical characterization of recombinant mutant mt-PheRS proteins showed that mutations can affect synthetase function in different ways, such as by reducing the binding of ATP, of phenylalanyl, or of tRNA, and/or by affecting synthetase folding and stability (Elo *et al.*, 2012). Further investigations were performed for the mt-PheRS, demonstrated that mutated mt-PheRS on p.P85A charges tRNA very similarly to WT, but p.H135D demonstrated failure in aminoacylation activity (Walker *et al.*, 2016).

In vitro aminoacylation assays of a recombinant p.Gly191Asp *YARS2* displayed a 38-fold loss in catalytic efficiency compared to the wild-type mt-TyrRS (Riley *et al.*, 2013). The authors predicted reduced mitochondrial protein synthesis, as it had been previously shown with the p.Phe52Leu mutation on mt-TyrRS, where the reduction in aminoacylation efficiency resulted in defective mitochondrial protein synthesis (Riley *et al.*, 2010).

Investigations were done in two mutations of *LARS2* founded in the allelic composition of a patient presenting Sideroblastic Anemia (Riley *et al.*, 2016). Recombinant mt-LeuRS p.Thr522Asn and p.Ala430Val presented a 9- and 18-fold loss of catalytic efficiency, respectively. The authors suggested that the combined effect of the two mt-LeuRS variants on aminoacylation activity in the patient most likely results in reduced mitochondrial protein synthesis in affected organs and hence mitochondrial RC dysfunction. They supported this idea with data showing respiratory chain deficiencies in the muscle and liver (Riley *et al.*, 2016).

Here we show that, at least *in vitro*, the aminoacylation function of the mt-ArgRS is maintained when testing individual mutants. So far, the effect of pathological mutations using recombinant proteins and testing *in vitro* aminoacylation has been limited to the systems described above (mt-TyrRS, mt-AspRS, mt-PheRS, mt-LeuRS and mt-ArgRS in this work). They reveal distinct ranges of impacts. Further and complementary investigations on other mt-aaRSs seem necessary to provide valuable information in understanding how the mutations could produce dysfunctions on these proteins, if any.

5. Conclusion and perspectives

From the report of LBSL, the first mt-aaRS-related pathology with mutations on mt-AspRS, it has long been suggested that the cause of the diseases produced by mutations on mt-aaRSs is due to an impact on the ability of the enzyme to aminoacylate its cognate tRNA. The host laboratory and other teams have studied the effect of some mutations on the canonical function of the mt-aaRSs and the results are fluctuating. Since mt-aaRSs were supposed to be solely involved in the mitochondrial translation, this hypothesis of impact on the canonical function was conceivable. Given that disease-related mutations in mt-aaRS genes are present in all nuclear copies of the genome, a constitutive failure in mitochondrial translation in all organs could have been expected. However, this is not the case and it has been recalled at several occasions that although there is a target towards the central nervous system, the clinical manifestations extend to other anatomical systems.

Despite great efforts made to date, it was not yet possible to establish a mechanism for the pathogenicity of mutations on the mt-aaRSs that could explain the tissue specificity. The results of my work together with additional data obtained by others on other mt-aaRSs strongly suggest the absence of a common and unique mechanism correlating mt-aaRSs mutations and the reported diseases. Thus, different molecular mechanisms specific to each synthetase (or common to a few of them) might be conceivable. Additional experiments will be indispensable

before being able to reach conclusive outcomes. For instance, at this stage, a contamination with the *E. coli* ArgRS cannot be excluded. One possible experiment could be to design a new variant of the human mitochondrial ArgRS with a point mutation within one of the catalytic residues. This variant should be fully inactive and be considered as a negative control (meaning in case we observe activity similar to the one obtained for mt-ArgRS L72, this would demonstrate that the activity is exclusively provided by the contaminant *E. coli* ArgRS). In any situation, Northern blot experiments made on mitochondria (obtained from fibroblast from PCH6-related patients) that are under preparation should provide valuable complementary pieces of data and should confirm if the activity is affected or not. To mention, however, in order to be truly complementary, fibroblasts should need to be obtained from patients with a mutation identical to the one tested within our *in vitro* experiments.

Perhaps the manifestation of diseases, rather than the consequence of a specific event such as aminoacylation, represents a combination of incidents, such as the alteration of the components of mitochondrial translation that leads to an alteration of the production of ATP, and the possible alteration of some hitherto unknown other function of these enzymes. One can indeed hypothesize a possible participation of these enzymes in molecular mechanisms that would be tissue-specific (such as e.g. the formation of specific components of the central nervous system as neurotransmitter) or that would be specific to some developmental stages. Speculatively, the alteration of such mechanism would then lead to the disease. Due to the specific injuries to the neuronal tissue, studies of the possible integration of these enzymes in a neurophysiologic context, for example in the formation of myelin, or in the regulation of energy production in a neuronal environment, should help in the understanding of the reported pathological processes. To further explore the tissue-specific issue some strategies have been initiated in our laboratory searching a possible involvement of mt-aaRSs in local processes of the central nervous system. One of them is the study of effects on neural cells by LBSL-mutations (See annex).

Annexes

Annexes

1. Background: Release of the human mt-AspRS from mitochondria

A former PhD student Loukmane Karim demonstrated that under some conditions, the mt-AspRS display an extra-mitochondria localization. During the screening of chemicals to see whether the dual localization of mt-AspRS is dynamic, few of these chemicals (among which 10 mM of ATP) provoked the unexpected release of mt-AspRS from mitochondria into the extra-mitochondrial medium (See **Figure 25**). The applied protocol was as follows: enriched mitochondria were incubated for 10 min with different concentrations of ATP (0.1, 1, 2, 4, 6, 8, 10, 20 and 50 mM), and ADP (10 mM). After treatment, mitochondria were centrifuged 10 min at 16 000 g (4 °C), then the supernatant corresponding to the extra-mitochondrial medium (EM) was separated from mitochondrial pellet. EM were ultracentrifuged 30 min at 125 000 g (4 °C), then concentrated in order to be analyzed by western blot, dynamic light scattering (not shown here), mass spectrometry or electron microscopy (not shown here).

Mt-AspRS was detected in both soluble and membranes fractions after fractionation of untreated mitochondria, however mitochondria treated with either ATP or GTP (10 mM) displayed highly reduced intensities of mt-AspRS in soluble and membranes fractions, but significant intensities in the extra-mitochondrial media (EM) (**Figure 25**). This observation was specific to mt-AspRS since neither SOD2 nor Prohibitin were detected in the EM, but remained in their respective fractions, whatever is the applied treatment. These observations clearly showed the release of mt-AspRS outside mitochondria upon treatment with either ATP or GTP.

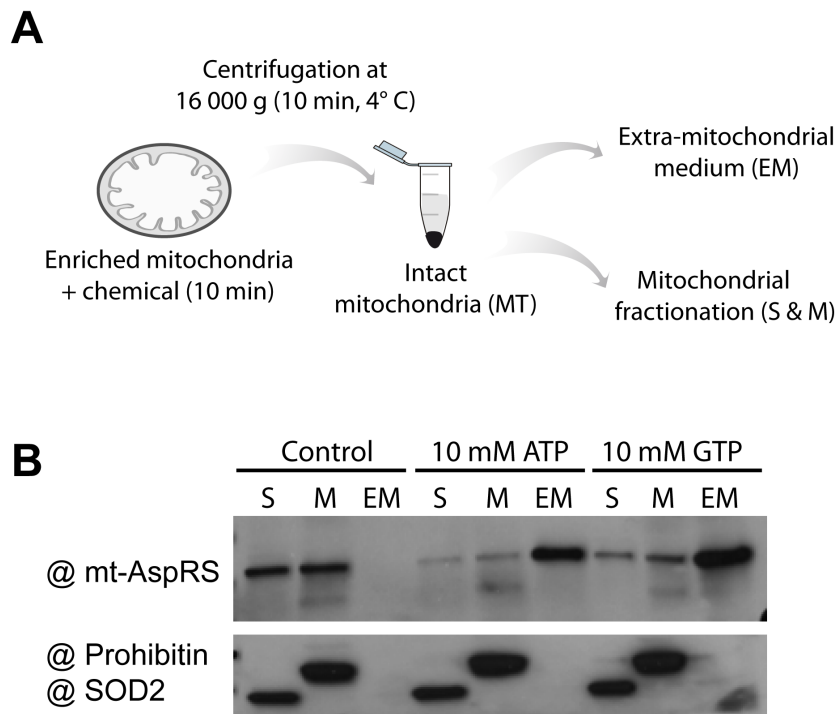


Figure 25. Analysis of the extra-mitochondrial medium (EM) after ATP or GTP treatment. Mitochondria were purified from HEK293T cells and treated with 10mM of ATP or GTP. **A.** Workflow of mitochondrial treatment and fractionation. **B.** Untreated mitochondria (control) or treated with 10mM of either ATP or GTP prior to fractionation were analyzed by western blot. Prohibitin and SOD2 were used as control to proof the purity of the S and M fractions, and the integrity of the mitochondrial upon treatment. S, and M, Soluble and Membrane fraction, respectively. Figure taken from L. Karim's Thesis - Oct, 2016.

Additional investigations revealed that other proteins, among which the Cyt *c*, are also released out of the enriched mitochondria under the same experimental conditions. These experiments haven't yet been pursued, and neither the biological relevance of the release of the mt-AspRS nor the coincidental release of the Cyt *c* (and thus a possible role of the mt-AspRS in cell death or survival (pro- or anti-apoptotic)) has been further investigated.

My contribution in the follow-up of this research was the application of the protocol established by L. Karim to mitochondria that had been previously transfected with plasmids expressing the mt-AspRS with LBSL-related mutation. The aim was to investigate whether LBSL-related mutations have an impact on the extra-mitochondrial re-location of the mt-AspRS. To do this, BHK 21 cells were transfected with the plasmid pBCJ-mt-AspRS, encoding for the WT and the mutants [c.133A>G (p.S45G), c.172C>G (p.R58G), c.406A>T (p.T136S), c.550C>A (p.Q184K), c.788G>A (p.R263Q), c.1013G>A (p.G338E), c.1837C>T (p.L613F) and c.1876T>A (p.L626Q)] versions of the enzyme. Results are shown in **Figure 26**.

We can appreciate that no matter which mutation was tested, the mt-AspRS always re-located to the EM fraction upon ATP treatment.

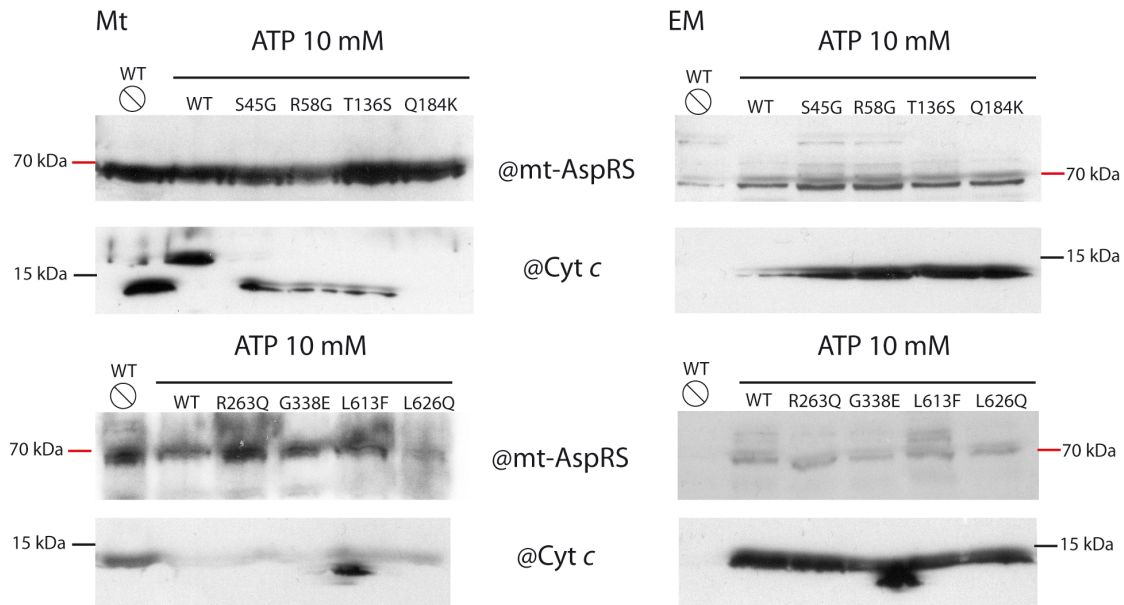


Figure 26. Extramitochondrial location of the mt-AspRS. BHK 21 cells were transfected with WT or LBSL-related mutant versions of the mt-AspRS. Mitochondria were enriched prior to treatment with ATP (10 mM), and the Mitochondrial (Mt) and extramitochondrial (EM) fraction were analyzed by western blot. WT ⊙: Mitochondrial with the WT mt-AspRS untreated (control). Cyt *c*, Cytochrome *c*.

This new location of the mt-AspRS in an extra-mitochondrial compartment goes in line with the so-called possibility of the involvement of the mt-aaRSs in additional or alternative processes others to aminoacylation.

