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“Aminoacyl-tRNA synthetases mutations in human mitochondrial diseases: contribution of yeast to understand the functional significance of novel variants and to approach the identification of therapeutical compounds”

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ABSTRACT

To date, genetic analysis with Next Generation Sequencing (NGS) approaches in patients affected by mitochondrial diseases (MD) has made possible the identification of new disease genes or new mutations in known disease genes, thus contributing to improve the diagnosis of mitochondrial diseases and to find the molecular defects causing pathological phenotype.

Mitochondrial aminoacyl-tRNA synthetases (mtARSs) are nuclear encoded essential enzymes for mitochondrial translation since they ensure the proper attachment of each amino acids to its cognate tRNA. Mutations in the genes encoding for mtARSs are associated to mitochondrial human disorders that can primarily affect central nervous but also other organs and systems.

Recently, in patients affected by MD, many novel variants have been found in mtARS genes and for this reason it becomes essential to assess their pathogenic significance.

Over the years, the simple eukaryote *Saccharomyces cerevisiae* demonstrated to be a good tool to understand the causative role of mutations associated to MD thanks to its ability to grow without a functional respiratory chain, if a fermentable carbon source is made available. Moreover, since most of mutations are found in compound heterozygosity, the yeast ability to exist in haploid or diploid status can help to investigate on the contribution of each mutation to the pathological phenotype.

Most of this PhD work has focused on the validation and functional analysis of novel variants, identified by our clinical collaborators, in mtARSs genes using *ad hoc* yeast models and to approach the identification of therapeutical molecules able to restore, at least partially, the pathological phenotype.

Specific yeast models for the different mtARS genes considered in this work (*DARS2*, *NARS2*, *RARS2*, *WARS2*, *VAR2*) have been constructed and, on mutant strains expressing the different allelic variants, several phenotypic characterizations have been performed.

Moreover, since there is currently no established treatment for pathologies caused by mutations in mtARSs this research has addressed also the identification of beneficial molecules. In particular it focused on the evaluation of the ability of amino acid supplementation to rescue the mitochondrial impairment showed by mutant strains and on the search of chemical compounds able to rescue the OXPHOS defective phenotype associated to mtARS mutations using drug repositioning as strategy.

Another part of this work concerned the evaluation of the functional conservation of pantothenate kinase (PanK) activity between human and yeast. PanK are the enzymes involved in the first step of biosynthesis of Coenzyme A, a key molecule involved in several metabolic pathways. For this purpose, the ability of the three different isoforms of PanK to rescue the pathological phenotypes of yeast model strains lacking for the unique gene encoding for PanK, *CAB1*, and carrying pathological mutations associated to PKAN has been evaluated.

PART 1

1. Introduction

1.1. Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases (ARSs) are a class of nuclear-encoded enzymes essential for the correct translation of the genetic code. The role of each enzyme is to ensure the proper attachment of each amino acids to its cognate tRNA during protein synthesis using a reaction divided into two steps [1]: the first step consists in the amino acid activation by ATP hydrolysis with the formation of an aminoacyl adenylate (aa-AMP) and the releasing of a molecule of pyrophosphate; the second step consists in the transfer of the aa-AMP on its corresponding tRNA [2](Figure 1.1).

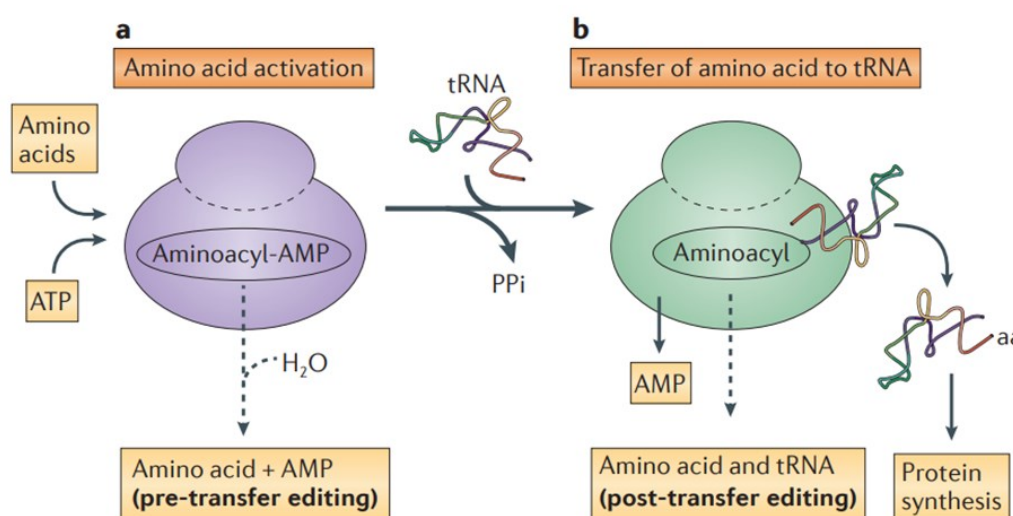


Figure 1.1 ARSs reaction. The reaction catalyzed by ARSs is divided into two steps: first step involves amino acid activation with the releasing of PPi (a); second step consist in the loading of the activated amino acid on the cognate tRNA (b)[3].

The tRNA charging is a high-fidelity process thanks to the proofreading activity given by an editing domain in the aminoacyl-tRNA synthetase protein sequence; this domain has the role to correct misaminoacylation by removing the wrong amino acid or the mischarged tRNA [4]. ARSs are divided into two families called class I and class II on the basis of the architecture of their active site [5]. ARSs belonging to class I are generally monomeric and possess in the folded protein conformation a Rossmann fold that consists in a five stranded β -sheet connected by α -helices [4]. In this structure two conserved motifs are present: HIGH (His-Ile-Gly-His) and KMSKS (Lys-Met-Ser-Lys-Ser). The first helps in the correct positioning of the adenine of ATP and makes interactions with phosphates. The role of the second motif is to bind the amino acid in the catalytic site, forming a loop that, in performing this action, is open; when the aa-AMP is formed, the status of the loop shifts to closed [6–8]. Class II ARSs can be dimeric or multimeric and possess a seven-stranded β -sheet conformation flanked by α -helices [4]. Their catalytic domain presents three conserved motifs that

have a critical role in the enzymatic activity: in particular, motif 3 has a highly conserved Arg that binds ATP, while motif 2 possess highly conserved Arg and Phe that are involved in the coupling between ATP and amino acid and in the transfer of the activated amino acid to the 3'- of the cognate tRNA. Conserved residue of motif 1, as well as motif 2, are also important in the enzyme dimerization [9]. Also in this case, during the reaction, the protein undergoes to a conformational change [4]. Another difference between class I and class II ARSs concerns on which adenosine of the tRNA the amino acid is placed: class I ARSs catalyze the binding of the amino acid on the 2' hydroxyl group while class II ARSs on 3' hydroxyl group and, after an esterification reaction, the amino acid is eventually shifted on the 2' hydroxyl group [9].

The ARSs genes are generally identified as *XARS1* or *XARS2*, where X indicates the amino acid charged by the specific ARS and 1 or 2 indicate if the protein acts in the cytosol or mitochondria, respectively; this gene family includes 17 genes that encode for the cytosolic isoforms, 17 genes encoding for mitochondrial isoforms, 2 genes that encode for both cytosolic and mitochondrial isoforms, *GARS* and *KARS*, that use a different start codon and an alternate splicing site, respectively [10,11] and 1 gene that is a fusion gene encoding for the glutamyl-prolyl tRNA synthetase [12] (Figure 1.2).

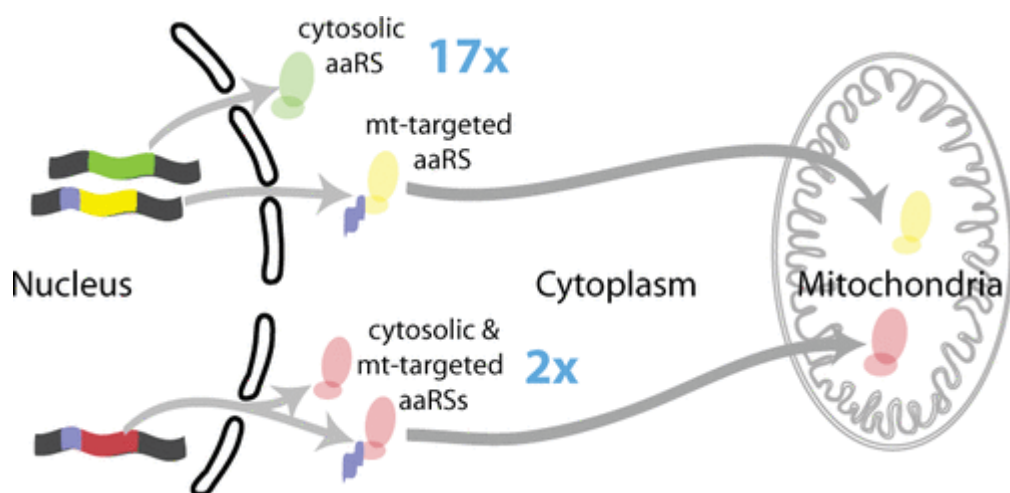


Figure 1.2 Representation of genes encoding for cyt- and mt-ARSs [13].

1.2. Mitochondrial diseases associated to mutations in mitochondrial aminoacyl-tRNA synthetases (mtARSs)

Mitochondrial diseases (MD) are a group of clinically heterogeneous pathologies with an incidence of 1 in 5000 births making MD among the most common genetic disorders. This kind of diseases is associated with mutations not only in mitochondrial genes but in every nuclear gene having a role in mitochondrial function/biogenesis.

Because of the variety of the symptoms associated to MD, it is difficult to give a precise definition of this pathology, to make diagnosis and to identify therapies and drug targets [14].

Mitochondria have a central role in several essential pathways, including ATP synthesis through oxidative phosphorylation (OXPHOS) carried out by respiratory chain complexes. They have their own DNA, mitochondrial DNA (mtDNA), that encodes for thirteen of the proteins of respiratory chain complexes synthesized inside the organelle, while the other additional proteins are encoded by nuclear genes, synthesized in the cytoplasm and imported into mitochondria. This genomic distribution also applies to molecules of the mitochondrial translation machinery, since the two rRNAs and the 22 tRNAs are encoded by mtDNA, but all the other proteins, such as ribosomal proteins, tRNA modifying enzymes, translation factors, aminoacyl-tRNA synthetases, etc. are encoded by the nuclear genome [15].

Therefore, it is no wonder if mutations affecting the mitochondrial translation machinery lead to human pathologies [16–18].

Mutations in mitochondrial genes encoding for functions that are needed for mitochondrial protein synthesis were identified in patients since the late 80's and pathologies associated with these mutations were called “mitochondrial translation disorders” which include several clinical presentations in which muscular and neurological systems were particularly involved [19–22]. More recently, it has been assessed that also mutations in nuclear genes encoding proteins that play a role in mitochondria translation are linked to MD. Between nuclear genes encoding mitochondrial located proteins, several mutations in mtARS genes were found to be associated to mitochondrial diseases. The disorders derived from these mutations include a wide range of symptoms and can be characterized by defined symptoms and/or neuroradiological features (e.g. LBSL), isolated clinical signs (e.g. nonsyndromic hearing loss) or described syndromes (e.g. Perrault syndrome). On the basis of which organ system is involved in the symptomatology, four main groups were distinguished: mtARSs with mutations leading to clinical manifestations (i) exclusively in the central nervous system (CNS); (ii) in the CNS *and* another system; (iii) in the CNS *or* another system, and (iv) a system other than the CNS (Table 1.1) [11].

Gene	Protein	Main phenotype	Main affected organ	Age on onset
mtARSs correlated with clinical manifestations in exclusively the CNS				
Encephalopathies				
<i>CARS2</i>	mt-CysRS	Mitochondrial epileptic encephalopathy		Infancy-Childhood
<i>FARS2</i>	mt-PheRS	Alpers encephalopathy		Infancy

<i>NARS2</i>	mt-AsnRS	Alpers' syndrome Leigh syndrome Non-syndromich hearing loss (NSHL)	Brain	Infancy-Childhood
<i>PARS2</i>	mt-ProRS	Alpers' syndrome		Infancy
<i>RARS2</i>	mt-ArgRS	Pontocerebellar Hypoplasia type 6 (PCH6)		Infancy
<i>TARS2</i>	mt-ThrRS	Fata mitochondrial encephalopathy		Infancy
Leukoencephalopathies				
<i>DARS2</i>	mt-AspRS	Leukoencephalopathy with brainstes, spinal cord involvement and lactate elevation (LBSL)	Brain	Childhood-Adulthood
<i>EARS2</i>	mt-GluRS	Leukoencephalopathy with thalamus and brainstem involvement and high lactate elevation (LTBL)		Infancy
<i>MARS2</i>	mt-MetRS	Autosomal recessive spastic ataxia wuth leukoencelopathy (ARSAL syndrome)		Childhood-Adulthood
<i>WARS2</i>	mt-TrpRS	Intellectual disability		Infancy-Childood
mtARSs correlated with clinical manifestations in the CNS and other system				
<i>VARs2</i>	mt-ValRS	Fatal mitochondrial encephalocardio-myopathy	Brain and heart	Infancy
<i>AARS2</i>	mt-AlaRS	Leukoencephalopathy with ovarian failure	Brain and ovaries	Childhood-Adulthood
<i>HARS2</i>	mt-HisRS	Perrault syndrome	Brain and ovaries	Childhood-Adulthood
<i>LARS2</i>	mt-LeuRS	Perrault syndrome	Brain and ovaries	Childhood-Adulthood
<i>IARS2</i>	mt-IleRS	CAGSSS Leigh syndrome	Brain and musculoskeletal	Infancy-Childhood
mtARSs correlated with clinical manifestations in the CNS or other system				
<i>SARS2</i>	mt-SerRS	Progressive spastic paresis	Brain	Infancy
		Hyperuricemia, Pulmonary hypertension, Renal	Kidney	Infancy

		failure in infancy and Alkalosis (HUPRA)		
mtARSs correlated with clinical manifestations in a system other than the CNS				
<i>AARS2</i>	mt-AlaRS	Hypertrophic cardiomyopathy	Heart	Infancy
<i>YARS2</i>	mt-TyrRS	Myopathy, Lactic Acidosis, Sideroblastic Anaemia 2 (MLASA2)	Muscle/Bone marrow	Childhood-Adulthood

Table 1.1 mtARSs classification on the basis of the system involved [11].

In the first group are classified mtARSs whose mutations lead to clinical manifestations only in the CNS. This group is further divided into epileptic encephalopathy as main symptom, that was observed in patients with mutations in *CARS2*, *FARS2*, *NARS2*, *PARS2*, *RARS2*, and *TARS2* genes, and leukoencephalopathy, observed in patients with mutations in *DARS2*, *EARS2*, *MARS2*, and *WARS2*. For the first subgroup, epilepsy in all its forms is the commonest feature while mutations affecting genes belonging to the second subgroup manifest in patients as changes in the white matter as well as ataxia, particularly in the lower limbs.

The second group includes some patient with mutations in *AARS2* and all patients with mutations in *HARS2*, *LARS2*, *IARS2*, and *VARS2*. It has been suggested that the secondary symptoms showed by the other system different from CNS are due to primary defects in the CNS itself. For instance, the ovarian failure observed in patients with mutations in *HARS2*, *LARS2* and *AARS2* is possibly caused by disfunctions in the pituitary gland, the hormonal center employed in the correct ovarian function, as well as for mutations in *IARS2*, where impairment in the musculoskeletal system is associated to defects in growth hormone production (at the level of CNS) [23]. Also, in patients with mutations in *VARS2*, cardiomyopathy is suggested to be caused by encephalopathy [24].

Only patients with mutations in *SARS2* belong to the third group, in which was observed that the symptomatology caused by mutations in this gene can affect the brain, manifesting as progressive spastic paresis, or the kidney, manifesting in most of cases with renal failure [25,26]. Despite the mutations affect the same gene, there is no overlapping between the clinical symptoms described above.

In the last group are classified patients with mutations in *YARS2* and *AARS2* that are affected by myopathies and cardiomyopathies, respectively, with no affection of CNS (Figure 1.3).

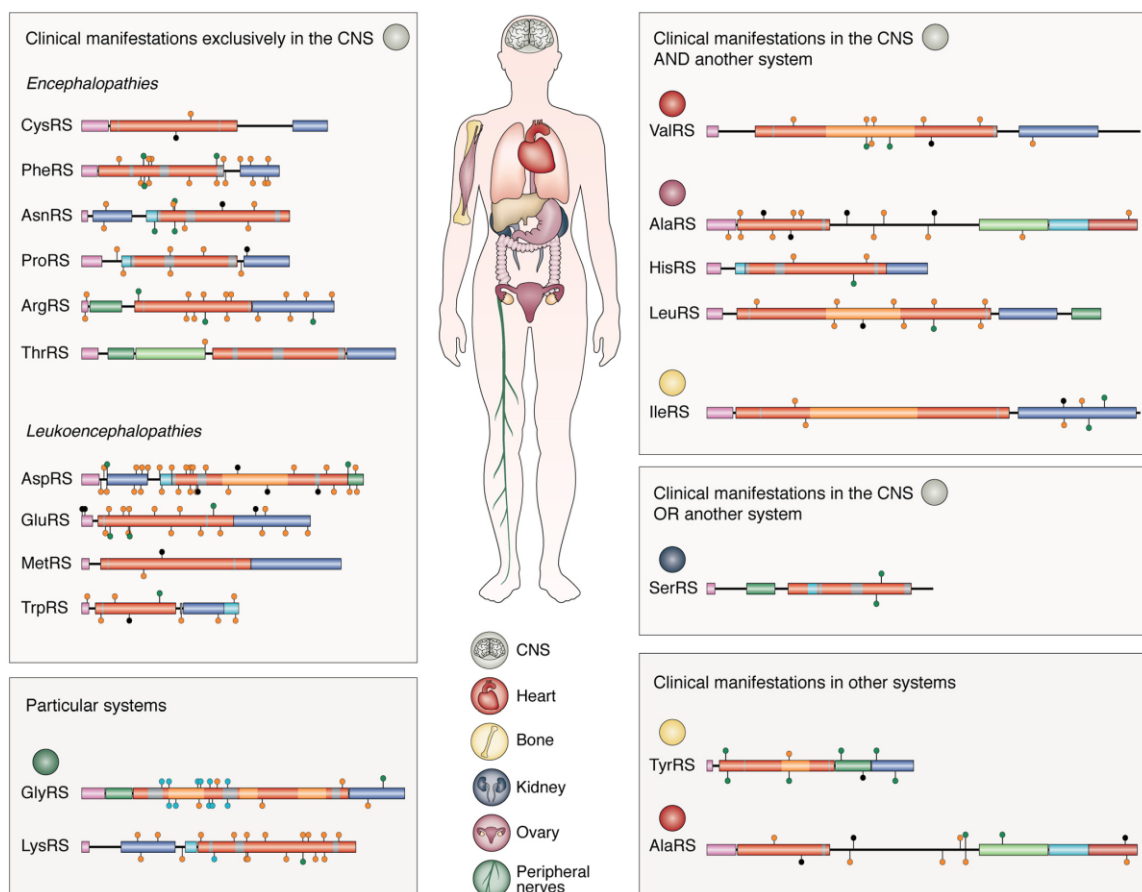


Figure 1.3 Classification of mtARSs following the systems involved in the symptomatology [11].

Another aspect that needs to be underlined about diseases caused by mutations in mtARSs is that the severity of the symptomatology is associated with the age of onset; in particular, earlier a patient manifest symptom, severer is the clinical development of the disease. For example, patients affected by Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and Lactate Elevation (LBSL), due to mutations in *DARS2* gene, can develop symptoms during the childhood but also in the adulthood, manifesting mainly spasticity and ataxia and require, in the more serious cases, the use of a wheelchair. Patients affected by Pontocerebellar hypoplasia type 6 (PCH6), due to mutations in *RARS2*, on the contrary, develop symptomatology very early after birth and the severely affected patients never reach the developmental milestone [27,28].

1.3. Amino acid supplementation as therapeutical strategy

The wide range of clinical manifestations given by mutations associated to MD represents a challenge for the medical community, above all for the development of therapeutic treatments. To date, there is not an effective cure for this kind of pathologies and the treatment of patients is limited to symptomatic treatment and nutritional supplementation. Compounds as dichloroacetate, vitamins A and E, creatine, lipoic acid, L-arginine and CoQ are frequently used in patients affected by mitochondrial diseases but just as treatment of the symptomatology and not

as a cure. Newer therapeutical approaches were developed, like gene therapy consisting in the use of adeno-associated viral (AAVs) employed to correct the mutations or CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) used for genome editing, and good results have been obtained in animal models, but their efficacy in humans was not tested yet. An important requirement for the development of new therapeutical approaches is the understanding of the molecular insights characterizing the pathologies and, in the case of mtARSs, this has not been assessed [29].

Being mitochondrial protein synthesis essential for life, mtARSs mutations identified in MD patients does not lead to complete loss of function but they have to maintain partial enzymatic activity. In the hypothesis that increased availability of the aa substrate specific to a given mtARS could ameliorate the efficiency of the mutant enzyme, one of the strategies under investigation for the treatment of patients affected by MD caused by mutations in both genes encoding for cytosolic and mitochondrial ARSs, is the supplementation of the specific amino acid substrate of the defective ARS.

Beneficial effect for the supplementation of some amino acids was observed in yeast models (e.g. valine); oxidative growth of a yeast strain expressing a *VAR2* mutation in the yeast ortholog *VAS1* resulted increased when valine was added in the media, suggesting that the analyzed mutation could affect a residue important for the binding of the aminoacid [30].

Moreover, there are reports about benefits of aa supplement in diverse cell lines [31,32]. However, when the same approach was used in different mtARS *in vitro* models no beneficial effect has been observed, suggesting that the response to aa supplementation is gene or mutation-specific.

As recently hypothesized by Kok et al. [33], aminoacylation in ARS patients could be sufficient for cellular functions under normal conditions but not in situation in which amino acid availability is decreased, for example during vomiting or starvation, or when an increased translation activity is requested, like during illness or tissues formation or rapid growth. Studies performed in patients fibroblasts, with mutations in *LARS* and *FAR2B* (encoding for subunit beta of PheRS) genes, demonstrated indeed a strong decrease of aminoacylation activity when incubated at feverish temperature (38,5-40°C), probably due to protein unfolding. This effect was especially observed in variants that decrease protein stability [34]. In the same study, amino acid supplementation was also used directly on patients and was demonstrated that amino acid supplementation could improve patients symptoms only if some residual enzymatic activity is left [34].

1.4. mtARSs interactions and non-canonical functions

The principal role of ARSs is to charge each amino acid to its cognate tRNA and they can perform their role as free-standing enzymes or interacting with other proteins, the aminoacyl-tRNA synthetase-interacting multifunctional proteins (AIMPs), to form the MSC complex (Multi-Synthetase Complex). The MSC complex was observed in humans, where is called MARS complex as well as in yeast, where it is known as AME complex. However, under specific stress conditions or post-translational modification, the ARS associated with the complex can be released and act as a free-standing enzyme [35].

Despite their principal role, ARSs during the evolution acquired supplementary domains and insertions that conferred them other functions [36].

Most of the non-canonical functions are associated to cytosolic ARSs that however possess the same domains of the mitochondrial ones; thus, possibly the supplementary function could be executed by cytosolic as well as mitochondrial ARSs.

Just to give some examples, in human, the WHEP domain, found in in tryptophanyl-, histidinyl-, glycyl-, and glutamyl-prolyl-tRNA synthetase was demonstrated to stimulate the IFN γ (interferon gamma), by inducing a cascade reaction leading to the inhibition of translation of the mRNA targeted with GAIT complex (Gamma Interferon Inhibitor of Translation) [37–40].

The human glutaminyl-tRNA synthetase can also perform an antiapoptotic action, by interacting with the catalytic domain of an essential factor for apoptosis, the Apoptosis Signal-regulating Kinase 1 (ASK1) [35]; and studies on arginyl-tRNA synthetase in mammals reported that the binding with AIMPs protein is given by the presence of two leucine-zippers in the protein N-terminal extension [41–43].

Despite these functions given by specific protein domains, some role was associated specifically to mitochondrial aminoacyl tRNA-synthetases [36]. This is the case of yeast mitochondrial LeuRS that has been demonstrated to be involved in the splicing of group I introns of mitochondrial mRNA (encoding for the mitochondrial cytochrome b and a subunit of cytochrome c oxidase) [44]. For the splicing activity, the interaction between mtLeuRS and a maturase protein called b14 is requested, but in absence of the latest, mutations in a specific domain of mtLeuRS (the suppression site) reprimarize the splicing function, demonstrating that the synthetase alone can be sufficient for this supplementary activity [45].

In *Neurospora crassa* a second function of mitochondrial TyrRS was observed. The protein is involved in the splicing of mitochondrial rRNA: in fact, it interacts with particular domains of the mt-rRNA itself, helping in the correct folding and the correct creation of the active splice site [46].

In *Saccharomyces cerevisiae* another role of mitochondrial LysRS was demonstrated. In this organism, three iso-acceptor tRNA for the lysine are present: tRNA^{Lys}₁; tRNA^{Lys}₂; tRNA^{Lys}₃. The 5% of the first one is imported into mitochondria (95% remains in the cytosol) [47], the second is found only in the cytosol, the third (encoded by mitochondrial DNA) is exclusively in mitochondria. That 5% of tRNA^{Lys}₁ need to be linked with the pre-mtLysRS to be imported into mitochondria: it was demonstrated that tRNA^{Lys}₁ binds another protein (Eno2p) that helps in the successive binding with the pre-mtLysRS before the translocation in the organelle. Once in the mitochondria, pre-mtLysRS and tRNA^{Lys}₁ dissociate, the MTS of pre-mtLysRS is cut off making the protein able to perform the aminoacylation activity binding tRNA^{Lys}₃, while the tRNA^{Lys}₁ is requested for mitochondrial translation only at elevated temperatures [48,49].

1.5. *Saccharomyces cerevisiae* as model to study mitochondrial diseases caused by mutations in mtARSs

The use of Next Generation Sequencing (NGS) approach has allowed the identification of an increasing number of mutations in mtARSs in patients affected by mitochondrial diseases. To date, 400 between missense and nonsense mutations in all mtARSs were discovered, 233 of whom in different positions (<http://misynpat.org/>, accessed on December 10, 2022).