2. Human mt-ArgRS is not released from mitochondria

To continue with this study, we then wanted to know how the mt-ArgRS would react to the same ATP treatment. Florian Pierre (Laboratory Engineer) applied the previously established protocol, using enriched mitochondria from HEK293T cells, on which 10 mM of ATP were applied prior to the separation onto the two fractions (mitochondria and extramitochondria). Mt-AspRS was systematically identified in the western blot analysis, as a control of a protein that relocates to the extramitochondria fraction. We can appreciate (see **Figure 27**) that contrary to the relocation profile shown for mt-AspRS, the mt-ArgRS remains in the "mitochondrial" fraction when subjected to 10 mM of ATP.

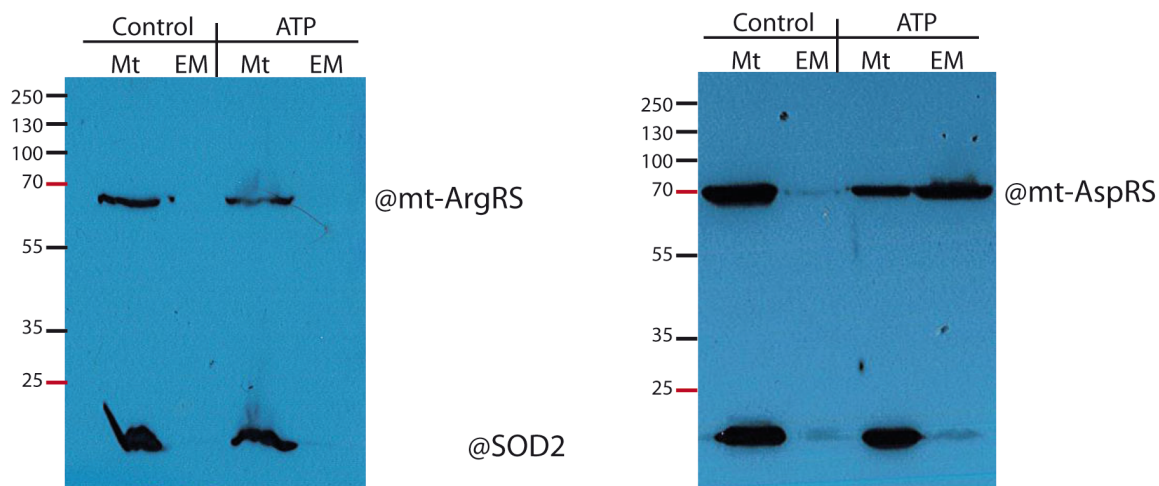


Figure 27. Analysis of the extramitochondrial medium (EM) after ATP treatment. Mitochondria purified from HEK293T cells are treated with ATP (10mM). Proteins from mitochondria and extramitochondrial media are analyzed by western blot. Left panel) @mt-ArgRS detection exclusively in the mitochondrial fraction (Mt) in both conditions: without ATP (control) *versus* incubation with 10 mM ATP (ATP). Right panel) @mt-AspRS detection in both Mt and EM fraction when using ATP, and just in the mt fraction in the control. SOD2 detection was used as control to proof of the fractions.

These results are astonishing; a distinct behavior of these two proteins, the mt-AspRS and the mt-ArgRS has already been highlighted and discussed throughout this thesis work. Here we add an additional distinguishing feature between these two enzymes, for which (we remind once again) the sole yet known function is to participate to the mitochondrial translation. Although these observations further extend our understanding of the mt-aaRS, they also raise more questions. Why the mt-AspRS is the only synthetase released from the mitochondria in stress condition? Could the fact that the ArgRS is membrane-anchored an impediment to the relocation into other compartments? Is this relocation an exclusive property of the mt-AspRS? Or do other mt-aaRS also relocated under some stress conditions? What is the biological relevance of this new location of the mt-AspRS outside the mitochondria? There are certainly directions that are waiting to be explored. Surely, as these answers will be found the global picture of mt-aaRS will expand the participation of the mt-aaRSs beyond their role in mitochondrial translation.

3. Search for mt-AspRS neuron-specific partner protein(s)

Several arguments led us to propose a non-canonical function/property (likely neuronal-specific) of the human mt-AspRS that will need to be searched for. Those arguments are i) the absence of a clear and/or common impact(s) of LBSL-related mutation on the canonical function of the mt-AspRS; ii) the observation that this enzyme has peculiar cellular properties [dual intra-mitochondrial location (see Chapter II), release out of mitochondria under some

stress conditions (see above)]; iii) the observation that LBSL-related mutations are mainly situated at the dimeric interface (not shown).

Also, the classification made in Chapter I identified three mt-aaRSs (mt-AspRS, mt-GluRS and mt-AlaRS) for which mutations lead to a leukodystrophy. Additional reports from the literature correlate as well mutations within the cytosolic AspRS to a similar leukodystrophy (Wolf *et al.*, 2014). When looking at the degree of conservation of the affected positions (using misynpat.org) for the three mitochondrial enzymes (see **Table 4**), we suggest that leukodystrophies linked to mutations in mt-GluRS or mt-AspRS (at non-conserved positions) are likely due to a molecular mechanism different from the one leading to the leukodystrophy linked to mutations of mt-AlaRS (at highly conserved positions) for which aminoacylation deficiencies are strongly suspected (Sissler *et al.*, 2017). In the former two cases, however, it has been shown that, when considering exclusively the subphylum of mammals, all reported mutations impact positions highly/strictly conserved ((Sauter *et al.*, 2015) and unpublished data). This observation suggests for these two enzymes a selective pressure restricted to mammals.

		Conservation of affected position	
Related leukodystrophy		throughout phylogeny	in mammals subphylum
Mt-AlaRS	ALSP	+++	+++
Mt-AspRS	LBSL	∅	+++
Mt-GluRS	LTBL	∅	+++

Table 4. Schematic representation of the conservation in affected positions either throughout phylogeny, or in the subphylum of mammals.

Mt-AspRS, mt-GluRS and the cytosolic AspRS have in common the aspartate and the glutamate, amino acids that are essential precursors in the formation of the N-Acetyl-L-Aspartate Glutamate (NAAG), a neurotransmitter of the central nervous system. One hypothesis, currently investigated in the laboratory, is to search for a possible link between mt-AspRS and Asp-NAT (L-aspartate N-acetyltransferase). The L-aspartate N-acetyltransferase (Asp-NAT, encoded by the *NAT8L* gene) is a mitochondrial inner membrane-bound enzyme, highly specific to L-aspartate and exclusively expressed in the neurons (Truckenmiller *et al.*, 1985; Wiame *et al.*, 2009). It synthesizes N-acetyl L-aspartate (NAA), one of the most concentrated molecules in the CNS, from N-acetyl Coenzyme A and L-aspartate (Moffett *et al.*, 2007). NAA is transferred to the oligodendrocytes (the neuroglial cells responsible for creating the myelin sheath), where the aspartoacylase, aminoacylase II (ASPA) hydrolyzes it

and uses the acetate moiety for lipid and myelin syntheses (Madhavarao *et al.*, 2005). NAA is also the precursor of N-acetyl L-aspartate glutamate (NAAG), a major neurotransmitter in the brain (Moffett *et al.*, 2007) (**Figure 28**). An alteration of both the myelin sheath of the neurons and of the NAA concentration in the cerebrospinal fluid has been reported in patients with leukodystrophy due to mt-AspRS mutations (van der Knaap *et al.*, 2003).

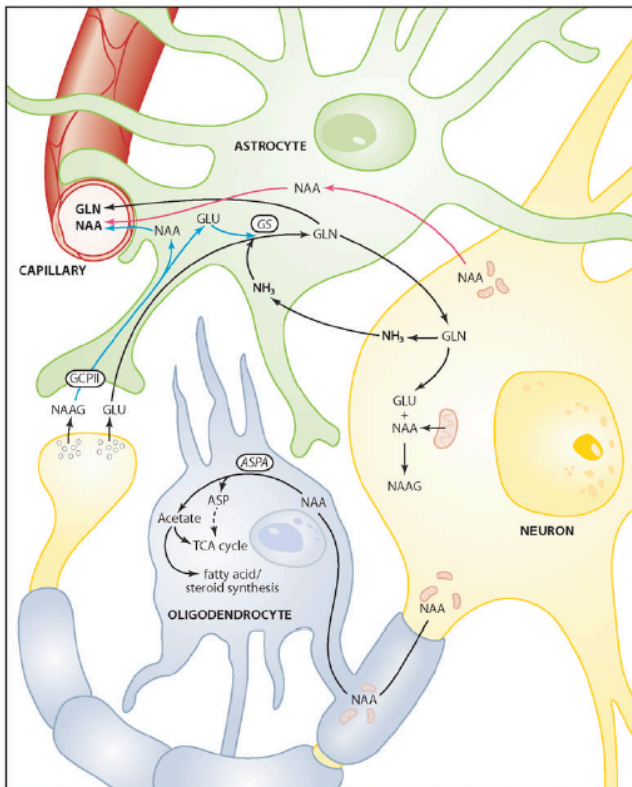


Figure 28. NAA synthesis and metabolism in the brain. NAA is synthesized in neuronal mitochondria, and can then either be transported to oligodendrocytes for fatty acid synthesis and energy production, or can be used for the synthesis of NAAG in neurons. Taken from (Moffett *et al.*, 2007).

3.1. Asp-NAT expression in HEK293T cell line

The two enzymes, Asp-NAT and mt-AspRS, share the aspartate as a substrate. A novel hypothesis is mt-AspRS has a role in the process of NAA synthesis (see above). We thus propose to search for a possible link between mt-AspRS and Asp-NAT. Asp-NAT being exclusively expressed in neurons, an impairment of such process by LBSL-related mutations might then explain the tissue-specificity of the disease. Of note, NAA synthesis was demonstrated in SH-SY5Y cells suggesting the expression and functionality of Asp-NAT in neuroblastoma (Arun *et al.*, 2009); and transfected HEK cells expressing Asp-NAT (not naturally expressed in kidney) were shown to artificially synthesize NAA (Wiame *et al.*, 2009). SH-SY5Y, Asp-NAT-transfected HEK and non-transfected HEK cells are thus good models to

investigate co-localization and physical interaction between Asp-NAT and mt-AspRS. If such an interaction exists, it should be visible in the two first cell types, but not in the last one.

In order to test the possible association between the mt-AspRS and Asp-NAT, we first transfected HEK293T cells with a pCI plasmid encoding to the Asp-NAT (34kDa) protein. Given that Asp-NAT is only expressed in neurons it was necessary to verify whether the protein was expressed, correct translocated to the mitochondria and well located in the mitochondrial membrane (**Figure 29**).

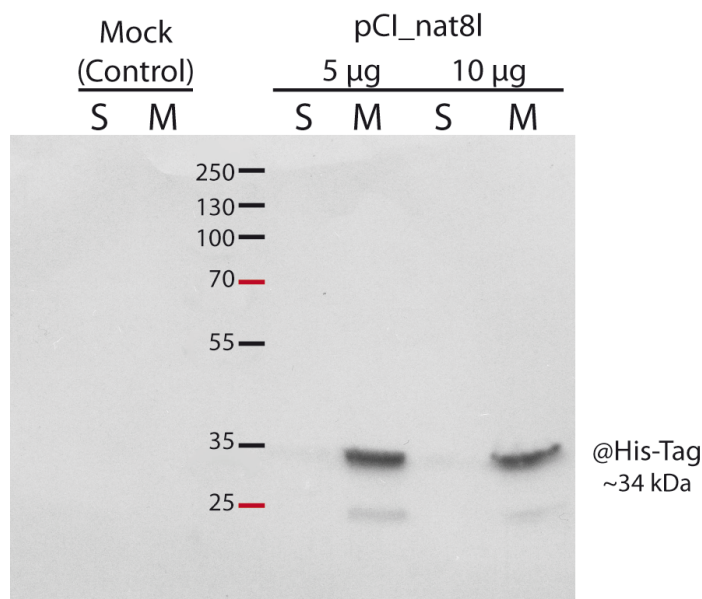


Figure 29. Asp-NAT detection in HEK293T cell. Enriched mitochondria were transfected with the pCI plasmid containing the gene *nat8l* encoding for the Asp-NAT protein prior to mitochondrial fractionation to verify the correct expression and location of Asp-NAT by western blot analysis. The detection was done using an anti-His-tag encoded by the plasmid, at the C-terminus of the protein. S, soluble; M, membrane fraction.

We wonder whether the expression of Asp-NAT display an effect on mt-AspRS double location inside mitochondrial. In order to evaluate this, HEK293T cells were transfected with pCI plasmid containing *nat8l* gene followed by a sequence coding for an His-Tag. The, western blots analysis was performed first, to confirm Asp-NAT expression and second to check on mt-AspRS location. Mt-AspRS was mainly detected in the membrane fraction in both transfected and non transfected cells (**Figure 30**).