When novel variants are identified a major challenge is to determine if these are the cause of the pathology. Bioinformatic tools can certainly help but they are not sufficient to prove unequivocally the causality, that must be evaluated biologically through functional analyses. Fibroblasts obtained from patients often did not show any easily measurable readout; for these reasons models are very useful and among these the yeast *Saccharomyces cerevisiae*. Thanks to its genetic tractability this simple eukaryote is one of the most intensively investigated model organisms in which several basic cellular mechanisms have been elucidated. Moreover, thanks to significant conservation in gene function between yeast and humans, this single cell organism proved to be an important tool to model human diseases. Owing to its good fermentation capacity, it can survive the loss of OXPHOS and for this reason it was established as a suitable genetic system for the study of pathological mutations in nuclear genes associated with mitochondrial diseases; it succeeds, indeed, in maintaining the mitochondrial function being able to survive without a functional respiratory chain. Therefore, in yeast mutations affecting the mitochondrial functions results in a respiratory deficient phenotype. Moreover, since yeast can exist in haploid or diploid status the contribution of individual mutations to the pathogenesis can be investigated [50–54]. For validating mutations in yeast, different approaches can be used.

When a human disease-linked gene does not have an ortholog in yeast, cDNA of the human gene of interest and the mutant alleles can be expressed in yeast under the control of an easily regulatable promoter, in order to control the expression of the human protein. On the contrary, if the human disease-linked gene has an ortholog in yeast, mutations can be introduced directly in human cDNA, if this is able to complement a yeast strain lacking for the endogenous gene (heterologous complementation), or in the yeast orthologue gene if the mutated residue in human counterpart is conserved between human and yeast protein (homologous complementation) [53].

Regarding *ARS* genes, as recently reviewed [54], in most of cases in yeast two genes encoding for mtARSs exist, one encoding for the cytosolic isoform, the second for the mitochondrial one. However, it is not a restrictive rule, since in some case only one gene encodes for both isoforms. Based on this feature, mtARSs were classified into four groups reflecting the presence of one or two genes in human and in yeast. The four groups are the following (Table 1.2):

Cases	Human	Yeast
Two to Two	mtARS, cytARS	mtARS, cytARS
Two to one	mtARS, cytARS	ARS
One to One	ARS	ARS
One to Two	ARS	mtARS, cytARS

Table 1.2 Genes encoding for ARSs in human and yeast. Each organism can possess two gene encoding for mitochondrial and cytoplasmic isoform (mtARS and cytARS, respectively) or one single gene encoding for both isoforms (ARS) [54].

Besides these four categories, other cases exist in which the aminoacylation of the tRNA of some ARS causes the addition of different amino acids to the nascent protein because of dissimilarities in the genetic code between human and yeast.

Mutations in several mtARSs have been previously validated in yeast model, as reported in Table 1.3, using different approaches.

Human Gene vs Yeast Gene	Protein	Human Gene	OMIM Phentoype	Yeast Gene(s)
Two to Two	mt aspartyl-tRNA synthetase	<i>DARS2</i>	#611105: leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation, LBSL	<i>MSD1</i>
	mt phenylalanyl-tRNA synthetase	<i>FARS2</i>	#614946: combined oxidative phosphorylation deficiency 14; COXPD14 #617046: spastic paraplegia 77, autosomal recessive, SPG77 #203700: mitochondrial DNA depletion syndrome 4A (Alpers type); MTDPS4A	<i>MSF1</i>
	mt isoleucyl-tRNA synthetase	<i>IARS2</i>	#616007: cataracts, growth hormone deficiency, sensory neuropathy, sensorineural hearing loss, and skeletal dysplasia; CAGSSS #256000: Leigh syndrome; LS	<i>ISM1</i>

	mt methionyl-tRNA synthetase	<i>MARS2</i>	#611390: spastic ataxia 3, autosomal recessive; SPAX3 #616430: combined oxidative phosphorylation deficiency 25; COXPD25	<i>MSM1</i>
	mt asparaginyl-tRNA synthetase	<i>NARS2</i>	#618434: deafness, autosomal recessive 94; DFN894 #203700: mitochondrial DNA depletion syndrome 4A (Alpers type); MTDP54A #616239: combined oxidative phosphorylation deficiency 24; COXPD24	<i>SLM5</i>
	mt prolyl-tRNA synthetase	<i>PARS2</i>	#618437: developmental and epileptic encephalopathy 75; DEE75	<i>AIM10</i>
	mt seryl-tRNA synthetase	<i>SARS2</i>	#613845: hyperuricemia, pulmonary hypertension, renal failure, and alkalosis; HUPRAS	<i>DIA4</i>
	mt tryptophanyl-tRNA synthetase	<i>WARS2</i>	#617710: neurodevelopmental disorder, mitochondrial, with abnormal movements and lactic acidosis, with or without seizures; NEMMLAS	<i>MSW1</i>
	mt tyrosyl-tRNA synthetase	<i>YARS2</i>	#613561: myopathy, lactic acidosis, and sideroblastic anemia 2; MLAS2	<i>MSY1</i>
One to One	mt and cyt glycyl-tRNA synthetase	<i>GARS</i>	#601472: Charcot-Marie-Tooth disease, axonal, type 2D; CMTD2 #600794: neuronopathy, distal hereditary motor, type VA; HMN5A #619042: spinal muscular atrophy, infantile, James type; SMAJI	<i>GRS1</i>
Two to One	mt alanyl-tRNA synthetase	<i>AARS2</i>	#615889: leukoencephalopathy, progressive, with ovarian failure; LKENP #614096: combined oxidative phosphorylation deficiency 8; COXPD8	<i>ALA1</i>
	mt cysteinyl-tRNA synthetase	<i>CARS2</i>	#616672: combined oxidative phosphorylation deficiency 27; COXPD27	<i>CRS1</i>
	mt histidyl-tRNA synthetase	<i>HARS2</i>	#614926: Perrault syndrome 2; PRLTS2	<i>HTS1</i>
	mt valyl-tRNA synthetase	<i>VAR2</i>	#615917: combined oxidative phosphorylation deficiency 20; COXPD20	<i>VAS1</i>
One to Two	mt and cyt lysyl-tRNA synthetase	<i>KARS</i>	#613641: Charcot-Marie-Tooth disease, recessive intermediate, B; CMTRIB #613916: deafness, autosomal recessive 89; DFN889 #619196: deafness, congenital, and adult-onset progressive leukoencephalopathy; DEAPLE #619147: leukoencephalopathy, progressive, infantile-onset, with or without deafness; LEPID	<i>KRS1</i> <i>MSK1</i>
Others	mt leucyl-tRNA synthetase	<i>LARS2</i>	#615300: Perrault syndrome 4; PRLTS4 #617021: hydrops, lactic acidosis, and sideroblastic anemia; HLASA	<i>NAM2</i>
	mt arginyl-tRNA synthetase	<i>RARS2</i>	#611523: pontocerebellar hypoplasia, type 6; PCH6	<i>MSR1</i>
	mt threonyl-tRNA synthetase	<i>TARS2</i>	#615918: combined oxidative phosphorylation deficiency 21; COXPD21	<i>THS1</i> <i>MST1</i>
	mt glutamyl-tRNA and mt glutamyl-tRNA ^{Gln} synthetase	<i>EARS2</i>	#614924: combined oxidative phosphorylation deficiency 12; COXPD12	<i>MSE1/GUS1*</i>
	GatTCAB complex	<i>QRSL1</i>	#618835: combined oxidative phosphorylation deficiency 40; COXPD40	<i>HER2</i>
		<i>GATB</i>	#618838: combined oxidative phosphorylation deficiency 41; COXPD41	<i>PET112</i>
		<i>GATC</i>	#618839: combined oxidative phosphorylation deficiency 42; COXPD42	<i>GTF1</i>

Table 1.3 Human mtARSs and corresponding yeast ortholog genes [54].

Nine genes belong to the *Two to Two* group (*DARS2*, *FARS2*, *IARS2*, *MARS2*, *NARS2*, *PARS2*, *SARS2*, *WARS2*, *YARS2*) and the ability of human cDNA to complement the yeast null mutant was tested only

for *IARS2*, that reached only partial complementation. Yeast model for validating mutations were performed for *DARS2* (performed for the first time in this research work and already published [55]), *WARS2* and *YARS2* using the homologous complementation approach, thus introducing mutations in the corresponding ortholog yeast gene *MSD1*, *MSW1* and *MSY1*, respectively [55–59].

One gene belong to the *One to One* group, *GARS*, that encodes for both cytosolic and mitochondrial glycyl-tRNA synthetase, as well as its yeast counterpart, *GRS1*. Even if it is supposed that mutations in *GARS* could affect both cytosolic and mitochondrial translation, it was actually demonstrated that some mutation can influence more one translation system than the other; in fact, cognate cyt-tRNAs and mt-tRNAs can be differently concentrated and the mutations identified in this gene are associated with Charcot-Marie-Tooth disease type 2D or distal hereditary motor neuropathy type VA, with no involvement of mitochondrial apparatus [60]. Besides *GRS1*, another gene codifying for GlyRS exist in yeast, *GRS2*, and it is expressed under stress conditions; it possess 12 amino acids more that have a role in the mitochondrial activity of the protein [61]. Models in yeast were performed using both homologous complementation and heterologous complementation, since it was observed complementation by *GARS*, truncated in MTS and WHEP-TRS domains, in a yeast strain deleted in *GRS1* [62–67].

Four genes belong to *Two to One* group (*AARS2*, *CARS2*, *HARS2*, *VAR2*); for these, two different genes encode for cytosolic and mitochondrial isoforms while only one gene in yeast encodes for both isoforms. Except for *CARS2*, yeast models were constructed for the other three genes. In yeast, mitochondrial and cytosolic isoforms are translated by different translation starts in the mRNA sequence of each of these mtARSs. To analyze mutations affecting *Two to One* genes, one of the approaches used was to deduce mutations severity observing mutant alleles complementation in strain totally lacking for yeast ortholog genes, *HTS1* [68] and *ALA1* [69]. This approach has however the limit to evaluate the cytosolic translation more than the mitochondrial. A second yeast approach used for *AARS2* and *VAR2* mutations [30,70,71] is to investigate on the effect of mutations in strains lacking for the endogenous ortholog gene and carrying a cytosolic copy of the same, in order to avoid effects due to cytosolic metabolism.

The only mtARS belonging to *One to Two* group is *KARS*, the only human gene codifying for cytosolic and mitochondrial LysRS, generated by alternative splicing [72,73]. In yeast two isoforms are present, *KRS1*, encoding for the cytosolic isoform and *MSK1*, encoding for the mitochondrial one. It was demonstrated that human cyt-LysRS cloned in a multicopy vector can complement the absence of *KRS1* in a yeast strain [74] and human mt-LysRS can complement the absence of *MSK1* [75]. Taking advantage of this feature it was possible to analyze mutations introducing them both in the cytosolic or mitochondrial isoform and assess on which isoform mutations had the major impact [75].

Besides these four cases, the remaining mtARSs (*LARS2*, *RARS2*, *TARS2*, *EARS2* and CatCAB complex genes *QRSL1*, *GATB* and *GATC*) can be considered as special cases. Regarding *LARS2* and *RARS2*, they have as yeast counterpart *NAM2* and *MSR1*. Yeast genes can perform their function but the cognate tRNA loaded by human and yeast protein is different because of dissimilarities in the genetic code, as schematized below (Table 1.4); in fact, the same codon specifies for the loading of different amino acids in human and yeast and, for this reason, yeast cannot exactly mimic human condition, especially for mutation affecting the anticodon binding domain.

Human/Yeast mtARS	Anticodon-Codon mt Human	Anticodon-Codon mt Yeast	aa Codified in Human	aa Codified in Yeast
RARS2/Msr1	UCG-CGN -	UCG-CGN UCU-AGR	Arg Ter	Arg Arg
LARS2/Nam2	UAA-UUR UAG-CUN	UAA-UUR -	Leu Leu	Leu Thr
TARS2/Mst1	UGU-ACN -	UGU-ACN UAG-CUN	Thr Leu	Thr Thr

Table 1.4 Differences in codon/anticodon specificity in human and yeast[54].

Despite this feature, yeast was used as model to study mutations both in *LARS2*, using heterologous complementation [76], and in *RARS2*, introducing mutations in yeast *MSR1* [77].

TARS2 has two ortholog genes in yeast, *MST1*, strictly mitochondrial, and *THS1*, mainly cytosolic but its presence was assessed also in mitochondria where it can charge only the canonical tRNA (see table 1.4). Mutations in *TARS2* can not be performed introducing them in *MST1* gene, because it is considered an error prone synthetase since it lacks the post transfer N-terminal editing domain, reason for which could load the wrong amino acid. It cannot be possible introducing *TARS2* mutations directly in *THS1*, since it is not able to charge the non-canonical tRNA^{Thr}. However, modelling in yeast for *TARS2* mutations was performed; was, indeed, observed that human *TARS2* lacking the first 19 amino acids, specific for the mitochondrial targeting, can partially complement a strain lacking for *THS1* [78].

The last case to discuss regards the CatCAB complex that catalyze the amidation of mischarged Glu-tRNA^{Gln} to obtain the correct Gln-tRNA^{Gln}. In human three genes are involved in the creation of this proteic complex, *QRSL1*, *GATB*, *GATC*, as well as in yeast where the complex, called GatFAB, is encoded by *HER2*, *PET112* and *GTF1*. *HER2* and *PET112* are *QRSL1* and *GATB* ortholog, respectively, while there is no similarity between *GATC* and *GTF1* [54]. For this reason, only mutations in *QRSL1* and *GATB* can be studied in yeast, as in fact was done by Friedrich et al. in 2018 [79].