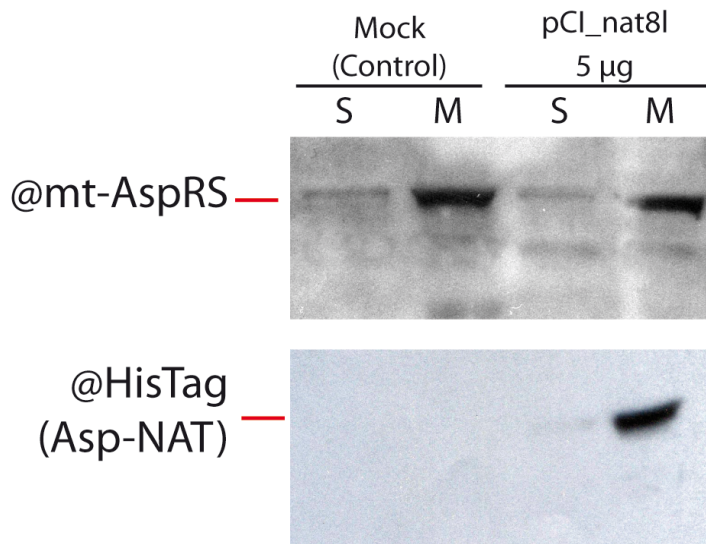


Figure 30. mt-AspRS location in Asp-NAT presence. HEK293T cells were transfected with pCI plasmid + *nat8l* gene. Mitochondrial fractionation was performed prior to western blot analysis of the soluble and membrane fraction. Mock (control), no Asp-NAT; pCI_nat8l, Asp-NAT expression; S, soluble and M, membrane fraction.

After verification of the correct expression of the Asp-NAT protein, translocation to the mitochondrial and correct insertion in the mitochondrial membrane, we next performed Crosslinking experiences for the purpose of possible interaction of mt-AspRS and Asp-NAT.

3.2. *In vitro* Crosslinking of mt-AspRS

To continue the research of a possible interaction between these two proteins, *in vitro* crosslinking was performed (as described in **2.4.4 Material and Methods**). Different conditions were tested. To test the influence of the presence of Asp-NAT, HEK293T cell were transfected with the pCI plasmid containing the sequence coding for the Asp-NAT followed of a His-Tag. Briefly, 100 µg of mitochondria protein from transfected and non-transfected (as a control) HEK293T were incubated with glutaraldehyde (at 0.02% final concentration) for 20 minutes under rotation at room temperature. After incubation, the crosslinking reaction was stopped by quenching with Tris-HCl 1.5 M (pH 8.8). Samples were concentrated to a final volume of 80 µl and supplemented with 20 µl of Laemmli dissociating buffer for SDS-PAGE and Western blot analysis.

As shown in **Figure 31**, the proteins are detected at their correct theoretical molecular weights and in the expected fractions. We refer to “control” when the mitochondrial used were from cells not transfected with pCI-*nat8l*-HisTag. As expected, Asp-NAT is not detected by the anti-His-Tag antibody in non-transfected HEK293T cells. In the situation “Transfected Asp-NAT” the protein was correctly expressed at the expected theoretical molecular weight. The detection of the mt-AspRS was done using a specific antibody addressed against this

protein. A change in the quantity of the mt-AspRS is shown when using the crosslinker, and the appearance of higher molecular weight complexes is evident (**Figure 31**). Since these are preliminary results, it is necessary to repeat these experiments in order to draw accurate conclusions. In previous studies (not shown), similar high molecular weight complexes were observed when performing cross-linking experiments with Glutaraldehyde on recombinant mt-AspRS, expressed in *E. coli* and purified. We thus hypothesize that those high molecular weight forms correspond to an auto-association of the protein, leading to dimers and tetramers of mt-AspRS. What is puzzling is the mt-AspRS is not detected in the condition where Asp-NAT is expressed. Additionally, the antibody against the His-Tag detect of protein of ~70 kDa (left panel) in the same condition, indicating that the Glutaraldehyde either promotes the dimerization of the Asp-NAT or that this protein is truly cross-linked with a mitochondrial protein partner. Additional experiments are required before being able to reach any conclusion.

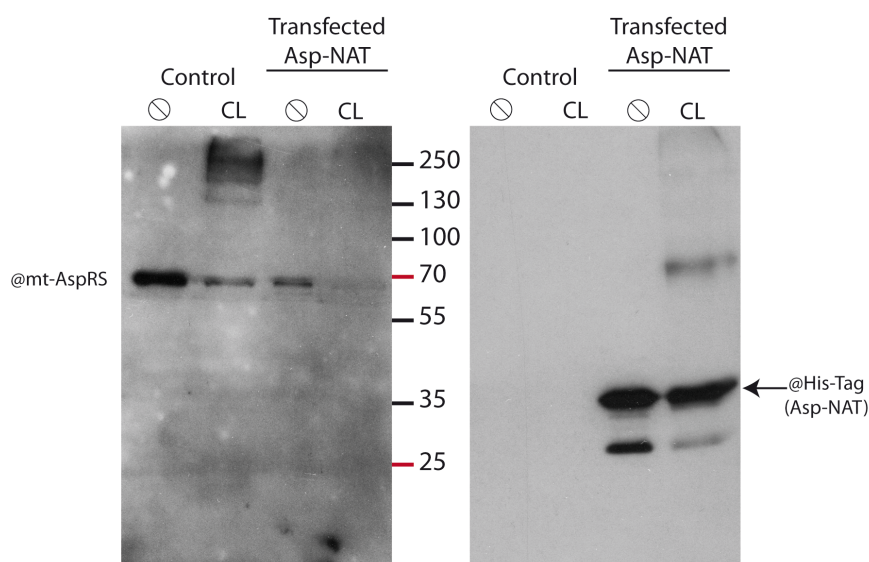


Figure 31. *In vitro* crosslinking of mitochondrial proteins by glutaraldehyde. Protein extracts from mitochondria from non-transfected (Control) or transfected (Transfected Asp-NAT) HEK293T cells were submitted to *in vitro* crosslinking. Glutaraldehyde was incubated during 20 min to 100 μ g of protein mitochondria (CL), washing buffer was used as negative control of the crosslinking (\odot). Western blot analysis was done using @mt-AspRS antibody and @His-Tag antibody (for the Asp-NAT).

4. Conclusions

The search for alternative functions for the mt-aaRSs has been an emerging topic of study over the past few years. The compendium of a series of characteristics dealt with in this thesis manuscript prompts us to propose research strategies aimed at seeking the involvement of at

least our two best known proteins, the mt-AspRS and the mt-ArgRS, in other physiological processes. We have highlighted at numerous occasions that mutation in patients of these enzymes lead to tissue-specificity impairments; mainly focus to the central nervous system. The observation of the double location for the mt-AspRS or of its release to the extramitochondrial environment under some stress conditions was the inspiration for the research proposed in this thesis annex.

More clues must be found, more strategies established. So, and to give continuity to the hypotheses raised here, other work proposals have been drawn up: *i) Biochemical characterization of a possible partnership in vitro*: Asp-NAT is membrane-bounded in mitochondria *via* transmembrane and proline-rich domains. Its production as an active recombinant enzyme in bacterial strain was recently established (Wang *et al.*, 2016b). Recombinant Asp-NAT could be prepared following the provided protocol, and a possible interaction with mt-AspRS may be characterized using classical biochemical approaches (mobility shift on native PAGE, gel filtration, among others). In case of a significant interaction with the wild-type mt-AspRS, impact of the LBSL-related mutations on the partnership would be characterized by investigating recombinant mutant mt-AspRSs. *ii) Characterization of partnership in cellulo and impact of LBSB-associated mutations*: The mt-AspRS was shown to have dual localization in HEK cells, and to be released out of purified mitochondria under stress conditions (see above). The aim here would be to investigate a possible co-localization between mt-AspRS and Asp-NAT *in cellulo*. As already mentioned, NAA synthesis was demonstrated in SH-SY5Y cells (Arun *et al.*, 2009), and, transfected HEK293T cells expressing Asp-NAT (not naturally expressed in kidney) were shown to artificially synthesize NAA (Wiame *et al.*, 2009). SH-SY5Y, Asp-NAT-transfected HEK293T and non-transfected HEK293T cells should be thus good models to investigate co-localization and physical interaction between Asp-NAT and mt-AspRS. If such an interaction exists, it might be visible in the neurons cell type and transfected HEK cells, but not in non-transfected HEK293T cells.

In all case, we do believe that in order to understand the physiopathological basis of the diseases produced by these proteins it is necessary the integration of these molecules in processes where their activity may be indispensable, and their dysfunctions accompanied by consequences translated into the manifestation of pathologies.

Conclusions and perspectives

Conclusions and perspectives

Scientific context. The first description of a correlation between a mutation affecting a mt-aaRS and a human disease dates back to a decade, when mutations within the *DARS2* gene (coding for the mt-AspRS) were associated with a leukodystrophy (Scheper *et al.*, 2007). This first description attracted the attention of the medical community so that many other disease-associated mutations have been described since. Today, mutations in all the 19 mt-aaRS-encoding genes have been reported and the related diseases are now described at an increased frequency and revealing to be complex (Diodato *et al.*, 2014a; Konovalova and Tyynismaa, 2013; Moulinier *et al.*, 2017; Rötig, 2011; Schwenzer *et al.*, 2014b; Sissler *et al.*, 2017). The complexity is not only due to the variety of phenotypic expressions, with tissue-specific phenotypic imprints, but also due to the absence, for the moment, of clear mechanistic explanations for most of the systems. This leads to an emerging demand for basic knowledge on functional properties and organization of mt-aaRSs. In this context, one of the main goals of our research group is to understand the fundamental mechanisms of the human mitochondrial translation machinery, focusing the interest on the mt-aaRSs and mt-tRNAs. In the past years, the host laboratory has particularly focused on the human mt-AspRS and established some functional (Bonfond *et al.*, 2005a; Fender *et al.*, 2012; Fender *et al.*, 2004; Messmer *et al.*, 2009; Neuenfeldt *et al.*, 2013; Schwenzer *et al.*, 2014a) and structural (Gaudry *et al.*, 2012; Neuenfeldt *et al.*, 2013) properties/peculiarities of this enzyme; as well as investigated its role in mitochondrial diseases (Gonzalez-Serrano *et al.*, 2018; Messmer *et al.*, 2011; Sauter *et al.*, 2015; Scheper *et al.*, 2007; van Berge *et al.*, 2013). The above-mentioned works plus the contributions of many other groups have rapidly revealed that the human mt-aaRSs have numerous functional and structural peculiarities (recalled in the introduction) that distinguish them from the classical view/knowledge, now of text-book type, established for aaRSs from other organisms. Those peculiarities and the lack of deep and comprehensive fundamental knowledge is a strong obstacle for the understanding of the related human disorders.

My contribution. The work performed during my PhD follows the long-standing efforts of the host laboratory but was aimed at expanding the interest and the knowledge towards an unexplored system: the human mitochondrial arginyl-tRNA synthetase. This enzyme was poorly characterized despite an increasing number of descriptions of patients, mutated in this enzyme and suffering from a severe neurodevelopmental disease. My PhD work was a significant contribution to this new orientation to the team's interests, and the major outcomes from my work are summarized here.

The clinical analysis of the phenotypes presented by the patients provided a more comprehensive vision of the mitochondrial pathologies related to mt-aaRS. This allowed the identification of groups of mt-aaRS that affect a same anatomical organ system: those affecting exclusively the central nervous system (CNS), affecting the CNS AND an other system, affecting the CNS OR an other system and the last one affecting other system than the CNS. Among those affecting the CNS, we could highlight differences in clinical manifestations, the severity of these as well as the age of onset of the disease. This was the case with LBSL and PCH6, which represent our comparative systems for two synthetases that cause damage to the CNS. **This categorization allowed an overview of the situation regarding the clinical manifestations of pathologies correlated with the mt-aaRSs.**

The analysis of the cellular properties of the mt-ArgRS was also essential and widened the collection of peculiarities so far described for human mt-aaRSs. Whereas mt-AspRS had been identified double localized in the mitochondria (in the soluble and in the membrane mitochondrial fractions), I demonstrated that mt-ArgRS is exclusively located in the membrane fraction and its anchoring is done by hydrophobic interactions. The observation of distinct locations for two enzymes presumably involved in the same cellular translation process suggests that these proteins have distinct cellular properties, thus probably allowing additional unveiled functions. The analysis is currently being extended to other mt-aaRSs in the laboratory, and so far now, it has been shown that two other enzymes, LysRS and mt-LeuRS, are present only in the soluble fraction. Thus, we establish the sub-mitochondrial localization of four mt-aaRSs and demonstrate that **regardless of their common role in the aminoacylation of cognate tRNAs, the mt-aaRSs have distinct intra-mitochondrial localizations and distributions.**

A further investigation during my PhD work was to assess the impact of some PCH6-causing mutations first *in cellulo* on properties of mt-ArgRS, and second *in vitro* on the aminoacylation function of the protein, not explored so far. I show that none of the PCH6-mutations has an impact on the location or on the aminoacylation property of the mt-ArgRS, whereas a strong impact was anticipated. I also investigated the impact of LBSL-related mutations on the release of the mt-AspRS out of the mitochondria under specific chemical conditions, observed by a previous PhD student. I show that none of the tested mutants affects this release. All these contributions further reveal that disease-related mutations have either no or only minor but distinct impacts on the canonical properties of the investigated mt-aaRSs. This indicates that, **at least for the mt-ArgRS and the mt-AspRS, the housekeeping role of the mt-aaRS in the protein synthesis is not the general target of the mutations.** This

is finally in concordance with the absence of patients with multi-systemic affections and prompts us to envision new perspectives.