All the analyses performed in yeast through the years demonstrated that this simple organism can be successfully used to study the impact of mutations affecting mtARSs on the mitochondrial metabolism. *S. cerevisiae* helped in understanding the severity of each mutation, that is difficult to

assess in patients since mutations are usually in compound heterozygosity and the severity of mitochondrial impairment observed in yeast often correlate with human clinical phenotype [54].

Moreover, yeast model of MD has been extensively used as preliminary step in the discovery of new beneficial drugs able to decrease the impairment induced by mutations in genes known to be associated to MD.

Regarding yeast model of MD associate to mutations in mtARSs it is possible also to test the amino acid supplementation as potential therapeutical approach and yeast already demonstrated to be a evaluable tool to evaluate amino acid supplementation beneficial effect [30].

Therefore, yeast can be considered a good model for quickly and efficiently analysis of mutations in mtARSs thanks to the similarities shared between human and this organism.

1.6. *Saccharomyces cerevisiae* as model for discovering new drugs for mitochondrial diseases

Unfortunately, there is currently no effective treatment for mitochondrial diseases; for this reason, there is an urgent need to find new compounds that could exert a therapeutical effect.

Drug discovery is a complex and multidisciplinary process that has the purpose to find new molecules able to rescue partially or totally the function of a protein or a cellular pathway altered in the human disease. The drug discovery process includes a screening system in order to find small molecules able to modulate the activity of a previously validated target (usually a protein). The active compounds are then characterized and their efficacy, toxicity and side effects are tested in cell culture and/or animal models. Eventually, a series of clinical trials supervised by FDA are performed to investigate on the safety, efficacy and pharmacology of these molecules comparing their features with the ones owned by other molecules already used as treatments [80].

Recently, phenotype-based screenings were performed in yeast models of disease for discovering drugs able to recover the affected phenotype, in general the OXPHOS phenotype, associated with MD mutations [53].

One of the phenotype-based high throughput screening applied on yeast is called “Drug drop test” [81,82]; this test allows the analysis of a large number of molecules in relatively brief time. To perform the screening, mutant yeast cells defective in oxidative growth are initially spread on solid respiratory medium and then exposed to the chemical compounds, spotted on small sterile filters placed on the agar plate. After incubation, the active compounds are identified through a halo of enhanced growth around a filter. This assay was firstly developed by Couplan et al. [82] to discover active drugs against NARP syndrome (neuropathy, ataxia, and retinitis pigmentosa), a human mitochondrial disease affecting ATP synthase. In that case a yeast model deleted in *FMC1* nuclear gene, encoding for a protein essential for the assembling of F1 of ATP synthase, was constructed and ~12.000 molecules were tested as described before. Two molecules resulted having a beneficial

effect and further studies demonstrated that both molecules were active on human cells also (Figure 1.4) [82].

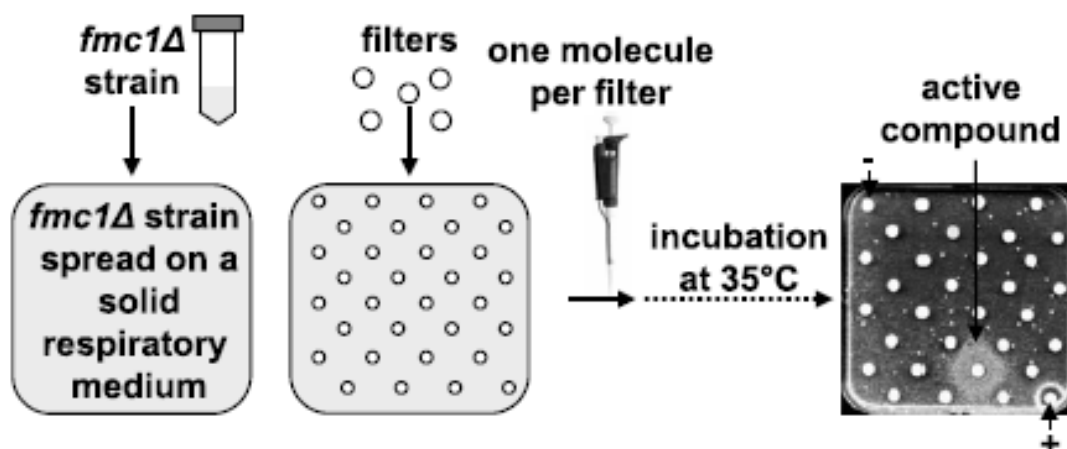


Figure 1.4 Schematic representation of “Drug drop test” technique [82].

Some examples that illustrate the potential of this approach and that validate this organism as useful tool in discovering promising chemical therapeutics and insights into disease mechanisms are reported below.

Thanks to Drug drop test technique, two antibiotics, pentamidine and clarithromycin, were found to rescue the OXPHOS phenotype in yeast cells carrying missense mutations in *BCS1*, ortholog of *BCS1L* human gene and encoding for a protein translocase and chaperone required for Complex III assembly [83]. It was also demonstrated that these antibiotics alter the assembly of complex III targeting the mitochondrial rRNA specific for the translation of the mitochondrial subunit.

Another molecule identified as beneficial performing this assay is the clofilium tosylate (CLO), able to improve the respiratory growth and to avoid the loss of mtDNA in all the *mip1* (encoding for mitochondrial polymerase gamma) mutants analyzed by Pitayu et al. [84]. Experimental procedures performed on *Caenorhabditis elegans* and patients fibroblast and recently in zebrafish confirmed the beneficial role of the compound [85].

The same assay was used to identify two active drugs, nalidixic acid and 5,7 dichloro-8-hydroxyquinoline, able to restore the mitochondrial function and other phenotypes of a yeast model of a neurodegeneration associated to a deficit in pantothenate kinase activity [86].

Recently, molecules able at correcting the biochemical alterations associated with *OPA1* mutations associated with dominant optic atrophy (DOA) were identified as effective in the yeast model were also able to improve the energetic functions or the mitochondrial network dynamics of MEFs [87,88]. The chemical libraries used to perform this kind of screening are collections of thousands of drugs FDA-approved which allows a drug repurposing approach that means that existing drugs can be exploited for their use for other pathologies [89,90]. In this case the findings obtained in yeast, if

confirmed in more advanced models, can lead to a rapidly translation into clinical trial for mitochondrial diseases [88].

2. Aim of the research

To date, genetic analysis with Next Generation Sequencing (NGS) approaches in patients affected by mitochondrial diseases (MD) have made possible the identification of new disease genes or new mutations in known disease genes thus contributing to improve the diagnosis of mitochondrial diseases and to find the molecular defects causing pathological phenotype [91].

Recently, an increasing number of variants in genes encoding for mitochondrial aminoacyl-tRNA synthetases (mtARSs) have been identified in MD patients. mtARSs are the enzymes charging amino acids on its cognate tRNA and mutations in *mtARSs* genes are usually associated with early onset encephalo and/or cardiomyopathies. There is not an effective cure for these pathologies; for this reason, it becomes necessary understanding the molecular mechanisms involved in the diseases, in order to find a way to treat at least the symptoms.

The research work that I carried out during my PhD fits into a project, financed by the Ministry of Health, “Mitochondrial aminoacyl tRNA synthetases: implementation of the genetic diagnosis and evaluation of amino acid supplementation as potential therapeutic approach”, which involves, in addition to the Parma Unit, the Bambino Gesù Hospital (Rome) and Carlo Besta Institute (Milan). In particular, this project is aimed to i) improve genetic diagnosis in MD patients, ii) to evaluate the effect of amino acid supplementation as potential treatment and iii) to assess the pathogenic significance of mtARS variants identified in MD patients using *ad hoc* yeast models.

In the major cases mutations in mtARSs are found in compound heterozygosity; therefore, it becomes essential to understand their causative role in the pathologies and to investigate on the contribution of each mutation to the defective phenotype. *Saccharomyces cerevisiae* proved to be a good model system to study the consequences of alleged pathological human mutations associated to MD thanks to its ability to survive without a functional respiratory chain, in presence of a fermentable carbon source; this yeast feature makes possible the evaluation of the impact of mutations on mitochondrial activity through the analysis of respiratory related phenotype. Moreover, yeast can exist in haploid or diploid status and this can help to investigate the contribution of individual mutations on the phenotype [53].

The principal aim of this research was to create specific yeast models of mitochondrial pathologies associated with mutations in human mtARS genes to assess the pathogenetic significance of novel missense mutations found in MD patients, lacking a genetic diagnosis, in *DARS2*, *NARS2*, *RARS2*, *WARS2*, *VAR2* genes, encoding for mitochondrial aspartyl-, asparaginyl-, arginyl-, tryptophanyl- and valyl-tRNA synthetases, respectively. The homologous complementation approach was used for the strains construction, by inserting the mutations in the corresponding residue of the ortholog yeast proteins, Msd1, Slm5, Msr1, Msw1 and Vas1. One mutation affecting the cytosolic *AARS1* gene was also investigated, introducing it in the ortholog yeast gene *ALA1*.

As mentioned before, a real cure for pathologies caused by mutations in mtARSs does not exist and the therapeutic approach for patients is limited to symptomatic treatment and nutritional supplementation [29]. For this reason, the second aim of this research work was the identification of beneficial molecules and, in particular, the evaluation of the ability of amino acid supplementation, the specific substrate of mitochondrial aminoacyl-tRNA synthetase, to rescue the mitochondrial impairment showed by mutant strains. Moreover, to identify molecules able to rescue the mitochondrial defective phenotypes associate to mtARS mutations, a phenotypic screening of FDA-approved chemical libraries was undertaken. Finding potential compounds able to rescue the mutation associated phenotype represents the starting point to find potential new treatments and improve patients conditions.

3. Results

3.1. mtARS genes mutant strains construction

In order to assess the functional impact of variants in mtARSs genes found in compound heterozygosity in MD patients still lacking genetic diagnosis, *ad hoc* yeast models for each mtARSs were constructed using the *Saccharomyces cerevisiae* strain W303-1B {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*}. Since it is known that the deletion of mtARSs genes in yeast leads to mitochondrial DNA instability [92], to create the yeast models the plasmid shuffling strategy has been utilized to avoid the mtDNA loss.

To this end, each mtARS gene was first cloned in the centromeric plasmid pFL38 (*URA3* marker), the recombinant construct was then transformed into yeast and the corresponding mtARS gene was disrupted by “one step gene disruption” technique.

For each mutation under analysis in a specific mtARS gene, a mutated allele was constructed. Starting from the plasmid pFL38mtARS, site-directed mutagenesis using Overlap PCR or Quick-Change PCR technique was performed with appropriate primers to obtain the allelic variants into the pFL39 vector (*TRP1* marker). The strain W303-1B *mtars*Δ/pFL38-mtARS was then transformed with pFL39- harbouring the *mtars* mutant alleles, previously obtained, or with the empty vector pFL39 and transformants carrying both plasmids were selected. In order to investigate on the effect of the mutant alleles, wild type allele containing plasmid was counter selected through plasmid shuffling on 5-FOA supplemented medium. The construction of the strain is described in detail in Materials and Methods. The mutated strains, constructed as described above, were used to investigate the effects of alleged pathological mutations on mitochondrial phenotypes.

3.2. Study of missense variants in *DARS2/MSD1* encoding mitochondrial aspartyl-tRNA synthetase

In human, *DARS2* gene encodes for mitochondrial aspartyl-tRNA synthetase, the enzyme charging the aspartate on its cognate tRNA [93]. Mutations in this gene are associated with an autosomal recessive disease called Leukoencephalopathy with Brainstem and Spinal cord involvement and Lactate elevation (LBSL) (OMIM 611105) [94]. At the Emma Children Hospital in Amsterdam, mutations were found through Whole Exome Sequencing (WES) in compound heterozygosity in 15 patients. The patients showed a different MRI pattern, for this reason were divided into two groups: 9 patients showed early severe cerebral hypoplasia/atrophy and were classified as Group 1, 6 patients showed white matter abnormalities without long tract involvement and were classified as Group 2 [55]. For each missense mutation, pathogenicity predictions were assessed by considering the location of the affected residues on mtAspRS protein structure and in case some functional region

was interested by mutation, by examining interspecies conservation of affected residues and by calculating free energy change ($\Delta\Delta G$) (Table 3.1).