Major outcomes and towards new questions to solve. Some of the major outputs from this present work are: i) that mt-aaRSs have different cellular properties which suggests different functional roles that will need to be unveiled; ii) all the mt-aaRS are correlated with pathological disorders; iii) until now, there is no common combination of molecular mechanisms that explain the various phenotypic expressions observed in patients leaving unsolved the physiopathology of these disorders; iii) the pathologies have a target for high energy-demand tissues. All these observations may reflect the integration of mitochondrial translation process within cellular homeostasis; these may occur through specific crosstalk(s) between the cellular program and cellular energy demands, especially in neuronal cells. It calls for the necessity of a better comprehension of how the mitochondrial translation is indeed integrated within different cells and tissues. This should be done taking into account the notion of space (possible modulations or peculiarities that would be tissues-specific), of time (possible modulations or peculiarities that would vary during the different developmental stages), and under different conditions (physiological and/or physiopathological).

The following are some of the ideas that emerge from the integration of information from previous work by our team and others, along with the information obtained from my research work. I have listed below perspectives not previously mentioned at the end of each chapter. I contributed to initiate those currently under investigations in the laboratory.

Possible perspectives, among others.

The host laboratory has previously developed a knowledge-based repository coupled with a web server ([http:// misynpat.org](http://misynpat.org)), which at the moment integrates and links clinical, genetic, and structural data on disease-related mutations of human mt-aaRSs (Moulinier *et al.*, 2017). One important contributions of my present work is the clinical categorization of the mt-aaRSs-related diseases. We believe that the development of a database (or the extension of the existing one) where the medical community can systematically update each new diagnosed case, add a detailed clinical description, with complementary medical analyses, associated genetic studies (of the patient and the relatives concerned) and, above all, where a chronological follow-up of the clinical history of patients with mt-aaRSs disorders must fulfilled, would be an additional useful tool for both fundamental researchers and physicians. To further complete this initiative, the creation of a bank of biopsies (from living or post-mortem patients) where

the materials obtained would be accessible to the scientific community involved in these mitochondrial pathologies, would contribute a further value to the advancement of global research in this field. Nowadays, the clinical management of patients with mt-aaRSs disorders is still limited to symptomatic treatment. The fundamental understanding of mt-aaRSs at a first glance, followed by shedding new lights on of the particularities that each mt-aaRSs could perform in specific organ- tissue- or cells will be the key to establish clear and concrete explanations of the pathological mechanisms related to these diseases.

In order to increase the fundamental knowledge of the human mt-aaRSs, we shall for example recall that the MTS, besides the fact that it creates difficulties in the design of soluble and functional recombinant proteins, also constitutes a pool of diversity for some mitochondrial proteins. This was shown for the mt-AspRS, but is probably also the case for mt-ArgRS and/or other mt-aaRSs (Carapito *et al.*, 2017). The existence of different forms of mt-aaRSs after *in organello* maturation and cleavage would, if their existences are confirmed and biological relevance established, represents a valuable breeding ground for protein multifunctionality. This is an aspect that has not yet been investigated and should be taken into account.

Regarding the cellular integration of the mitochondrial translation and considering the possible modulations or peculiarities that would be tissues-specific, perspectives remain challenging. Several hypotheses have been evoked when trying to understand the tissue specificity of some mt-aaRS-related diseases. For instance, it has been suggested that tissue-specific differences in mitochondrial chaperone activities have a participation in determining the stability or the instability of some mutants (Konovalova and Tynymmaa, 2013). In the case of LBSL patients, most of them have a mutation in intron 2 of *DARS2*, which affects the splicing of the third exon. Using a splicing reporter construct, van Berge *et al.* find cell-type-specific differences in the sensitivity to these mutations: the mutations have a larger effect on exon 3 exclusion in neural cell lines, especially neuronal cell lines, than in non-neural cell lines (van Berge *et al.*, 2012). Other hypothesis regarding the tissue-specificity was proposed by Götz *et al.* when studying the clinical manifestations of the cardiomyopathy produced by mutations in mt-AlaRS. They hypothesized that the tissue-specific manifestation of the cardiomyopathy could be explained by variable amino acid concentrations in different tissues, especially those of glycine and serine, which in the case of proofreading-deficient mt-AlaRS could influence the misincorporation rate of serine and glycine in RC complexes. Their results did not show considerable differences in glycine or serine concentrations between tissues, but increased levels of alanine in the patient heart and muscle, but not in the liver. The authors

proposed that given that alanine can be secreted from RC-deficient skeletal muscle for gluconeogenesis in the liver, the high alanine might be an unspecific response to RC defect (Götz *et al.*, 2011).

Another way to tackle this issue is to consider the implication of the mt-aaRSs into alternate functions. Several examples of such alternate function have been provided for human cytosolic aaRSs, but only a few are reported for the mt-aaRS. It is only recently that a pro-angiogenic function of the rat mt-TrpRS (Wang *et al.*, 2016a) and a cysteine polysulfidation activity of the mice and the human mt-CysRSs (Akaike *et al.*, 2017) have been identified. We should unveil the environmental contributions that either modify or modulate mitochondrial translation from those involved in moonlighting activities. Following these lines, we initiated a project, whose aim is to uncover a CNS-specific role(s) of the mt-AspRS. To do so, we first consider a possible implication of the mt-AspRS in the process of N-acetyl L-Aspartate (NAA) synthesis by a neuron-specific transferase (literature-based hypothesis), knowing that the two enzymes use aspartate as a substrate. The rationale of this project, as well as the experiments that are currently performed and the corresponding perspectives are provided in the Annex. Meanwhile, a complementary strategy has been initiated in collaboration (Hélène Puccio, IGBMC, Illkirch). Neuronal (SH-SY5Y) and oligodendrocyte (MO3.13) cellular models (conditional mutants) will be generated using CRISPR-Cas9 approaches to investigate the impacts of LBSL-related mutations on the cellular function, with a particular focus on mitochondrial function. Our rationale is to focus on missense disease-related mutations identified in patients that have kept canonical aminoacylation properties (See **Table 5**). We hypothesize that these mutations have a common impact on the neuronal-specific alternate role/property of the mt-AspRS (visible only in SH-SY5Y-derived cells), in addition to the distinctive impacts on the canonical properties of the enzyme, visible in all investigated cell types (SH-SY5Y, M03.13 and HEK293T).

Mutations	Expected impact of mutation on the function	Expected phenotype in HEK 293	Expected phenotype in Neurones	Expected phenotype in Oligodendrocytes
R263X	Complete KO – Loss of canonical and moonlighting function	no growth	no growth	no growth
Q184K	Loss of canonical and moonlighting function	Mild/severe	Severe	Mid/severe
R263Q	Loss of canonical and moonlighting function	Mild/severe	Severe	Mild/severe
R58G	Loss of moonlighting function	No effect	Severe	No effect
T136S	Loss of moonlighting function	No effect	Severe	No effect
L613F	Loss of moonlighting function	No effect	Severe	No effect
L626Q	Loss of moonlighting function	No effect	Severe	No effect

Table 5. Choice of mutations that we will use to generate cellular models and their possible impact in different cell types based on in vitro data and our hypothesis. All the selected mutations are reported in LBSL-patients.

Regarding the cellular integration of the mitochondrial translation, the specific case of neuronal cells should be mentioned. Indeed, neuronal cells present a great diversity of morphologies and an extremely high-energy demand. Due to these extremely varied morphological features, neurons face exceptional challenges to maintain the energy homeostasis. Neurons require specialized mechanisms to efficiently distribute mitochondria to far distal areas where energy is in high demand, such as synaptic terminals, active growth cones, and axonal branches. Functional mitochondria must thus be efficiently transported to distal regions of neuronal cells where specific quality-control mechanisms can remove dysfunctional mitochondria. Thus, one can wonder if the mitochondrial translation itself undergoes specific pathways in response to such exceptional situation.

Now, considering the cellular integration of the mitochondrial translation taking in a notion of time, and considering possible modulations or peculiarities that would vary during the different developmental stages, the perspectives are even more challenging. We indeed have referred that the mutations on mt-aaRSs may participate at some point in the development of embryonic tissues. It is easy to realize that the verification of this idea is not easy to accomplish, at least in human. The production of conditional tissue-specific animal models for different mt-aaRSs could provide an insight into the pathological mechanisms and the selective tissue involvement caused by mutations in these ubiquitously expressed enzymes. The cessation of the embryonic process at different stages, followed by the verification of each affected organ,

would allow elucidating the possible involvement of the mt-aaRSs in specific embryonic development of an organ or tissue. One of the characteristics highlighted during the clinical analysis of mutation-related pathologies in mt-aaRS (chapter I) was the onset-age of the clinical manifestations. Pontocerebellar hypoplasia type 6 (PCH6) is one of the ideal models to investigate the notion of time. PCH6 is produced by mutations in *RARS2* (Edvardson *et al.*, 2007) and has a very early onset, in most cases within few hours after birth (Zhang *et al.*, 2018), suggesting that damages might occur during the embryonic development. The hallmark sign of this pathology is a hypoplasia of the pons (Kastrissianakis *et al.*, 2013), a sign easy to identify using neuroimaging analysis. The creation of animal models carrying the PCH6 genotypic compound, followed by cessation of the gestation in different stages may allow the determination of pathologic changes in the brain structures. This would allow us to establish if there is a specific moment during embryogenesis that the participation of mt ArgRS is more essential than in another.

All this together, we point out that there is still much to do, that each new piece of information opens up more perspectives for research. In any case, we believe that the contribution of this work will promote more lines of investigations, and that in the end, the integration of a clinical and a fundamental research will allow us to continue expanding the knowledge of this family of enzymes. Above all, to understand the pathologies and later in the future to be able to develop therapeutic strategies.

Materials and Methods

Materials and Methods

1. Materials

1.1. Chemical products

Acetic acid	Roth
Acrylamide/bisacrylamide (Rotiphorese Gel 30/40)	Carl Roth
Ammonium chloride (NH ₄ Cl)	Sigma-Aldrich
Ammonium sulfate	Roth
Ammonium persulfate (APS)	Carl Roth
Ampicillin	Sigma-Aldrich
Arginine, L-[2,3,4 -3H]	Hartmann Analytic
Calcium chloride (CaCl ₂)	Roth
Chlorophorm	Carl Roth
ECL Western blotting Substrate	Fisher Scientific
Ethanol VWR	
Ethidium bromide	ICN
Ethylenediaminetetraacetic acid (EDTA)	Euromedex
Glucose Sigma-Aldrich	
Glutaraldehyde	Sigma
Glycerol Carl Roth	
HEPES Euromedex	
HIS-Select [®] HF Nickel Affinity Gel	Sigma-Aldrich
Hydrochloric acid	AnalaR NORMAPUR
Imidazole	Sigma-Aldrich
IPTG Euromedex	
Isopropanol	Sigma-Aldrich
K ₁ – Potassium phosphate dibasic (H ₂ PO ₄ ⁻ , K ⁺)	Merck Millipore
K ₂ – Potassium phosphate monobasic (HPO ₄ ²⁻ , 2K ⁺)	Merck Millipore
L-Arginine	Sigma-Aldrich
LB Carl Roth	
Magnesium chloride (MgCl ₂)	VWR
Methanol	VWR
Mini-Protean [®] TGX Precast polyacrylamid gels	BioRad
n-Dodecyl-b-D-Maltopyranoside (DDM)	CliniSciences
Na ₁ – Sodium phosphate dibasic (NaH ₂ PO ₄)	Merck Millipore
Na ₂ – Sodium phosphate monobasic (Na ₂ HPO ₄)	Merck Millipore
Phenol Carl Roth	
Potassium carbonate	Sigma-Aldrich
Potassium chloride (KCl)	Panreac
Potassium hydroxide (KOH)	Sigma-Aldrich
ProteaseArrest [™] Protease Inhibitor Cocktails	G-Biosciences
Sodium acetate	Carl Roth
Sodium carbonate	Sigma-Aldrich
Sodium chloride (NaCl)	VWR

Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Sodium hydroxide (NaOH)	Sigma-Aldrich
Spermidine	Sigma-Aldrich
β -Mercaptoethanol	Carl Roth
TBE Euromedex	
TEMED Acros	
Trans-Blot Turbo PVDF system	BioRad
Trichloroacetic acid (TCA)	Carl Roth
TriReagent	Sigma-Aldrich
Tris-base	Sigma-Aldrich
Tween 20	Sigma-Aldrich
Urea Sigma-Aldrich	

1.2. Cell culture

BHK-21	ATCC
HEK293T	Invitrogen
SH-SY5Y	Sigma-Aldrich
Fibroblasts	Imagine Institute (Paris)
Hepatocytes	CarMeN Laboratory (Lyon)
Myotubes	CarMeN Laboratory (Lyon)
Dubelcco's Modified Eagle's Medium (DMEM)	Gibco
Fetal Calf Serum (FCS)	Eurobio
Glasglow-MEM (GMEM)	Gibco
Modified Vaccinia Ankara (MVA-EM21)	IGBMC (Strasbourg)
Penicillin-streptomycin (pen-strep)	Gibco
Phosphate Buffered Saline (PBS)	Gibco
Polyethlenimine (PEI)	Polyscience
Trypsin EDTA	Gibco
Tryptose phosphate broth	Gibco
Roswell Park Memorial Institute (RPMI) 1640 Medium	Gibco

1.3. Bacterial Strains

<i>E. coli</i> One Shot® TOP 10	Invitrogen
BL21(DE3) Competent <i>E. coli</i>	New England Biolabs®

1.4. Enzymes

DpnI	New England Biolabs®
FastAP	Thermo Scientific
NdeI	New England Biolabs®
NotI	New England Biolabs®

Phusion High Fidelity DNA Polymerase
 SalI
 T₄ DNA ligase
 Taq polymerase
 XhoI
 XmaI

Thermo Scientific
 New England Biolabs®
 Thermo Fisher Scientific
 IBMC production
 New England Biolabs®
 New England Biolabs®

1.5. Plasmids

pCI Mammalian Expression Vector
 pDEST14
 pBCJ749.77

Promega
 Thermo Fisher Scientific
 (Jester *et al.*, 2011)

1.6. Primers

Primers were purchased lyophilized, from Integrated DNA Technologies (IDT®). Upon arrival primers were resuspended in H₂O mQ to a final concentration of 100 μM and stored as stock solution at -20°C.