Group 1 - severe cerebral atrophy										
LBSL nr	DARS2 variant	Amino acid change	Protein Polyphen ^a	SIFT ^b	Russell ^c	$\Delta\Delta G^d$	Bacterial variant	$\Delta\Delta G^d$	Functional region ^e	Predicted effect
285	c.172C>G ^g	p.(Arg58Gly)	0.992	0.30	-2	-0.10	Arg11Gly	1.22	tRNA ^{Asp} binding	May impair tRNA binding
	c.742C>T	p.(Gln248*)	na	na	na	ne	ne	ne	ne	-
281	c.748C>G	p.(Leu250Val)	0.996	0	1	3.22	Leu207Val	2.99	Asp-AMP binding, homo-dimerization	Destabilizes structure near the catalytic and dimerization regions
	c.1452C>G	p.(Phe484Leu)	1	0	0	2.09	Phe426Leu	0.92	Asp-AMP binding	Alters multiple interactions with surrounding residues near the catalytic region
292	c.90C>A	p.(Tyr30*)	na	na	na	ne	ne	ne	ne	-
	c.785C>T	p.(Ala262Val)	0.997	0	0	-2.11	Ala219Val	-1.15	Asp-AMP binding, homo-dimerization	Causes important structural reorganization and effects on catalysis and homo-dimerization
287	c.90C>A	p.(Tyr30*)	na	na	na	ne	ne	ne	ne	-
	c.469T>A	p.(Phe157Ile)	0.995	0.15	0	1.74	Phe108Ile	2.78	tRNA ^{Asp} binding	May alter protein structure and interaction with tRNA
294	c.228-20 - 12delinsCCCCCCCCG ^g	p.(Arg76Serfs*5)	na	na	na	ne	ne	ne	ne	-
	c.407C>A	p.(Thr136Lys)	1	0.01	-1	5.15	Thr87Lys	6.39	tRNA ^{Asp} binding	May alter protein structure and interaction with tRNA
286	c.492+2T>C ^g	p.(Met134_Lys165del)	na	na	na	ne	ne	ne	ne	-
	c.172C>G ^g	p.(Arg58Gly)	0.992	0.30	-2	-0.10	Arg11Gly	1.22	tRNA ^{Asp} binding	May alter tRNA binding
293& 295	c.535C>T	p.(Arg179Cys)	1	0	-3	3.54	Arg136Cys	4.93	tRNA ^{Asp} binding, homo-dimerization	Destabilizes homo-dimerization and tRNA binding regions
	c.1789A>G	p.(Ser597Gly)	1	0.08	0	1.56	Ser541Gly	3.37	tRNA ^{Asp} binding, Asp-AMP binding	Significant structural changes at catalytic and tRNA binding regions
284	c.382G>C	p.(Gly128Arg)	1	0.02	-2	-0.32	ne	ne	tRNA ^{Asp} binding	Alters interactions with tRNA
	c.1441G>A	p.(Val481Met)	1	0.01	1	3.69	Val423Met	2.06	Asp-AMP binding	Causes significant structural reorganizations affecting the catalytic region
Group 2 - severe white matter abnormalities										
LBSL nr	DARS2 variant	Amino acid change	Protein Polyphen ^a	SIFT ^b	Russell ^c	$\Delta\Delta G^d$	Bacterial variant	$\Delta\Delta G^d$	Functional region ^e	Predicted effect
288	c.1762C>G	p.(Leu588Val)	1	0.04	1	3.56	Leu532Val	1.55	Asp-AMP binding	May affect the Asp-AMP binding without affecting main protein structure
	c.562C>T	p.(Arg188*)	na	na	na	ne	ne	ne	ne	-
291	c.228-20_21delTTinsC ^g	p.(Arg76Serfs*5)	na	na	na	ne	ne	ne	ne	-
	c.1817A>T	p.(Lys606Met)	1	0	-1	-0.35	Lys550Met	-1.00	homo-dimerization	May stabilize the homodimer
289	c.228-15C>A ^g	p.(Arg76Serfs*5)	na	na	na	ne	ne	ne	ne	-
	c.492+2T>C ^g	p.(Met134_Lys165del)	na	na	na	ne	ne	ne	ne	-
290& 297	c.1762C>G	p.(Leu588Val)	1	0.04	1	3.56	Leu532Val	1.55	Asp-AMP binding	May affect the binding of the ligand without affecting main protein structure
	c.1A>C	p.?	na	na	na	ne	ne	ne	ne	-
280	c.228-20 - 16delinsCCCCG ^g	p.(Arg76Serfs*5)	na	na	na	ne	ne	ne	ne	-
	c.1273G>T (paternal) ^g	p.(Glu425*)	na	na	na	ne	ne	ne	ne	-
	c.536G>A (paternal) ^g	p.(Arg179His)	1 ^h	0 ^h	0 ^h	6.23 ^h	Arg136His	6.98 ^h	tRNA ^{Asp} binding, homo-dimerization ^h	Destabilizes homo-dimerization and tRNA binding region ^h

Table 3.1 DARS2 variants and pathogenicity predictions. Bioinformatic pathogenicity predictions made using different website and $\Delta\Delta G$ calculation. nr, number; na, not applicable; ne, not evaluated; (a) Scores indicate probably damaging. (b) Score < 0.05 is predicted deleterious. (c) A lower Russell score indicates a more disfavored amino acid change. (d) Positive and negative $\Delta\Delta G$ values indicate, respectively, destabilization and stabilization of the local protein structure. (e) Functional regions: tRNA Asp binding, Asp-AMP = aspartyl-adenylate, homodimerization. (g) Variants seen before in different combination, Van Berge et al. (h) Predictions for the missense variant of LBSL280 are not taken into account when comparing groups 1 and 2, as it is present on a null-allele. ([55], modified).

Bioinformatic predictions suggested that all the missense mutations could cause protein structure alterations that are allegedly pathogenic. Regarding Group 1 missense mutations, were predicted to be severe since they occur near the tRNA^{Asp} binding site and could cause important structural changes nearby the aspartyl-adenylate binding site. Missense mutations of Group 2 are predicted to be less severe since the aminoacidic changes are quite conservative and could stabilize the protein folding and homodimerization. The mutations affected different Dars2 domains with most of the residues falling in the catalytic domain (Leu250Val, Phe484Leu, Ala262Val, Ser597Gly, Val481Met, Leu588Val, Lys606Met), others in the anticodon binding domain (Arg58Gly, Phe157Ile, Thr136Lys, Gly128Arg) and one in an undefined domain (Arg157Cys) (Figure 3.1).

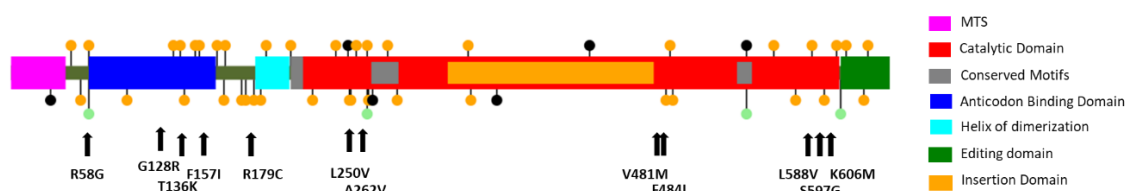


Figure 3.1 Dars2 domains affected by mutations (<http://misynpat.org/>). Dots represents mutations positions already found in patients; green, orange and black colours indicate homozygous, compound heterozygous missense mutations and nonsense mutations, respectively.

To assess the impact of these variants on mitochondrial activity we took advantage of the presence of the orthologous yeast gene *MSD1*. In order to evaluate which mutations could be studied in yeast, ClustalΩ alignment of Dars2 and Msd1 proteins was initially performed (Figure 3.2).

DARS2	MYFPSWLSQLYRGLSRPIRRTTQPIWGSLSYRSLQSSQRRPEFS-----SFVVRT	51
MSD1	-----MLARSVCLQTITRRLADFFPEANAIAKKKFLFRKDT	35
	: * ** : ** : *	
DARS2	NICGELSSHLGQEVTLGWIQYRRQN----TFLVLRDFDGLVQVIIPQDESAASVKKI	106
MSD1	STIKQLGLSSGQKIVLNGWIEQKPKRVGKNLIFGLLRDSNGDIIQLVDN-----KSL	88
	. * : * . ** : * ** : : : * : ** : * : : : *	
DARS2	LCEAPVESVQVSGTVISRPAQENPKMPGEIEIKVKTRALLNACKKLPFEIKNFVKKT	166
MSD1	LKGFTELDVQVAVGILSLKR--KLSNEDDEYEVQLEDITVLNASNKKPQMQDFKL-S	144
	* : * . * : : : : * : * : : : * : * : : : *	
DARS2	EALRLQYRYLDLSFQMQYNLRLRSQVMVMKREYLCNLHGFDIETPTLFKRTPGGAKEF	226
MSD1	AIYPPFERYLQLRNPKYQDFLKKRSSISKEIRNS-FNNFDTEVETPMLFKATPEGAREF	203
	: ** : * . : * : * : : : * : * : : * * * * * *	
DARS2	LVPSRE----PGKFYSLPQSPQQFKQLMVGGLDRYFQVRCYRDEGSRPDRQPEFTQI	281
MSD1	LVPTRTKRSDGKPSFYALDQSPQQYKQLMASGVNKYQMRCFRDEDLRADRQPEFTQV	263
	*** : * : * : * * * * * * : * : * : * : * : * * * * *	
DARS2	DIEMSFVDQTGIQSLIEGLLQYSWPN-----KDPVVPFPTMTFA	322
MSD1	DMEMAFANSEVVMKIEKTVSGVWSKFSKKRGLLTLDKGTLPVPAKKENGTVSIFRMTYE	323
	* : * : * . : : : * : * : * : * : * : * : *	
DARS2	EVLATYGTDKPDRFG-MKIIDISDFERNTEIGFLQDALSKEPHGTVAICIEPAGAKYLKR	381
MSD1	QAMTSYGDIDKPDRLAPDLKIINLGEFNAFSH---L-----NKKFPVFEVILRSA--FSNM	374
	: : : * * * * * : * : * : : : : * : * : * : * : * : *	
DARS2	KDIESIRNFAAD--HFNQE---ILPVF--LNANRNWNSPVANFIMESQRLELIRIMETQE	434
MSD1	EEYKERWSFLTNNSNYRVPVLPVPIENDEQANSNWFFENFHAIAITFENPHLITKFLKLKK	434
	: : . * : : : * : * : * : * * * . . : : : : : : *	
DARS2	EDVLLTAGEHNK----ACSLGKLRLECADLLETGR-VVLRDPTLFSFLWVDFPLFLP	489
MSD1	GDIVCGCTREPNSHIFENPTPLGRRLQLVL--QSEHGKNIYHAVNKDVASWVDFPLFSP	492
	* : * : * : : * : * : * : * : * : * : * : * : *	
DARS2	KEE-----NPRELESAHHPTAPHPSDIHLLYTEPKKARSQHYDLVLNGN	534
MSD1	VIIEDKSGKKEKLAYPEYKDRLCSTHHPTFMVKLDYKLEKTEPEKCLGRHYDLVNVG	552
	: * * : * * : * : * . * . * : * : * : * : *	
DARS2	EIGGGSIRIHNAELQRYILATLKED--VKMLSHLLQALDYGAPPHGGIALGLDRICILV	592
MSD1	ELGGGSIRIHDPRQLQYIFEDILKIDNAYELFGHLLNAFDMGTPPHAGFAIGFDRMCAMI	612
	* : * * * * * : * * * : * * : : : * : * : * : * : * : *	
DARS2	TGSPSIRDVIAFPFSFRGHDLMSNTPDSVPPEELKPYHIRVSKPTDSKAERAH	645
MSD1	CETESIRDVIAFPFSITGADLVVKSPPVIPESILEPYNIKYSNSKK*-----	658
	: * : * * * * * : * * : : * : * : * : * : * : *	

Figure 3.2 Alignment between human and yeast mt-AspRS. The result of the alignment between Dars2 and Msd1 by ClustalQ. Marked in green the conserved residues, marked in yellow the not conserved residues, marked in red the not analyzed residues.

As shown in the protein alignment the identified variants affect both conserved and not conserved residues between human and yeast proteins: in particular six Dars2 amino acid residues are conserved Leu250, Phe484, Ala262, Ser597, Lys606, Arg179 and correspond to the Leu232, Phe487, Ala244, Ser617, Lys626 and Arg157 amino acidic residues, respectively; 4 resulted not conserved in Msd1 Val481, Leu588, Arg58, Phe157 corresponding to residues Ile484, Met608, Lys42 and Ala136, respectively. In this case, in addition to the pathological allele, also the humanized allele was constructed, in which the wild type amino acid residue present in the yeast protein was replaced by the corresponding wild type amino acid in the human protein in order to use it as humanized control. The remaining two Thr136 and Gly128 were not studied: the first because falls in a non-conserved region, the second because was not aligned. Start codon, nonsense mutations and variants affecting splice sites were not analyzed (Table 3.2). Pathological and humanized alleles and mutant strains were constructed as described in Materials and Methods.

Group 1 - severe cerebral atrophy			
LBSL nr	DARS2 variant	Amino acid change	Yeast position residue
285	c.172C>G ^a	p.(Arg58Gly)	yLys42 non-conserved
	c.742C>T	p.(Gln248*)	ne
281	c.748C>G	p.(Leu250Val)	yLeu232 conserved
	c.1452C>G	p.(Phe484Leu)	yPhe487 conserved
292	c.90C>A c.785C>T	p.(Tyr30*) p.(Ala262Val)	ne yAla244 conserved
287	c.90C>A c.469T>A	p.(Tyr30*) p.(Phe157Ile)	ne yAla136 non-conserved
294	c.228-20_-12delinsCCCCCCCCG ^a c.407C>A	p.(Arg76Serfs*5) p.(Thr136Lys)	ne yAla115 region non-conserved
286	c.492+2T>C ^a c.172C>G ^a	p.(Met134_Lys165del) p.(Arg58Gly)	ne yLys42 non-conserved
293&295	c.535C>T c.1789A>G	p.(Arg179Cys) p.(Ser597Gly)	yArg157 conserved ySer617 conserved
284	c.382G>C c.1441G>A	p.(Gly128Arg) p.(Val481Met)	y- region non-conserved yIle484 non-conserved
Group 2 - severe white matter abnormalities			
LBSL nr	DARS2 variant	Amino acid change	Yeast position residue
288	c.1762C>G	p.(Leu588Val)	yMet608 non-conserved
	c.562C>T	p.(Arg188*)	ne
291	c.228-20_21delTTinsC ^a c.1817A>T	p.(Arg76Serfs*5) p.(Lys606Met)	ne yLys626 conserved
289	c.228-15C>A ^a c.492+2T>C ^a	p.(Arg76Serfs*5) p.(Met134_Lys165del)	ne ne
290&297	c.1762C>G	p.(Leu588Val)	yMet608 non-conserved
	c.1A>C	p.?	ne
280	c.228-20_-16delinsCCCCCG ^a c.1273G>T (paternal) ^a c.536G>A (paternal) ^a	p.(Arg76Serfs*5) p.(Glu425*) p.(Arg179His)	ne ne yArg157 conserved

Table 3.2 Aminoacidic corresponding positions between human and yeast proteins. ([55], modified).