I. Amplification of hm_mt-ArgRS from pBCJ739.14	
Type	Sequence 5' → 3'
Fw	AAATCTCGAGATGGCGTGC GGCTTTTCG
Rv	TTTGCGGCCGCTTAGTGGTGGTGGT

II. Mutagenesis PCR for mutants of mt-ArgRS in pCI		Purpose
Fw	GCTTTCGCCGCGCTGTTGCTTGCCAGC	p.I9V
Rv	GCTGGCAAGCAACAGCGCGCGAAAGC	
Fw	GCTATTGCTTGCCGGCTTCCAGAGTG	p.Q12R
Rv	CACTCTGAAAGCCGGCAAGCAATAGC	
Fw	GCACTTTCAGTGGCAAAAAATTTTCG	p.W241R
Rv	CGAAATTTTGCCTCAGTCAAAGTGC	
Fw	CACTGTGGCAAAAAATTTTCAGGACTTGAGCATTGAAG	p.R245Q
Rv	CTTCAATGCTCAAGTCCGAAATTTTGCACAGTG	
Fw	CATTCGGGTTTACAAGCATCTGGGAGTATATTTTG	p.R258H
Rv	CAAAAATACTCCAGATGCTTGTAACCCGAATG	
Fw	GGTCTTAAAGTTGCAGGAGAGTAAAGGAC	p.L283Q
Rv	GTCCTTACTCTCCTGCAACTTTAAGACC	
Fw	GCTGCTATAGATGGAATGGACAAG	p.R333G
Rv	CTTGTCCATTCGATCTATAGCAGC	
Fw	GGACAAGTATAATTTTGATACAGTGATATATGTGACAGATAAAGG	p.M342V

Rv	CCTTTATCTGTCACATATATCACTGTATCAAAATTATACTTGTCC	
Fw	GAGATTCAATTAAGGAAGCTACAGAACATGGC	p.M404K
Rv	GCCATGTTCTGTAGCTTCCTTAATTGAATCTC	
Fw	CACACACGCCCACCTCCACAGTTG	p.R469H
Rv	CAAACGTGGAGGTGGGCGTGTGTG	
Fw	GGAAGAGACTTTTAGATGTGGGTACC	p.G478R
Rv	GGTACCCACATCTAAAAGTCTCTTCC	
Fw	CCTTCTAACTTTAAGTTATCTTGCAGCTGTGG	p.H530Y
Rv	CCACAGCTGCAAGATAACTTAAAGTTAGAAGG	

III. Cloning different N-Termini of mt-ArgRS in pDEST		Purpose
Fw	GGCGCGCATATGAACCTGCCGCCAGAGAATCTGATTACGTCGATCTCCGCCGTGCCCATCTCGAAAAGGAAGAGG	N-Term N18
Fw	GGCGCGCATATGTCGATCTCCGCCGTGCCCATCTCGAAAAGGAAGAGGTAGCAGATTTTCAGCTGTCGGTGG	N-Term S27
Fw	GGCGCGCATATGCTTCGCTGCGATACCGTCGTC	N-Term L72
Fw	GGCGCGCATATGTGCGATACCGTCGTCCTCTGAGATCTCTACTGGACAGCGCACTGTCAACTTTAAAATCAACCGCGAGC	N-Term C74
Fw	GGCGCGCATATGATCGTTGTGGAATTCAGTAGTCCCAATGTGGCG	N-Term I127
Rv	ATTTTACTCGAGTCAGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGCATACGGCAAACCTGGCGTAATACC	All_reverse

IV. Mutagenesis PCR for mutants of L72_mt-ArgRS in pDEST		Purpose
Fw	CGTTGTGGAATGCAGTAGTCCC	p.F131C
Rv	GGGACTACTGCATTCCACAACG	
Fw	GGCAGAAATTCCAGGATCTGAGTATCG	p.R245Q_1
Rv	CGATACTCAGATCCTGGAATTTCTGCC	
Fw	GGCAGAAATTCCACGATCTGAGTATCG	p.R245Q_2
Rv	CGATACTCAGATCGTGAATTTCTGCC	
Fw	GGTGCTGAAACTCAAGAAAGTAAGGG	p.L283Q_1
Rv	CCCTTACTTTCTTGAAGTTTCAGCACC	
Fw	GGTGCTGAAACTCTAGAAAGTAAGGG	p.L283Q_2
Rv	CCCTTACTTTCTAGAAGTTTCAGCACC	
Fw	GTATACGCATGCGCATCTTCATTCGCTCG	p.R469H
Rv	CGAGCGAATGAAGATGCGCATGCGTATAC	
Fw	CTTTTAAACGCTTTCTTATCTGGCCGCGGTC	p.H530Y
Rv	GACCGCGGCCAGATAAGAAAGCGTAAAAAG	

V. Northern Blot Hybridization	
Type	human mt-tRNA ^{Arg} nucleotides 10421-10441
Fw	GAGTCGAAATCATTTCGTTTTG
human mt-tRNA ^{Leu} nucleotides 3275-3293	
Fw	CAGTCAGAGCTTCAATTCC

1.7. Antibodies

Protein		Antigen	Specificity	Clonality	Source	Dilution	Reference
Mitochondrial aspartyl-tRNA synthetase	mt-AspRS	486-497 aa	Human	Polyclonal	Rabbit	1/40000	IGBMC (Illkirch)
Mitochondrial arginyl-tRNA synthetase	mt-ArgRS	377-406 aa	Human	Polyclonal	Rabbit	1/1000	Abcam
Prohibitin	PHB	100-200 aa	Hu, Ms, Rt	Polyclonal	Rabbit	1/30000	
Superoxide dismutase	SOD2	Full length	Hu, Ms, Rt	Polyclonal	Rabbit	1/10000	
Anti-Flag	Flag-Tag HRP	DYKDDDDK	-	Monoclonal	Mouse	1/2000	
Anti-His tag	His-Tag HRP	HHHHHH	-	Polyclonal	Rabbit	1/30000	
Anti-Rabbit secondary antibody	Rabbit-HRP	-	Rabbit	Polyclonal	Goat	1/15000	BioRad

Table 6. List of all used commercial antibodies.

1.8. Patients' features

Fibroblasts were isolated from healthy, LBSL- and PCH6-diagnosed patients (skin biopsy) at the Institute IMAGINE (Paris). Informed consent for diagnostic and research studies was obtained for all subjects in accordance with the Declaration of Helsinki protocols and approved by local Institutional Review Boards in Paris.

LBSL patient is compound heterozygous for two *DARS2* variants, a missense variant (c.1013G>A; p.G338E) and a previously reported nonsense truncating variant (c.228-21_228-20delinsC; p.R76SfsX5) (Scheper *et al.*, 2007)

PCH6 patient is compound heterozygous for two *RARS2* variants, a missense variant (c.29C>T; p.A10V) and a splicing variant (c.298G-1>A) causing a frameshift and a premature termination codon (p.T100Cfs*5)

2. Methods

2.1. Cell Culture

2.1.1. Cell lines

2.1.1.1. HEK 293T

Human Embryonic Kidney 293T cells (HEK293T, Invitrogen) were grown in DMEM supplemented with 10% FCS and 1% Penicillin-Streptomycin at 37°C in a 5% CO₂ humidified incubator. For cell splitting (~every 2 days), cells at 80% of confluence were washed with PBS (Phosphate Buffered Saline, Gibco) and trypsinized (0,25% Trypsin-EDTA) for two minutes. Then, 10 ml of medium containing FCS were added to stop the trypsin reaction. Cells were seeded in new plates at dilution 1/10. For cryopreservation, cells (~2.10⁶ cells/ml) were frozen in growth medium containing 10% DMSO at -80°C using Mr. Frosty™ Freezing Container (Nalgen®) for one day, then cells were stored in liquid nitrogen.

2.1.1.2. SH-SY5Y

Human neuroblastoma SH-SY5Y cells (SH-SY5Y, Sigma Aldrich) were grown in RPMI supplemented with 10% FCS and 1% Pen-Strep at 37°C in a 5% CO₂ humidified incubator. For cell splitting, cells at 80% of confluence were washed with PBS and trypsinized for two minutes. Then, 10 ml of medium containing FCS were added to stop the trypsin reaction. Cells were seeded in new plates at dilution 1/8. Growth medium was changed every 2 days. For cryopreservation, cells (~2.10⁶ cells/ml) were frozen in growth medium containing 10% DMSO at -80°C using Mr. Frosty™ Freezing Container for one day, then cells were stored in liquid nitrogen.

2.1.1.3. Fibroblasts derived from patients

Human skin fibroblasts were from the Imagine Institute (Paris). Fibroblasts were grown in DMEM supplemented with 10% FCS and 1% Pen-Strep at 37°C, in a 5% CO₂ humidified incubator. Growth medium was changed every 2 days and cells were split approximately once a week. Fibroblasts at 60-70% of confluence were washed with PBS and trypsinized for two minutes. Then, 10 ml of medium containing

FCS were added to stop the trypsin reaction. Cells were seeded in new plates at dilution 1/5. For cryopreservation, cells ($\sim 2 \cdot 10^6$ cells/ml) were frozen in growth medium containing 10% DMSO at -80°C using Mr. Frosty™ Freezing Container for one day, then cells were stored in liquid nitrogen.

2.1.1.4. BHK21

Baby Hamster Kidney (BHK21) cells were grown in GMEM medium supplemented with 10% FCS, 5% Tryptose Phosphate Broth and 1% Pen-Strep at 37°C , in a 5% CO_2 humidified incubator. Cells at 80% of confluence were washed with PBS and trypsinized for two minutes. Then, 10 ml of medium containing FCS were added to stop the trypsin reaction. Cells were seeded in new plates at dilution 1/10. Cell splitting was done every two days. For cryopreservation, cells ($\sim 2 \cdot 10^6$ cells/ml) were frozen in growth medium containing 10% DMSO at -80°C using Mr. Frosty™ Freezing Container for one day, then cells were stored in liquid nitrogen.

2.2. BHK21 cell infection/transfection and mt-AspRS production

2.2.1. BHK21 infection/Vaccinia Virus expression system

An engineered mammalian expression system composed of Modified Vaccinia virus Ankara (MVA) and Baby Hamster Kidney cells (BHK21) was developed in the lab and published (Jester *et al.*, 2011). This method is based on the controlled expression of recombinant proteins using the T7 RNA polymerase provided by the MVA. The original vector pBCJ739.14, designed and prepared by Brian Jester (Jester *et al.*, 2011) contains the sequence coding for the wt mt-AspRS under the dependence of T7 promotor. The mutated sequences of mt-AspRS were prepared by L. Karim (Sauter *et al.*, 2015).

2.2.2. mt-AspRS production in BHK21

Transfection of BHK21 cells was performed as described in Sauter *et al.* 2015. (Sauter *et al.*, 2015). Briefly, for one 150-cm^2 plate of BHK21 previously seeded with

4.10⁶ cells, cells were washed with PBS and infected with modified vaccinia virus (MVA) expressing IPTG-inducible T7 polymerase. Infection was performed by adding 50 µl of MVA in 1 ml of GMEM (without FCS) per plate of 150-cm². Every 10 minutes, plates were tilted to avoid drying and to spread virus over cells. After 30 minutes of incubation with MVA, 14 ml of GMEM (supplemented with 10% FCS, 5% TPB and 1% Pen-Strep) were added to cells. Then, cells were transfected using polyethylenimine (PEI) with plasmids expressing either the wild-type mt-AspRS (pBCJ749.77) or the LBSL-related mutants. Expressed proteins correspond to full-length enzymes including the N-terminal mitochondrial targeting sequence (MTS) and the C-terminal Flag-Tag sequence (DYKDDDDK). Protein expression is under the dependence of T7 promoter. Plasmids/PEI complexes were prepared in a ratio of 1:4.5 (w/w) and incubated for 15 minutes at room temperature prior to transfection (5 µg DNA per plate 150-cm²). After 36 hours of transfection, cells were harvested for further studies.