3.2.1. Mitochondrial function characterization: spot assay analysis and Oxygen consumption rate

To study the impact of these mutations on Dars2 functionality, the first analysis performed was the evaluation of the oxidative growth of the mutant strains. W303-1B *msd1Δ* carrying pFL39 containing wild type-*MSD1*, humanized, mutant alleles or the empty vector were spotted with serial dilution on solid media supplemented with ethanol, as oxidative carbon source, or glucose, as control as described in Materials and Methods.

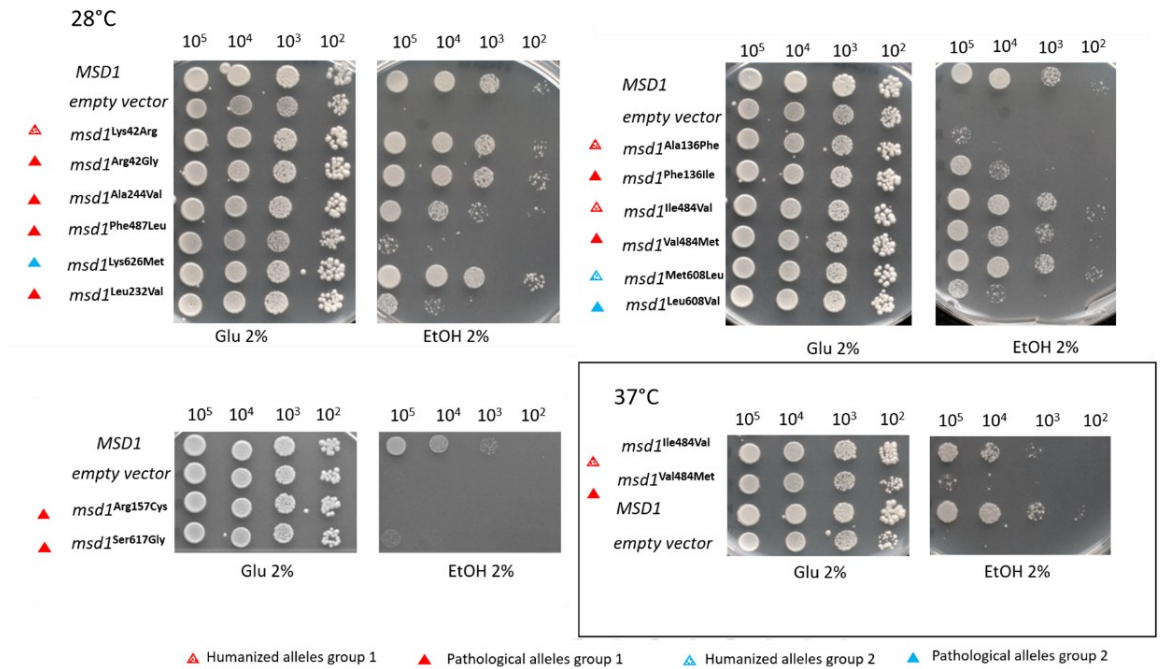


Figure 3.3 Spot assay. Ten-fold dilution spots for each strain, starting from 10^5 cells, were spotted on solid media added with glucose or ethanol. The growth was scored after 72 hours [55].

The results, reported in Figure 3.3, showed that the strains carrying the humanized alleles *msd1*^{Lys42Arg}, *msd1*^{Ala136Phe}, *msd1*^{Ile484Val} and *msd1*^{Met608Leu} were able to complement the absence of the endogenous *MSD1*, except *msd1*^{Ala136Phe} and, for this reason, the mutation Phe136Ile was not analyzed. The oxidative growth of the strains harboring the mutant alleles *msd1*^{Arg157Cys}, *msd1*^{Leu232Val}, *msd1*^{Phe487Leu}, *msd1*^{Leu608Val}, and *msd1*^{Ser617Gly} was affected in a severe manner. Also the allelic variant *msd1*^{Ala244Val} reduces the oxidative growth while the strains expressing the mutated alleles *msd1*^{Arg42Gly}, *msd1*^{Lys626Met} and *msd1*^{Val484Met} did not show phenotypical growth defect on ethanol. When the same spot assay analysis was repeated at 37°C, the latest mutant strain showed a severe growth defect, indicating that this variant affects the mitochondrial functions in a thermosensitive way; *msd1*^{Arg42Gly}, *msd1*^{Lys626Met} did not show a defective growth either at 37°C.

To further investigate the impact of the variants on the oxidative phosphorylation, the functionality of the mitochondrial respiratory complexes was evaluated by measuring the oxygen consumption rate (OCR) of cells grown at 28°C. Cells were cultured and respiratory rate measured as described in Materials and Methods. As shown in Figure 3.4, all the allelic variants affected, although at different extent, the respiration activity. The strain harboring *msd1*^{Arg157Cys}, *msd1*^{Leu232Val}, *msd1*^{Phe487Leu}, *msd1*^{Leu608Val}, and *msd1*^{Ser617Gly} allelic variants displayed a strong reduction of the respiratory rate comparable to that of *msd1Δ*; a 40% decrease was observed for the strain carrying *msd1*^{Ala244Val} mutant allele, while the strains carrying

msd1^{Arg42Gly}, *msd1*^{Lys626Met} and *msd1*^{Val484Met}, phenotypically similar to wild type strain in oxidative growth, showed a slight but significant reduction of the respiration activity (20%, 15% and 25%, respectively).

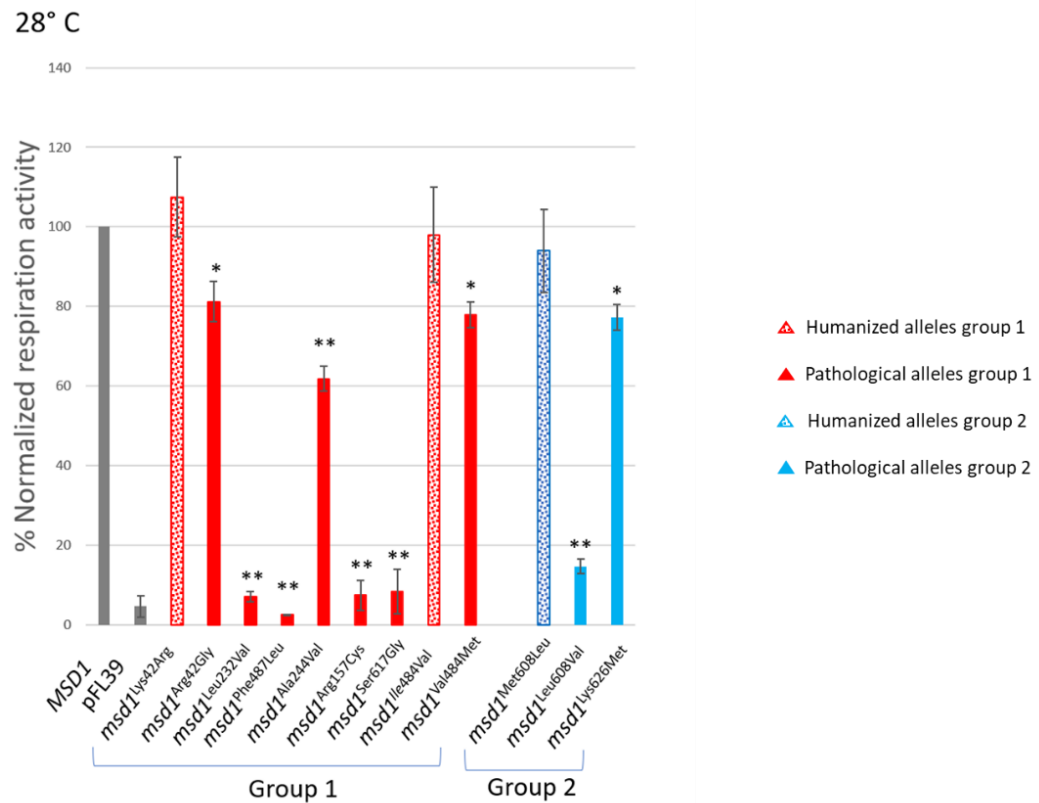


Figure 3.4 Oxygen Consumption Rate (OCR). Respiration activity measurements for *msd1Δ* harboring wild type, mutated, humanized alleles or empty vector grown in 0,6% glucose medium. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (* $p < 0,05$, ** $p < 0,01$) [55].

Taken together, the results obtained with both phenotypic analyses, confirmed an OXPHOS defect for all the mutations studied with mutations that induced serious defects and others that led to a less severe phenotype. The severity of the phenotypes observed in the mutant strains is almost always consistent with the bioinformatic prediction role of the affected residue in the protein and in many cases correlates with the severity of human clinical phenotypes of patients carrying the correspondent mutation [55].

3.2.2. Evaluation of Msd1 steady-state level

To evaluate if the metabolic alterations observed in the different mutant strains were due to an altered steady-state level of the mutated Msd1 proteins, a Western Blot analysis was performed, taking advantage of the presence of a HA tag at N-terminal of the protein.

Humanized, mutated and wild type strains were grown on media supplemented with 0,6% glucose and total proteins were extracted by TCA method as described in Materials and Methods. On these extracts Western blot was carried out and the Msd1 was detected using ANTI-HA antibody and as housekeeping control protein the mitochondrial porin (Por1) was used and detected employing an antibody ANTI-Por1. A representative image of one of the three independent experiments preformed is shown in Figure 3.5 together with the percentage of the normalized values, represented in the bar graph.

Phe487Leu and Leu608Val mutations led to a respectively ~50% and ~40% decrease in the steady-state level of Msd1 protein, suggesting that instability of the mutated protein contributes to mitochondrial defect and to a pathological phenotype. The proteins carrying Arg157Cys, Ser617Gly and Lys626Met mutations showed protein level 4, 5 and 2 times increased, respectively, suggesting a major protein stability. All the other amino acid substitutions in Msd1 protein do not affect protein level suggesting that the mitochondrial defect and consequently the pathogenetic mechanism were mainly caused by deficiency of enzyme activity rather than reduced protein level.

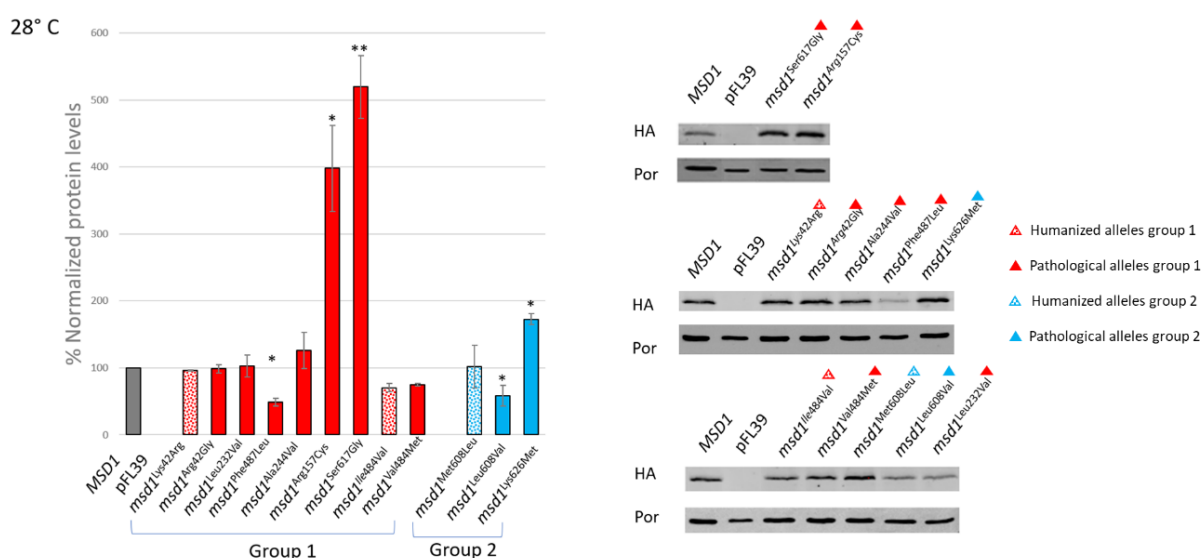


Figure 3.5 Msd1 steady-state level. Western Blot performed to detect Msd1 protein in the strains *msd1Δ* carrying humanized, mutated, wild type alleles or empty vector. Primary antibodies α -HA (1:1000 diluted) and α -Por1 (1:10000 diluted) were used. Secondary antibodies Dylight650 (1:2000) and StarBright520 (1:10000) were employed to recognize tagged Msd1 and Por1, respectively. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (* $p < 0,05$, ** $p < 0,01$) [55].