2.3. HEK 293T cell transfection and mt-ArgRS production

2.3.1. Vector

The pCI Mammalian Expression Vector (Promega) was used to express wild-type mt-ArgRS (corresponding to the full length of RARS2 including its MTS and followed by the sequence coding for the Flag Tag and the (His)₆-Tag at the C-Terminal) and mutants. The sequence coding for the wt mt-ArgRS was amplified from a plasmid available in the laboratory, previously prepared by Agnes Gaudry and named pBCJ-ArgRS-full length-Flag-Histag, and then cloned into the NdeI and XhoI sites of the pCI vector. Mutations c.25A>G (p.I9V), c.35A>G (p.Q12R), c.721T>A (p.W241R), c.734G>A (p.R245Q), c.773G>A (p.R258H), c.848T>A (p.L283Q), c.997C>G (p.R333G), c.1024A>G (p.M342V), c.1211T>A (p.M404K), c.1406G>A (p.R469H), c.1432G>A (p.G478R), c.1588C>T (p.H530Y) were introduced by site-directed mutagenesis using Phusion High-Fidelity DNA Polymerase (Thermo Scientific™) following the protocol described below. The primers used for mutagenesis are listed in **section 1.6, II.**

2.3.2. mt-ArgRS production in HEK293T cells

The expression of recombinant mt-ArgRS (WT and mutants) was performed by transfection of HEK293T cells using pCI mammalian expression vector. A 150-cm² plate of HEK293T cells at 50% of confluence was washed with PBS and 14 ml of DMEM (supplemented with 10% FCS and 1% Pen-Strep) were added one hour before transfection. Plasmids/PEI complex was prepared in a ratio 1:4.5 (w/w) in 1 ml of DMEM (without FCS) and incubated for 15 minutes at room temperature prior to transfection (5 µg DNA per plate 150-cm²). After 36 hours of transfection, cells were harvested for further studies.

2.4. Mitochondrial enrichment and fractionation

2.4.1. Mitochondrial isolation

Mitochondria were enriched from different cell lines using differential centrifugation steps. Cells were harvested and washed two times with PBS. Afterwards, cells were resuspended in an isotonic buffer (220 mM Mannitol, 70 mM Sucrose, 1 mM MgCl₂, 1 mM EDTA, 10 mM HEPES-KOH pH 7.5) and mechanically disrupted using 2 mm diameter ceramic beads in a FastPrep-24™ 5G Machine (MP biomedical). The lysate was centrifuged 10 minutes at 400 g at 4°C to remove the pellet containing residual intact cells, nuclei and debris. The supernatant (containing mitochondria) was centrifuged 10 minutes at 16 000 g at 4°C to collect crude mitochondria.

2.4.2. Sub-mitochondrial fractionation

Enriched mitochondria (~30 mg) were resuspended in 1 ml of isotonic washing buffer (300 mM Mannitol, 10 mM K₂HPO₄/KH₂PO₄ pH 7.5, 1 mM EDTA) containing 1x Protease inhibitor cocktail (Protease Arrest, G-Bioscience). Mitochondria were sonicated three times for 10 seconds (4W) on ice and then centrifuged at 16 000 g to remove unbroken mitochondria (residual fraction). The mitochondria lysate was subsequently ultracentrifuged at 125 000 g, 30 minutes at 4°C in 1,5 ml Microfuge Tube Polypropylene (Beckman Coulter®) to separate the soluble fraction (supernatant, containing molecules from the mitochondrial matrix and the inner mitochondrial space) and the membrane fraction (pellet, containing molecules from both inner and outer membranes). Membrane and residual fractions were solubilized in 80 µl of washing

buffer, while soluble fraction was concentrated to the same final volume (80 μ l) using Amicon[®] Ultra – 0.5 ml, exclusion size 10 kDa (Millipore).

2.4.3. Treatment of isolated mitochondria

2.4.3.1. Chemical treatments

Enriched mitochondria (~30 mg per experiment) were sonicated (three times for 10 seconds at 4W on ice) in the presence of different chemical agents (1M - 8M Urea, 0.1 M Na₂CO₃ M pH 11, 0.5 M KCl, 0.4 M DTT, or 1 M NH₂OH at pH 7 and pH 11) in 1 ml of washing buffer containing protease inhibitor cocktail. After sonication, mitochondria underwent sub-mitochondrial fractionation (**section 2.4.2**).

2.4.3.2. Incubation of mitochondria with ATP

Enriched mitochondria (~30 mg per experiment) were incubated with ATP (10 mM) in 1 ml of washing buffer containing a protease inhibitor cocktail for 10 min at 20 °C. After treatment, mitochondria were centrifuged 10 minutes at 16 000 g at 4°C so that to separate the supernatant (named extra-mitochondrial medium or EM) from mitochondrial pellet. This latter was either kept intact or fractionated. Extra-mitochondrial medium was ultracentrifuged 30 minutes at 125 000 g at 4°C, and the resulting supernatant is then concentrated to a final volume (80 μ l) using Amicon[®] Ultra – 0.5 ml, exclusion size 10 kDa (Millipore).

2.4.4. *In vitro* Cross-linking with Glutaraldehyde

Mitochondria enriched (~50 mg) from HEK293T cells were resuspended in 20 mM KH₂PO₄/K₂HPO₄ pH 7.5 (10 μ l/mg wet pellet) containing a protease inhibitor cocktail. After sonication 6 times for 10 seconds (4W) on ice, the mitochondrial lysate was centrifuged 10 minutes at 16 000 g at 4°C. The pellet was discarded and the proteins content within the supernatant was quantified following a standard Bradford assay (Bradford 1976). 100 μ g of mitochondrial proteins were incubated with glutaraldehyde (at 0.02% final concentration) for 5, 10 and 20 minutes in 1 ml of 20 mM KH₂PO₄/K₂HPO₄ pH 7.5 under rotation at room temperature. After incubation,

the cross-linking reaction was stopped by quenching with 100 μ l of 1.5 M Tris–HCl pH 8.8. Samples were concentrated to a final volume of 80 μ l using Amicon® Ultra – 0.5 ml, exclusion size 10 kDa (Millipore) and supplemented with 20 μ l of Laemmli dissociating buffer for SDS-PAGE and Western blot analysis.

2.5. DNA manipulation

2.5.1. Cloning

2.5.1.1. PCR-mediated amplification of mt-aaRS sequences

2.5.1.1.1. Gene amplification

Forward primer was designed to anneal the 5' extremity of mt-ArgRS sequence of the pBCJ-mt-ArgRS-full_length-Flag-HisTag and to add the NdeI unique restriction site. Reverse primer was designed to anneal the 3' of the same sequence and to introduce NotI unique restriction site. PCR reaction components and program are indicated in **section 1.6, I**. PCR products were separated on 1% agarose gel supplemented with 1/15 000 (v/v) GelRed™ dye (1 hour at 100 V). Corresponding bands were cut out and the DNA was recovered from the gel using the NucleoSpin Gel purification kit (Machinary and Nagel) following the manufacturer's instructions and DNA concentration was determined using NanoDrop™ spectrophotometer.

Component	Volume		
DNA 50 ng/ μ l	1 μ l	Denaturation 95°C	2 min
dNTP (5mM)	4 μ l	Denaturation 95°C	1 min
Forward primer (10mM)	2 μ l	Annealing Tm -2°C	1min
Reverse primer (10mM)	2 μ l	Extension 72°C	2 min 30 sec
Taq buffer (10x)	5 μ l	Final extension 72°C	10 min
Taq Polymerase	0.5 μ l	30 cycles	
H ₂ O	35.5 μ l		

Table 7. PCR reaction components and program

2.5.1.1.2. Enzymatic digestion

Five μl of PCR product (insert) and ~ 100 ng of pCI mammalian vector were separately double digested with 1 μl XhoI, 1 μl NotI, 2 μl of Buffer CutSmart 10x and H₂O MilliQ to reach a final volume of 20 μl of reaction. The reaction was performed during 3 hours at 37°C, afterwards insert and vector were separated on agarose gel 1% (1 hour at 100 V), then purified using NucleoSpin™ gel extraction kit and their concentrations were determined using NanoDrop™ spectrophotometer.

2.5.1.1.3. pCI mammalian vector dephosphorylation

50 ng of the vector were supplemented with 1.2 μl of Buffer CutSmart 10x and 1 μl of FastAP (Alkaline Phosphatase) and incubated 10 minutes at 37°C for dephosphorylation, then 5 minutes at 75°C to inactivate the enzyme.

2.5.1.1.4. Ligation of the insert in pCI mammalian vector

Insert (10 μl) was ligated into dephosphorylated vector (12 μl) in the presence of 1 μl of T4 DNA ligase and its appropriate buffer, in a final volume of 40 μl . The reaction was performed at 16°C overnight. Ligation product was directly used for bacterial transformation.

2.5.2. Directed mutagenesis

Substitution of nucleotides was performed according to PCH6-related mutations reported in patients (p.I9V, p.Q12R, p.W241R, p.R245Q, p.R258H, p.L283Q, p.R333G, p.M342V, p.M404K, p.R469H, p.G478R, p.H530Y). Self-complementary primers are listed in **section 1.6, II**. PCR reaction components and program are indicating in **table 8**.

Component	Volume
DNA 50 ng/μl	1 μl
dNTP (5mM)	4 μl
Forward primer (10mM)	2 μl
Reverse primer (10mM)	2 μl
Buffer HF 5x	10 μl
Phusion	0.5 μl
H ₂ O	30.5 μl

Denaturation 95°C	2 min	30 cycles
Denaturation 95°C	1 min	
Annealing T _m -2°C	1min	
Extension 72°C	2 min 30 sec	
Final extension 72°C	10 min	

Table 8. Components of PCR mutagenesis reaction and program

After the PCR reaction, 1 μl of DpnI (digesting exclusively methylated DNA, which corresponds to the template DNA, not mutated) was added to the reaction mixture (overnight at room temperature). The PCR product was directly used for bacterial transformation.

This same protocol was used to introduce PCH6-related mutations (p.F131C, p.R245Q, p.L283Q, p.R469H and p.H530Y) in the pDEST_N-ter_L72_RARS2_HisTag construction. The primers used for mutagenesis are listed in **section 1.6, IV**.

2.6. Bacterial transformation

Chemically competent One Shot® TOP 10 cells were transformed with either 5 μl of ligation mixture or with the mutagenesis PCR product following the given protocol:

- Thaw on ice a vial of competent cells One Shot® TOP 10 (50 μl)
- Add DNA to cells and incubate 30 minutes on ice
- Incubate cells 30 seconds at 42°C and then 2 minutes on ice
- Add to the vial 250 μl of prewarmed SOC (Super Optimal Catobolite) medium and incubate 1 hour at 37°C, shaking at 350 rpm
- Spread 80 and 160 μl of the vial content on 2 different LB-agar plates (supplemented with 100 μg/ml of ampicillin)
- Invert and incubate the plates overnight at 37°C

Colonies were picked and amplified in 5 ml of LB medium containing 100 μg/ml of ampicillin. After overnight incubation cells were centrifuged and the plasmid DNA was extracted using a MiniPrep kit (Macherey-Nagel). The concentration of DNA was

determined using Nanodrop™ spectrophotometer. Plasmids were then sequenced by GATC Biotect Company.

2.7. Plasmid amplification and purification

Bacterial strain containing the desired vector is grown in 250 ml of LB medium containing 100 µg/ml of ampicillin overnight at 37°C. Then, the plasmid DNA was purified using a MaxiPrep kit (Macherey-Nagel) following the manufacturer's instructions. The DNA was resuspended in H₂O milliQ and the concentration was determined using NanoDrop™ spectrophotometer.

2.8. Preparation of plasmids for the expression of N-terminal variants of the recombinant WT mt-ArgRS in bacterial strain

Agnès Gaudry previously cloned in the pDEST14 vector the RARS2 gene, with a sequence optimized for the bacterial expression. In this construction the sequence starts at the codon coding for the Leucine 17 in the putative MTS of the mt-ArgRS and is followed by the sequence coding for the (His)₆-Tag at the C-Terminus. Thus, the resulting plasmid is pDEST_N-ter_L17_RARS2_HisTag.

2.8.1.1. Design of the primers.

Forward primers were designed to anneal the 5' extremity of the mt-ArgRS sequence of the pDEST_N-ter_L17_RARS2_HisTag, to add the NdeI unique restriction site followed by the corresponding sequence coding for the respective different N-termini (starting at N18, S27, L72, or I127). Reverse primer (common for all constructions) was designed to anneal the 3' of the same sequence and to introduce XhoI unique restriction site. Primers are indicated in **section 1.6, III**. Then, a PCR was performed (same protocol 3.1.1.) to introduce the different inserts (corresponding to the different N-termini) in the pDEST vector.

2.8.1.2. Expression of recombinant mt-ArgRS in *E. coli*

The plasmid pDEST_N-ter_L72_RARS2_Histag expressing the mt-ArgRS starting at residue L72 and the mutants was expressed in BL21 DE3 bacterial strain as follows.

Preculture: BL21 DE3 cells containing the pDEST_N-ter_L72_RARS2_Histag were grown in 50 ml LB containing 50 µl of ampicillin (at 100 mg/ml) overnight at 37°C.