The results described below are published in the paper entitled “LBSL: Case Series and DARS2 Variant Analysis in Early Severe Forms With Unexpected Presentations” [55].

3.2.3. Analysis of Cox2 steady-state level and *in vivo* mitochondrial protein synthesis

To evaluate the effect of the mutations on the synthesis of mitochondrial proteins, two different analyses were performed. The first was a Western Blot to detect the subunit 2 of cytochrome c oxidase, Cox2, one of the proteins encoded by mitochondrial DNA.

Total proteins were extracted by TCA method as described in Materials and Methods and Western blot was carried out. Cox2 protein was detected using ANTI-Cox2 antibody and as housekeeping control protein the mitochondrial porin (Por1) was detected using an antibody ANTI-Por1.

The steady-state level of Cox2 reflected the severity observed in the previous analyses (Figure 3.6). In fact, except for the strains carrying the mutated alleles *msd1*^{Arg42Gly} and *msd1*^{Lys626Met}, that didn't show any alteration of the steady-state level of Cox2 protein, all the other mutations are associated to a significant reduction of the protein level/stability, indicating that the majority of Msd1 mutations negatively affect the synthesis of mitochondrial proteins.

The second analysis performed to evaluate the impact of Dars2 mutations on mitochondrial translation was the *in vivo* mitochondrial protein synthesis. The analysis was carried out using the ³⁵S-methionine, integrated in the new translated mitochondrial proteins instead of normal methionine. Mitochondrial translation is selected introducing cycloheximide, a compound able to inhibit the cytosolic translation and mitochondrial proteins pattern was observed after exposure of a photographic film (Figure 3.7). Surprisingly the results obtained showed that almost all the mutations did not impact on the *in vivo* mitochondrial protein synthesis; in fact, mitochondrial protein pattern looked like the one showed by the strain carrying wild type allele. Only in the strain harboring *msd1*^{Arg157Cys} pathological allele the mitochondrial protein synthesis was totally compromised.

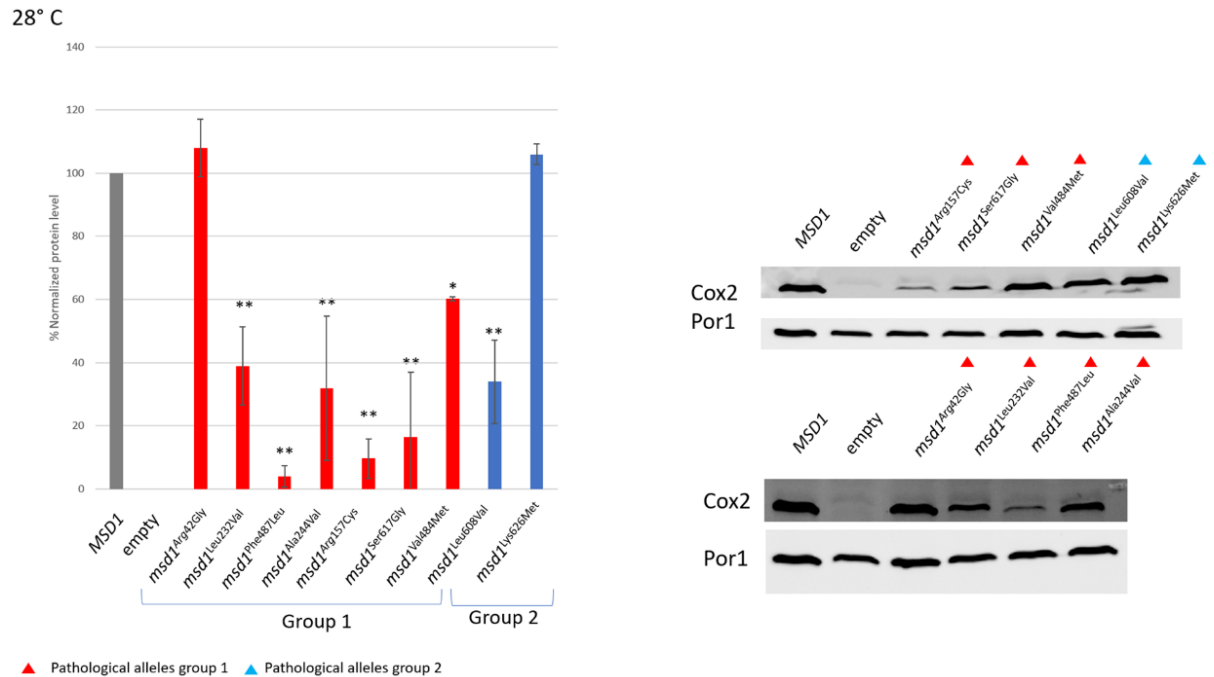


Figure 3.6 Cox2 steady-state level. Western Blot performed to detect Cox2 protein in the strains *msd1Δ* carrying mutated, wild type alleles or empty vector. Primary antibodies α -Cox2 (1:2000 diluted) and α -Por1 (1:10000 diluted) were used. Secondary antibodies StarBright520 (1:10000) was employed to recognize both marked proteins. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (* $p < 0,05$, ** $p < 0,01$).

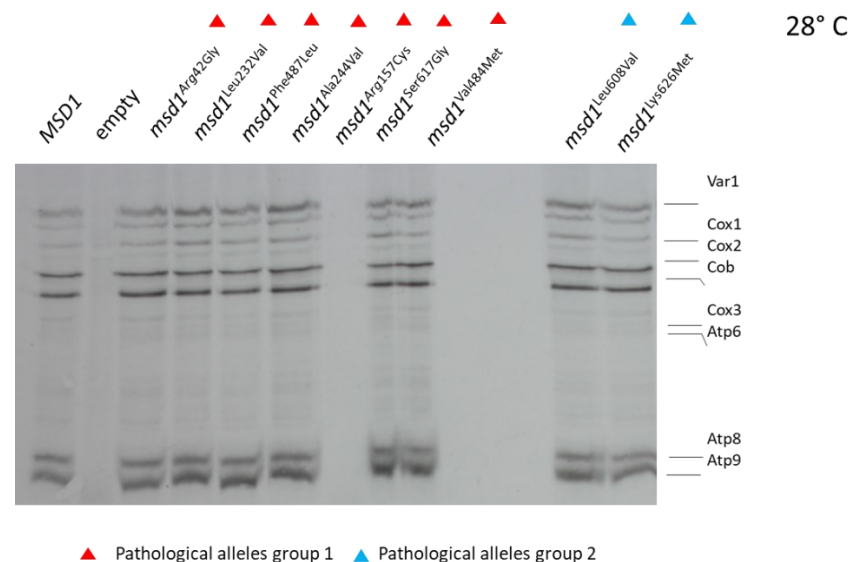


Figure 3.7 *In vivo* mitochondrial protein synthesis. *In vivo* mitochondrial protein synthesis performed in the strains *msd1Δ* carrying mutated, wild type alleles or empty vector. Mitochondrial genes products were labeled with [^{35} S]-methionine in whole cells in the presence of cycloheximide for 10 minutes at 28°C. Two of the several experiments performed are represented.

3.2.4. DARS2 mutations Arg125His and Gln184Lys

Regarding *DARS2* gene, two more mutations were found at Institute Carlo Besta, not belonging to the two groups previously described: Arg125His and Gln184Lys. These mutations were in compound heterozygosity in two different patients as reported in Table 3.3.

Patient	<i>DARS2</i> variant	Aminoacid change	Yeast position (Msd1)	
1	ivs2-20_21del TTinsC	----- p.Arg125His	----- Lys107	----- Not conserved
2	ivs2-20_21del TTinsC	----- p.Gln184Lys	----- Gln162	----- Conserved

Table 3.3 Aminoacidic corresponding positions between human and yeast proteins.

Arg125 residue was not conserved in yeast protein, corresponding to Lys107, therefore it was necessary to construct humanized and pathological alleles; Gln184 residue was conserved instead between human and yeast aminoacidic sequences, corresponding to Gln 162, thus only pathological allele was constructed (Figure 3.8).

DARS2	MYFSPWLSQLYRGLSRPIRRITQPIWGSLSYRSLQSSQRRIPFS-----SFVVRT	51
MSD1	-----MLARSVCLQITITRLADFPANAIAKKKFLFRKDT	35
	: * **: **: *	
DARS2	NTCGELRSSHLCQEVILCGWIQYRRQN-----TFLVLRDFDGLVQVVIIPQDESAASVKKI	106
MSD1	STIKQLKGLSSGQKIVLNGWIEQKPKRVGKNLIFGLLRDSNGDIIQLVDN-----KSL	88
	. * :*. : **:. * **: : :. * :*** : * : : : *	
DARS2	LCEAPVESVQVSGTVISRPAQGENPKMPTGEIEIKVKTAE LLNACKKLPFEIKNFVKKT	166
MSD1	LKGFTELDVVQAVGILSLKR---KLSNEDADEYEVQLEDITVLNASNKKPAQMQDFKL-S	144
	* :*.***. * : * : : : * :*: : : ***. : * * :*: * :	
DARS2	EALRLQYRYLDLRSFQMYNLRLRSQVMVMKREYLCNLHGFDIETPTLFKRTPGGAKEF	226
MSD1	AIYPPEFRYLQLRNPKYDFLKKRSSISKEIRNS-FNNFDTEVETPMLFKATPEGAREF	203
	:***:*. : : * : **.: :*: * ..:*** ** ** ** ****	
DARS2	LVPSRE----PGKFYSLPQSPQQFKQLLMVGGGLDRYFQVARCVRDEGSRPDQPEFTQI	281
MSD1	LVPTRTKRSDDGKPSFYALDQSPQYKQLLMASGVNKYQMARCFRDELDLRADQPEFTQV	263
	:* ..*: * **:***. :*: :*:***:***. * *****:	
DARS2	DIEMSFVDQTGIQSLIEGLQYSWPN-----KDPVVPFPFMTFA	322
MSD1	DMEMAFANSEDMVKIIEKTVSGVWSKFSKRGLLTLDKGTLPVPAKKGTVSIFRMTYE	323
	*:***:*. :. : :*** :. * : *	
DARS2	EVLATYGTDKPDTRFG-MKIIDISDVFRNTEIGFLQDALSKPHGTVKAICIEPGAKYLKR	381
MSD1	QAMTSYGIKPDRLRAPDLKIINLGEFNAFSL---L-----NKKFPVFEVILRSA--FSNM	374
	:. :*: ** **** * :***: :. :. * ..* . :. :. : *	
DARS2	KDIESIRNFAD--HFNQE---ILPVF--LNANRNWNSPVANFIMESQRLELIRLMETQE	434
MSD1	EEYKERWSFLTNNSNYNRYRPIVLPINDEQANSNWFENFHAIAITFENPHLITKFLKLR	434
	: :. . * : : : * :. : ** : ** ** . : : : : : :	
DARS2	EDVVLLTAGEHNK---ACSLGKLRLECADLLETRG-VVLRDPTLFSFLWVDFPLFLP	489
MSD1	GDIVCGCTREPNSHIFENPTPLGRRLQVL--QSEHGKNIYHAVNKDVASWIVDFPLFSP	492
	: : * * : : **:* . : * : : . * :***** *	
DARS2	KEE-----NPRELESAHHPFTAPHPSDIHLLYTEPKKARSQHYDLVLNGN	534
MSD1	VIIEDKSGKKEKLAYPEYKDRLCSTHHPFTMVKLDKYEKLEKTEPKCLGRHYDLVVG	552
	: . * ***** : * . * . * :. :*****:*	
DARS2	EIGGGSIRIHNAELQRYILATLLKED--VKMLSHLLQALDYGAPPHGGIALGLDRLICLV	592
MSD1	ELGGGSTRIHDRLQDYIFEDILKIDNAYELFGHLLNAFDMGTPPHAGFAIGFDRMCAMI	612
	*:*** ** :. * * : : * * : :.***: * :***. * : * : * : *	
DARS2	TGSPSIRDVIAFPKSFGRHDLMSNTPDSVPPEELKPYHIRVSKPTDSKAERAH	645
MSD1	CETESIRDVIAFPKSIITGADLVVKSPPSIVPESILEPYNIKYSNSKK*-----	658
	: *****: * **: :*: . : * . * :***: * : *	

Figure 3.8 Alignment between human and yeast mt-AspRS. The result of the alignment between Dars2 and Msd1 by ClustalΩ. Marked in green the conserved residues, marked in yellow the not conserved residues.

Spot assay was performed to evaluate the capability to grow on non-fermentable carbon sources and revealed that the strain carrying the humanized allele *msd1*^{Lys107Arg} is perfectly capable to grow on ethanol as well as the strain carrying the wild type allele. Both the strains harboring the two mutated alleles were defective in oxidative growth with different levels of severity, with the strain carrying *msd1*^{Gln162Lys} mutated allele more severe than the one carrying *msd1*^{Arg107His} (Figure 3.9 A).