Main culture: After dilution to 1:10 with 1000 mL of fresh LB medium containing 20 g of glucose and 1 ml of ampicillin 100 mg/ml in three separate flask of 5L (1L per flask), cells were grown at 37°C for 2.5 hours until an OD 600 of 0.7 was reached. Cells were then centrifuged at 4200 g for 20 minutes (4°C) to removed the glucose and the medium was replaced by fresh LB medium containing 100 µg/mL ampicillin and 10 µM/L IPTG, and cells incubation continued 4 hours at 37 °C. The cells were harvested by centrifugation at 4200 g for 20 min (4°C). Bacterial pellets were stored at -20°C.

2.9. Purification of mt-ArgRS

2.9.1. Affinity chromatography

BL21-DE3_pDEST_N-ter_L72_RARS2_Histag cell pellets were resuspended in 20 ml of buffer Ni-A (50 mM NaH₂PO₄/Na₂HPO₄ pH 7.5, 150 mM NaCl, 10 mM β-Mercaptoethanol and 10% Glycerol) containing a protease inhibitor cocktail before sonication on ice at 120 V for 40 seconds, 7 times. Lysed cells were then subjected to centrifugation at 30 000 g for 20 minutes (4°C). The supernatant was then ultracentrifuged at 45 000 g for 40 minutes (4°C) before loading onto a 5 ml column HIS-Select® HF Nickel Affinity Gel (Sigma-Aldrich). The column was equilibrated in buffer Ni-A (1 ml/min, the maximal pressure was always maintained at < 100 psi). Then, the sample (usually ~25 ml) were loaded onto the column and the affinity chromatography was performed as follows: I. Isocratic flow: 25 ml volume, 1 ml/min, (sample load; collect in 5 ml-fractions), II. Isocratic flow: 50 ml, 2 ml/min, buffer Ni-A (correspond to washing step of the column, until the DO becomes stable to zero again; collect in 5 ml-fractions), III. Linear gradient: 30 ml, 2 ml/min, buffer Ni-A to

Ni-B (buffer Ni-A containing 500 mM imidazole) (corresponding to the elution; collect in 1 ml-fractions) and IV. Isocratic flow: 10 ml, 2 ml/min, buffer Ni-B.

2.9.1.1. Dialysis and concentration

The enzyme-containing fractions, detected by the OD₂₈₀ profile and a SDS-PAGE analysis, were pooled and dialyzed for 2 hours against 1 L of buffer dialysis 1 (50 mM Hepes NaOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM β -Mercaptoethanol and 10 % Glycerol). The buffer was change by fresh buffer dialysis 1 and dialysis continued overnight at 4°C. In a final dialysis step, the buffer was replaced by 1 L buffer dialysis 2 (50 mM Hepes NaOH pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10 % Glycerol) and the protein was dialyzed for additional 2 hours.

The protein solution was concentrated by means of centrifugation using Amicon® Ultra – 0.5 ml, exclusion size 10 kDa at 5000 g. Protein concentration was determined using NanoDrop™ spectrophotometer considering the theoretical extinction coefficient of mt-ArgRS ($\epsilon = 59120 \text{ M}^{-1}\text{cm}^{-1}$).

2.10. Aminoacylation assay

In vitro arginylation was performed in 50 mM HEPES-KOH pH 7.5, 30 mM KCl, 8 mM MgCl₂, 8 mM ATP, 0.2 mg/ml BSA, 1 mM spermine, 25 μ M of isotopic mixture with Arginine L-[2,3,4-³H] (40 Ci/mM), different concentrations of total tRNA (routinely 40 pmoles, but this concentration changed depending of each experiment) from *E. Coli* (purified from bacterial cultures in the laboratory by A. Gaudry), and different concentrations of enzyme (50 nM, 100 nM, 200 nM, 250 nM, 400 nM, 800 nM and/or 1500 nM). The aminoacylation reaction mixture was equilibrated to 37°C before the addition of the enzyme to a final volume of 50 μ l. At different times, 10 μ l aliquots were spotted onto Whatman 3MM filter-papers previously equilibrated with 5% trichloroacetic acid (TCA) (v/v). The papers were immediately placed in cold 5% TCA to precipitate tRNA and terminate the reaction. Then the papers were washed three times (10 minutes) with 5% TCA and one time with ethanol before be dried under a heat lamp. The dried papers were placed in counting vessels and incorporation of

radioactive arginine was measured by liquid scintillation (Ecoscint 0TM, National diagnosis) counting using a scintillizer (LS6500, Beckmann Coulter).

2.11. Proteins preparation and analysis

2.11.1. Protein separation in SDS-polyacrylamide gel electrophoresis

The separation of proteins, depending on their size was performed on SDS-polyacrylamide gels (SDS-PAGE). The gels contained a 10% resolving gel solution (pH 8.8) and a 4% stacking gel solution (pH 6.8). Protein samples were supplemented with Laemmli dissociating buffer and heated at 95°C for 10 minutes. Samples were briefly centrifuged before loaded onto the gel. Electrophoresis was performed with 1x TGS running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) at 100V and 180V for the staking and resolving gel, respectively. To visualize the protein bands, the gel was stained SDS-staining solution for at least 30 minutes, and then washed with distaining buffer (25% Ethanol and 10% Acetic Acid)

2.11.2. Western blot analysis

After separation on SDS-PAGE gel, proteins were blotted on a PVDF membrane using the Trans-blot Turbo Transfer System from BioRad following the manufacturer's instructions. For immuno-detection, the PVDF membrane was blocked with 5% milk in 1x TBST for 1 hour. Incubation with the primary antibodies were done in the adapted dilution of each antibody with 1% milk in 1x TBST (**Table 6.**) overnight at 4°C. After incubation, the membrane was washed three times with 1x TBST (50 mM Tris, 150 mM NaCl and 0.1% Tween). If required, incubation with secondary antibody was done for 1 hour at room temperature. The membrane was again washed three times with 1x TBST, and dried on Whatman paper. Chemio-luminescent detection was carried out using the Pierce Detection Kit low and high sensitivity (ECL Western blotting Substrate, and SuperSignal West Femto Maximum Sensitivity Substrate, respectively) according to manufacturer's instructions, mixed at a 1:1 ratio and added to the membrane. After 1 to 2 minutes, PVDF membrane was dried and exposed to

radiographic film (Fuji Medical X-ray Film, Fujifilm). Autoradiographies were scanned using Epson Perfection 3490 Photo and the relative amount of proteins was estimated from band intensities using ImageJ software.

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Résumé en Français

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Introduction

Les mitochondries jouent un rôle primordial dans la respiration et le métabolisme énergétique cellulaires (voir **figure annexe**), et les dysfonctionnements mitochondriaux sont souvent associés à diverses pathologies. La mitochondrie possède sa propre machinerie de transcription/traduction. Le génome mitochondrial (mt) code chez l'homme pour 13 protéines de la chaîne respiratoire, 22 ARNt et 2 ARNr. Le reste des protéines, codé par le génome nucléaire, est importé après traduction dans le cytosol. Parmi les protéines importées figurent les aminoacyl-ARNt synthétases (aaRS), enzymes clés de la traduction mitochondriale, responsables de l'attachement spécifique de chaque acide aminé à son ARNt correspondant. Elles sont adressées à la mitochondrie grâce à la présence d'une séquence N-terminale (MTS pour "mitochondrial targeting sequence"), clivée après importation.

En 2007, le laboratoire d'accueil a contribué à la première description corrélant des mutations dans le gène *DARS2* (codant pour l'AspRS mt) à une leucoencéphalopathie (LBSL pour « Leukoencephalopathy with brainstem and spinal cord involvement with lactate elevation »). Les caractérisations fonctionnelle et structurale, ainsi que des études en vue de comprendre le lien entre les mutations et les phénotypes observés dans la maladie LBSL ont été faites en partie au laboratoire. Aujourd'hui, toutes les aaRS mt sont impliquées dans des maladies aux conséquences souvent sévères. Des mutations dans le gène *RARS2*, qui code pour l'ArgRS mt, sont corrélées à une maladie neurodéveloppementale sévère, PCH6 (pour "Pontocerebellar Hypoplasia type 6"). L'implication de ces deux protéines dans la traduction mitochondriale pourrait conduire à l'idée que une fois mutées, leurs dysfonctionnements produisent des conséquences similaires. Or ce n'est pas le cas et les présentations cliniques des maladies PCH6 et LBSL diffèrent phénotypiquement.

Le travail de cette thèse s'articule autour de 3 axes : (I) L'analyse des phénotypes cliniques des pathologies liées aux mutations dans les aaRS mt, (II) La caractérisation

des propriétés cellulaires de l'ArgRS mt, et (III) L'étude de l'impact de mutations « pathologiques » sur diverses propriétés de l'ArgRS mt.

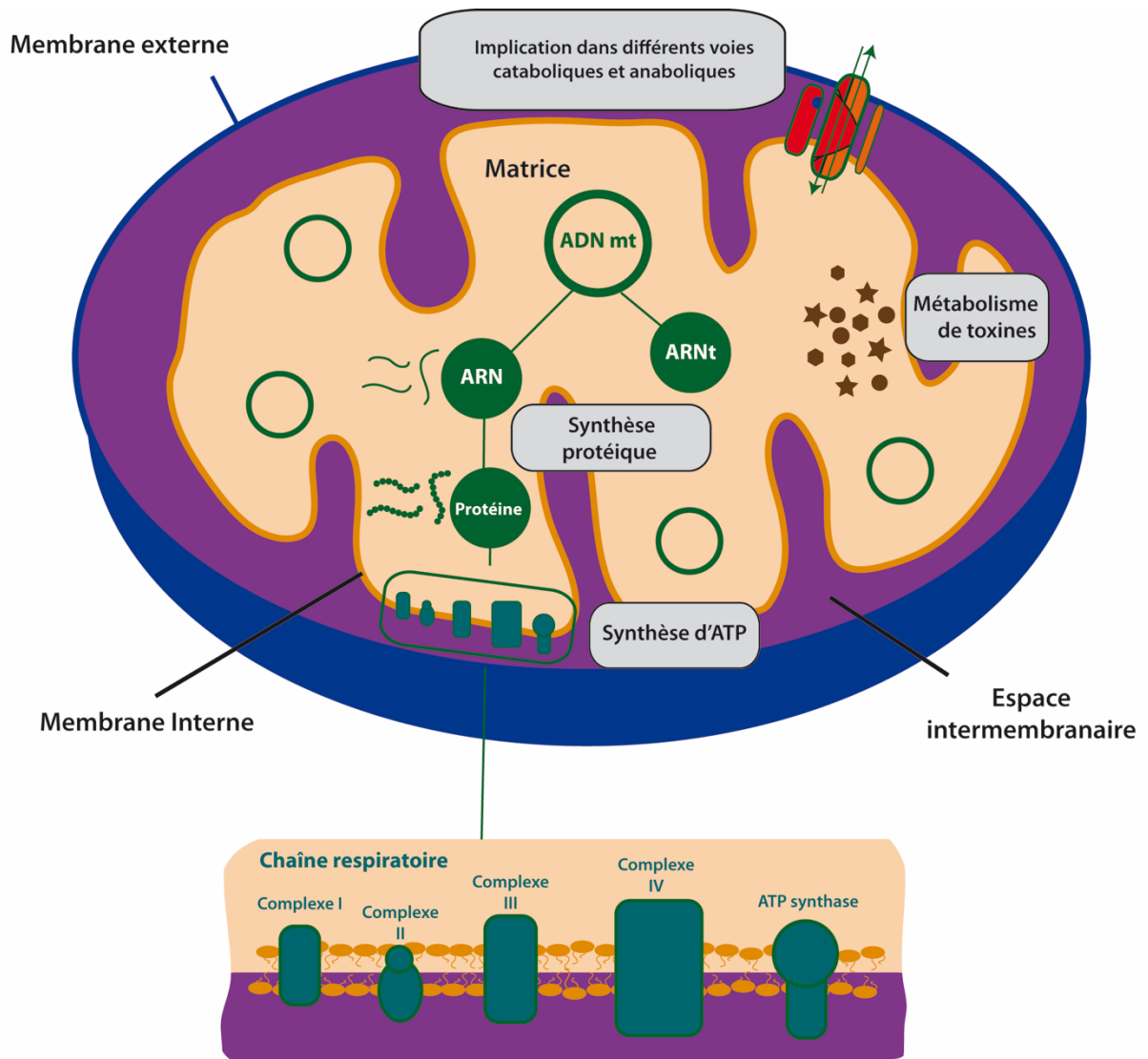


Figure annexe. Résumé graphique des différentes fonctions de la mitochondrie.

Résultats et discussion

(I) Analyse des phénotypes cliniques des pathologies liées aux mutations dans les aaRS mt

La diversité des présentations cliniques des pathologies liées aux aaRS mt mutées (décrites au cours des 10 dernières années) fait appel à la nécessité d'une analyse pour organiser ces maladies prenant en compte leurs phénotypes de présentation, les organes et/ou systèmes anatomiques affectés, l'âge de début des maladies ainsi que leurs sévérités cliniques. Les pathologies liées aux mutations dans les aaRS mt montrent une convergence d'affectation vers le système nerveux central (SNC) dans 80% des cas, avec cependant un nombre non négligeable d'exceptions. Ainsi, nous pouvons distinguer les aaRS mt qui conduisent i) à des affections uniquement du SNC, ii) à des affections du SNC et d'un autre organe, généralement le système reproductif, chez un même patient (e.g. mt-HisRS, mt-LeuRS et mt-AlaRS), iii) à des affections du SNC ou d'un autre organe chez des patients distincts (e.g. mt-AlaRS et mt-SerRS), et enfin iv) à des affections de seulement un autre système anatomique (e.g. mt-TyRS). La question soulevée est de savoir si ces différents sous-groupes d'aaRS mt ont des propriétés cellulaires distinctes, ou si les descriptions futures de nouveaux patients modifieront totalement cette catégorisation. En outre, il est observé à l'intérieur même d'une de ces catégories des différences significatives en termes d'expressions phénotypiques et de sévérité de la maladie : pour exemple, les mutations de l'AspRS mt et de l'ArgRS mt conduisent toutes les deux à des affections du SNC seulement : LBSL et PCH6, respectivement. La première est une maladie neurodégénérative, la seconde est une maladie du neurodéveloppement. Là encore, des différences de propriétés cellulaires des deux enzymes sont attendues. Cette analyse est publiée dans deux articles de revues.

(II) Caractérisation de propriétés cellulaires de l'ArgRS mt

Des travaux antérieurs du laboratoire ont permis de dévoiler des caractéristiques cellulaires spécifiques de l'AspRS mt humaine, tels que : la coexistence de deux formes de l'enzyme à l'intérieur de la mitochondrie, produits par coupures alternatives du MTS

après importation; l'établissement de la double localisation de l'AspRS mt, présente à la fois dans la fraction membranaire et dans la fraction soluble, et que son ancrage à la membrane se fait *via* des interactions électrostatiques ; la mise en évidence que l'AspRS mt sort de la mitochondrie dans certaines conditions de stress mitochondrial (ajout de 10 mM ATP). J'ai étendue cette étude à l'ArgRS mt et démontré qu'elle possède des propriétés cellulaires totalement distinctes.

Le processus de maturation et de clivage des séquences MTS est mal défini, et pourtant essentiel à l'acheminement précis et à la fonctionnalité des protéines mitochondriales. Par ailleurs, il est indispensable de connaître le site de coupure du MTS pour le design d'une protéine recombinante et son expression en système bactérien pour des études *in vitro*. Nous avons appliqué, en collaboration, la technique de spectrométrie de masse ("Doublet N-terminal Oriented Proteomics") utilisée pour définir le MTS de l'AspRS mt, et identifié différents sites possibles de coupure du MTS de l'ArgRS mt: N18, S27, L72, I127. Les quatre protéines recombinantes correspondantes ont été clonées, et leurs solubilités et stabilités analysées. La forme L72_ArgRS est la construction la plus soluble de l'enzyme et fût choisie pour la suite de expériences fonctionnelles *in vitro* (voir partie III).

Concernant la localisation intra-mitochondriale, j'ai démontré que l'ArgRS mt est localisée exclusivement dans la fraction membranaire, alors que aucun domaine transmembranaire ne lui est prédit. Grâce à l'ajout d'agents chimiques connus pour rompre les différents types de liaisons d'une protéine à la membrane, j'ai démontré que l'ArgRS mt n'est sensible qu'à des concentrations élevées d'urée, indiquant que la liaison à la membrane mitochondriale se fait *via* des interactions hydrophobiques. Cette localisation a été confirmée dans diverses lignées cellulaires issues de différents tissus humains. Par ailleurs, j'ai montré que l'ArgRS mt ne sort pas de la mitochondrie en condition de stress. Une partie de ces travaux a fait l'objet d'une publication, dont je suis co-premier auteur.

(III) Etude de l'impact de mutations « pathologiques » sur les propriétés de l'ArgRS mt.

Afin d'évaluer l'impact de mutations sur la localisation exclusivement membranaire de l'ArgRS mt, un système d'expression reposant sur la transfection de cellules HEK293T avec différentes constructions plasmidiques (contenant soit la séquence sauvage soit les séquences contenant les mutations corrélées à PCH6) a été utilisé. La première étape a été de valider ce système et de démontrer que l'adressage, l'internalisation, et la localisation membranaire de la forme recombinante contenant une étiquette poly-histidine de l'ArgRS mt se font correctement, de façon identique à l'ArgRS mt endogène.

Ce système d'expression a permis d'analyser individuellement l'impact de 12 mutations "pathologiques" sur la localisation de l'ArgRS mt *in cellulo*. Aucune des mutations n'affecte la localisation membranaire de l'ArgRS mt. En parallèle, des fibroblastes (hétérozygotes composites) issus d'un patient atteint de PCH6 ont été cultivés. Là encore, aucune délocalisation de l'ArgRS mt n'est observée.

En parallèle, j'ai étudié l'impact de 6 des mutations liées à PCH6 sur la propriété d'aminacylation de l'ArgRS mt *in vitro*. Pour ce faire, j'ai introduit chacune des mutations dans l'enzyme recombinante identifiée soluble (L72_ArgRS) après expression en système bactérien (voir ci-dessus). Les protéines sauvage et mutantes ont été produites dans *E. coli* et purifiées par chromatographie d'affinité. Les activités enzymatiques des mutants ont été établies, comparées à celle de l'enzyme sauvage. Aucun impact significatif n'est observé en dépit du fait que pour cette enzyme, les mutations touchent des positions très conservées, et de la gravité de la maladie. Ces deux observations étaient en faveur d'un impact fort sur la fonction canonique de l'ArgRS mt, or ce n'est pas le cas. Cette découverte soutient la possibilité d'une implication de l'ArgRS mt dans une fonction alternative, ce qui pourrait expliquer la tissu spécificité du phénotype clinique.

Conclusions et perspectives

Ce travail de thèse est une contribution importante dans la compréhension de propriétés fondamentales des aaRS mt humaines et des pathologies liées. Les

observations et résultats obtenus ouvrent de nombreuses perspectives. Celles initiées par moi et poursuivies au laboratoire sont indiquées ci-dessous.

L'analyse de phénotypes cliniques des patients permet une vision plus globale des pathologies liées aux aaRS mt, d'identifier des groupes d'aaRS mt qui ont des présentations cliniques similaires, et de ce fait, de proposer des hypothèses de travail. Par exemple, des mutations dans trois synthétases (mt-AspRS, mt-GluRS et AspRS cytosolique) conduisent à une leucodystrophie. Ces trois enzymes ont en commun l'aspartate et le glutamate, précurseurs indispensables au N-acetyl L-Aspartate Glutamate (NAAG), neurotransmetteur du système nerveux central. Une hypothèse en cours d'étude au laboratoire est de rechercher un lien possible entre l'AspRS mt et l'Asp-NAT (L-aspartate N-acetyltransferase). Cette enzyme est ancrée à la membrane mitochondriale interne, est spécifique du L-aspartate, est présente seulement dans le système nerveux central, et catalyse la synthèse de NAA (précurseur du NAAG). Or, le taux de NAA est significativement diminué chez les patients atteints de LBSL. Une seconde perspective en cours d'étude au laboratoire est la recherche de partenaire(s) d'aaRS mt qui soient neurone-spécifique(s). Pour ce faire, des expériences de "cross-link", initialement effectuées sur cellules HEK 293T ou HELA, sont poursuivies sur des cellules neuronales, telles les neuroblastomes SH-SY5Y non différenciées et différenciées.

Concernant l'établissement de propriétés cellulaires des aaRS mt humaines: alors que l'AspRS mt avait été identifiée doublement localisée dans la mitochondrie, j'ai démontré que l'ArgRS se retrouve uniquement au niveau des membranes et son ancrage se fait par des interactions de type hydrophobique. L'observation de localisations distinctes pour deux enzymes supposément impliquées dans le même processus cellulaire de traduction soulève immédiatement la possibilité que ces enzymes aient des propriétés cellulaires distinctes. L'analyse est actuellement étendue à d'autres aaRS mt au laboratoire, et il est montré qu'une troisième enzyme, la LysRS, est quant à elle présente dans la fraction soluble, uniquement. Ainsi, il sera important de définir la localisation à l'intérieur de la mitochondrie pour l'ensemble des aaRS mt, de vérifier les profils obtenus dans différents types cellulaires mais aussi dans des conditions physiologiques, ou pathophysiologiques distinctes. Il sera intéressant de voir si la

catégorisation établie corrèle avec celle des présentations cliniques observées. A plus long terme, il sera important de comprendre la raison biologique des différences observées: manière de moduler l'activité canonique d'aminacylation? Révélateur de fonction(s) alternative(s), éventuellement tissu-spécifique? Lien avec les différentes formes observées en particulier pour l'AspRS mt après l'étape de maturation *in organello*? L'ensemble de ces caractérisations futures permettra de mieux définir les propriétés fondamentales des aaRS mt, mais aussi de mieux comprendre comment la traduction mitochondriale est intégrée dans l'environnement cellulaire et quelles sont les modulations et/ou spécificités tissus-spécifiques.

L'identification des raisons moléculaires sous-jacentes aux pathologies liées aux mutations d'aaRS mt reste un défi important. Toutes les analyses effectuées précédemment au laboratoire sur l'impact possible des mutations liées à LBSL n'ont pas permis de mettre en évidence d'impact majeur sur les propriétés canoniques de l'AspRS. J'ai démontré, par mes travaux, que les mutations liées à PCH6 n'ont pas non plus d'impact sur la localisation ou la propriété d'aminacylation de l'ArgRS mt, alors qu'un impact fort était anticipé. Nous avons imaginé comme perspective de ce travail, de démontrer l'existence de fonction non canonique, neuronale spécifique de l'AspRS mt (dans un premier temps). Pour ce faire, des lignées cellulaires neuronales, dont les mutations liées à LBSL sont individuellement introduites par la technique de CrispR-Cas9 sont en cours de préparation au laboratoire (en collaboration). La démonstration d'un effet sévère sur les lignées neuronales et modérée sur des lignées non-neuronales sera un argument fort en faveur d'une fonction neurone-spécifique de l'AspRS mt, qui sera ensuite recherchée.

Mon travail de thèse est un apport à l'établissement des propriétés cellulaires des aaRS mt humaines et ouvre à de nouvelles voies d'investigation. La compréhension des mécanismes moléculaires sous-jacents aux pathologies liées aux mutations dans les aaRS mt ainsi que la tissu-spécificité, en particulier neuronale, des maladies liées ne sera probablement possible que après une meilleure compréhension de comment la traduction mitochondriale est intégrée/modulée dans un contexte cellulaire.

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Caractérisation de l'ArgRS mt humaine et contribution à la compréhension des pathologies liées aux mutations des aaRS mt

Résumé

Les aminoacyl-ARNt synthétases mitochondriales humaines (aaRS mt) sont des enzymes clés de la traduction mitochondriale. Elles catalysent l'amination des ARNt par les acides aminés correspondants. Des mutations dans leurs gènes sont corrélées à des pathologies avec un large spectre de phénotypes cliniques, mais aux mécanismes moléculaires sous-jacents encore incompris. L'objectif de ce travail de thèse s'intègre dans les axes scientifiques du laboratoire, mais élargit l'intérêt et les connaissances à un système encore peu exploré: l'arginyl-ARNt synthétase mitochondriale (ArgRS mt). Des mutations dans la ArgRS sont liées à une hypoplasie Pontocérébelleuse (PCH6), une pathologie neurodéveloppementale sévère. Le travail de cette thèse s'articule autour de 3 axes : (I) L'analyse des phénotypes cliniques des pathologies liées aux mutations dans les aaRS mt, (II) La caractérisation des propriétés cellulaires de l'ArgRS mt, et (III) L'étude de l'impact de mutations « pathologiques » sur diverses propriétés de l'ArgRS mt. Combinés avec les travaux précédents, les résultats obtenus sont une contribution importante à l'élargissement des connaissances fondamentales des mt aaRSs, et apportent un nouvel éclairage sur le lien entre les mt-aaRSs-mutations et la maladie.

Mots clés: traduction mitochondriale, mitochondrial aminoacyl-ARNt synthétase, aminoacylation, organisation sous mitochondriale, pathologies liées aux mutations.

Abstract

Human mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs) are housekeeping enzymes involved in the mitochondrial translation. They catalyze the aminoacylation of tRNAs with their cognate amino acids. Mutations in their nuclear genes are correlated with pathologies with a broad spectrum of clinical phenotypes, but with so far no clear explanations about the underlying molecular mechanism(s). The aim of this PhD work follows the long-standing efforts of the host laboratory but expands the interest and knowledge to an unexplored system: the human mitochondrial arginyl-tRNA synthetase (mt-ArgRS). Mutations in the mt-ArgRS lead to Pontocephalic hypoplasia type 6, a severe neuro-developmental pathology. I thus contributed to i) comprehensively analyze the clinical data reported in pathologies related to mutations on mt-aaRSs, resulting in a categorization according to the affected anatomical system; ii) decipher some cellular properties of the mt-ArgRS; and iii) investigate the impact of disease-associated mutations on mt-aaRSs properties. Combined with previous works, the present results expand the knowledge of the mt-aaRSs, shedding new light on the link between mt-aaRSs-mutations and disease.

Keywords: mitochondrial translation, mt-aaRSs, aminoacylation, sub-mitochondrial localization, pathology-related mutations.