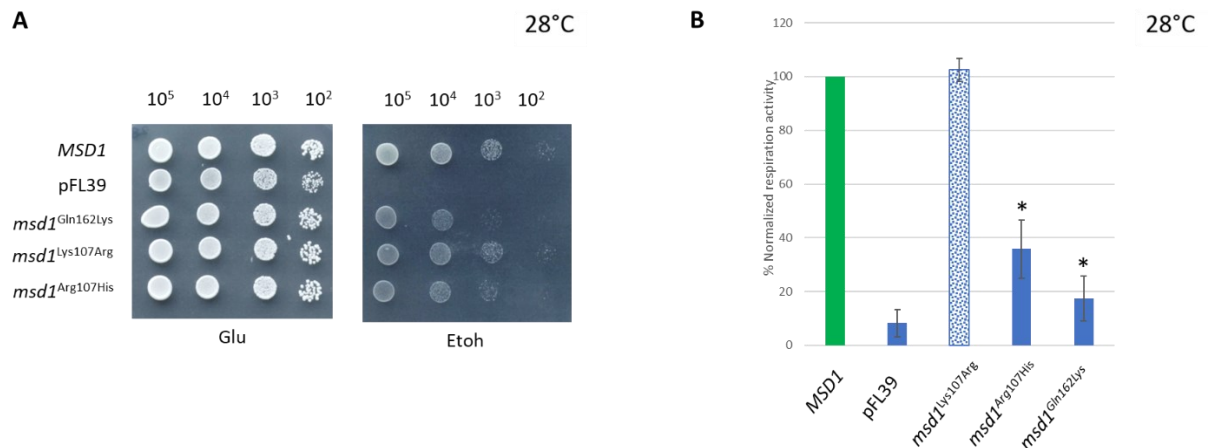


Figure 3.9 Spot assay and OCR. Ten-fold dilution spots for each strain, starting from 10⁵ cells, were spotted on solid media added with glucose or ethanol. Results scored after 72 hours (A). Oxygen Consumption Rate carried out on the same strains grown in liquid YNB DO-W with 0,6% of glucose added (B).

OCR analysis was conducted in order to further investigate on the OXPHOS phenotype of mutated strains and the results parallelized with what observed in the spot assay: in fact, the strain carrying the humanized allele showed normal respiration activity levels while the strain harboring *msd1*^{Arg107His} mutated allele exhibited a ~60% reduction in respiration activity and the strain harboring *msd1*^{Gln162Lys} mutated allele showed a ~80% reduction in respiration rate, indicating that both mutation impaired the mitochondrial activity (Figure 3.9 B).

Msd1 steady-state level was evaluated also for these two mutants and both mutations caused an increasing in protein levels (6 times increased for Arg107His mutation, 10 times increased for Gln162Lys), suggesting that both changes increased the stability of Msd1 protein and the defective OXPHOS phenotype observed could be mostly due to an unfunctional enzyme (Figure 3.10 A).

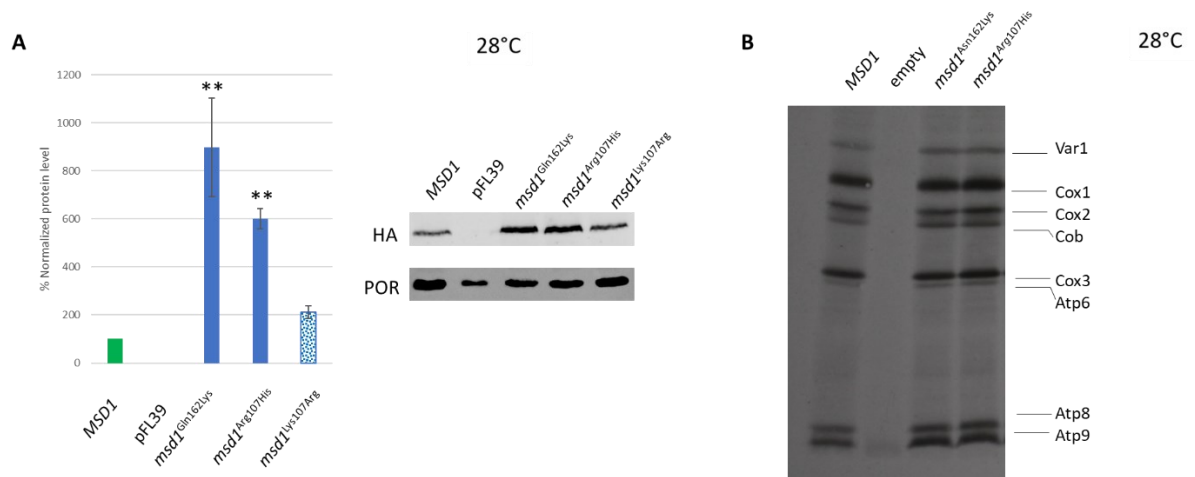


Figure 3.10 Msd1 steady-state level and *in vivo* mitochondrial protein synthesis. Western Blot performed to detect Msd1 protein in the strains *msd1Δ* carrying humanized, mutated, wild type alleles or empty vector. Primary antibodies α-HA (1:1000 diluted) and α-Por1 (1:10000 diluted) were used. Secondary antibodies Dylight650 (1:2000) and StarBright520 (1:10000) were employed to recognize tagged Msd1 and Por1, respectively. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (* $p < 0,05$, ** $p < 0,01$) (A). *In vivo* mitochondrial protein synthesis performed in the strains *slm5Δ* carrying mutated, wild type alleles or empty vector. Mitochondrial genes products were labeled with [³⁵S]-methionine in whole cells in the presence of cycloheximide for 10 minutes at 28°C (B).

To evaluate the impact of the mutations on the mitochondrial translation, the *in vivo* mitochondrial protein synthesis was performed. As for the other *msd1* mutant strains, these two mutations also showed to not influence the mitochondrial translation since the pattern observed was similar to the wild type one (Figure 1.10 B).

3.3. Study of missense variants in *NARS2/SLM5* encoding mitochondrial asparaginyl-tRNA synthetase

NARS2 is the gene encoding for asparaginyl-tRNA synthetase in human and mutations in this gene are associated with deafness, autosomal recessive 94 (DFNB94) (OMIM number: [618434](#)) and combined oxidative phosphorylation deficiency 24 (COXPD24) (OMIM number: [616239](#)). Two mutations were analyzed in this gene found in compound heterozygosity in one patient affected by mitochondrial disease, Met447Val and Phe265Ser, that affect one of the conserved motifs of the protein the first and catalytic domain the second (Figure 3.11). Mutations were identified at Carlo Besta Institute, in Milan, thanks to the use of NGS.

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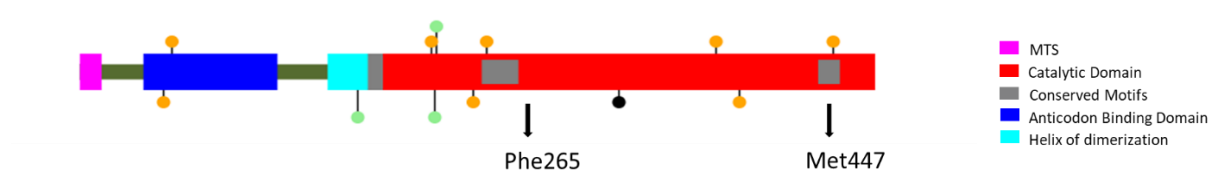


Figure 3.11 Nars2 domains affected by mutations (<http://misynpat.org/>). Dots represents mutations positions already found in patients; green, orange and black colours indicate homozygous, compound heterozygous missense mutations and nonsense mutations, respectively.

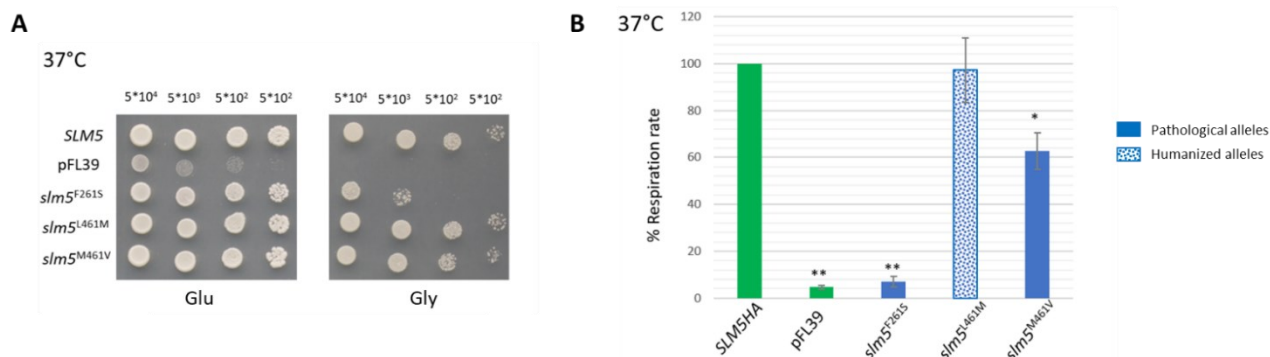
In order to study these mutations in yeast model, alignment using ClastalΩ between Nars2 aminoacidic sequence and the yeast ortholog Slm5 aminoacidic sequence was performed. Phe265 position is conserved between human and yeast sequences corresponding to Phe261 in yeast, thus only pathological allele was constructed; Met447 resulted to be aligned with Leu461 in yeast, thus humanized and pathological alleles were constructed (Table 3.4) (Figure 3.12).

Patient	NARS2 variant	Aminoacid change	Yeast position (Slm5)	
1	chr11 78147811 (hetero) T>C c.1339A>G	p.Met447Val	Leu461	Not conserved
	chr11 78204137 (hetero) T>C c.794T>C	p.Phe265Ser	Phe261	Conserved

Table 3.4 Aminoacidic corresponding positions between human and yeast proteins.

NARS2	MLGVRCLLRSVRFCCSAPFFPKHKPSAKLSVRDA-LGAQNASGERIKIQGWIRSVRSQKEV	59
SLM5	MFHAFTFLKGGRFYSSLT-----VKSLYEQVHHTSHDPIISINGWIKSIRLLKRI	49
	: . :: . ** ** *: . :*: : *: .*:***:.* *: .	
NARS2	LFLHVNVDGSSLESLOVVADSGLDSRELN-----GSSVEVQGQ-LIKSPSKRQNVELK-	111
SLM5	AFDLLDQDGTSVNPLRIVIPTNT-DEVQFLKILKTGGTSLISINATWQSTPNRKQPFELQI	108
	: .:*:*:*: *: ** *:** *: .*: . . :*:** *:**	
NARS2	---AEKIKVIGNCDAKDFPIKYKERHPLEYLRQYPHFRCRTNVLGSLILRIRSEATAAIHS	168
SLM5	KNPVKSIKLVPV-SENYPLO-KKYQTLRYLRSLPTLKYRTAYLSAILRLRSFVEFQFML	166
	.: .*:*: *: .*:*: *: : *.** . *: ** *: .*:** . : :	
NARS2	FFKDSGFVHIHTPIITSNDSEGADELFOLEPSGKLKVPENFFNVPAFLTIVSGQLHLEVM	228
SLM5	YFQKNHFTKVSPPILTSNDCEGAGELFQVSTNT--SPTASSYFGKPTYLTIVSTQLHLEIL	224
	: . . :: **:****.*****: . . : .*: .*:**.* ***:**	
NARS2	SGAFTQVFTFGPTFRAENSQSRRLHAEFYMIEAEISVDSLDQLMQVIEELFKATTMMVL	288
SLM5	ALSLSRCWTLSPCFRAEKSDTPRHLSEFWMLEVEMCVNSVNELTSFVETTIKHI----I	280
	: :*: :*:.* ***:***: ***:***:.*.*. :*:***: . .*: :*	
NARS2	SKCPEDV-----ELCHKFIAPGQKDRLEHMLKNNFLIISYTEAVEIL	330
SLM5	KACIDNQQLLPKQFISSQENNASSELSINQETQQIKTRWEDLINEKWHNITYTNAIEIL	340
	: : :* :* :* :* :* :* :* :* :* :* :* :* :* :* :*	
NARS2	KQA---SQNFFTFPEWGAADLRTEHEKYLVKHCGNIPVFVINYLTLKPFYMRDNEDGPQH	387
SLM5	KRRHNEVSHFKYPEKPGWQLQTEHEKFLAGEYFKSPVFVTYDPLRCKPFYMKQNS-TPDD	399
	: .: .*: ** *:*****: . : :*** :* *****: . *: .	
NARS2	TVAADVLLVPGVGELFGGGLREERYHFLEERLARSGLT--EVYQWYLDLRRFGSVPHGGF	445
SLM5	TVGCFDLLVPGMGEIIGGSLREDDYDKLCREMKARGMNRSGELDWYVSLRKEGSAPHGGF	459
	* . .*****:*: .*:***: * . * . . :* . :*:***: *******	
NARS2	GMGFERYLQCILGVDNIKDVIFFPRFPHSCLL-- 477	
SLM5	GLGFERFISYLGNNHIKDAIPFYRTSAESIDE* 492	
	*:*****: . : * ******	

3.3.1. Mitochondrial function characterization: spot assay analysis and oxygen consumption rate



The strain harboring *slm5*^{Leu461Met} humanized allele behaves as the wild type at both temperatures 28 and 37°C. No OXPHOS phenotype was observed for any mutant strains at 28°C whereas at 37°C the strain carrying the *slm5*^{Phe261Ser} mutated allele shows a severe oxidative growth defect and the strain expressing the *slm5*^{Met461Val} allele shows only a slight defect in oxidative growth. The OCR analysis, performed at 37°C, parallelizes the results obtained in the spot assay: in fact, the respiration rate of the humanized strain is similar to the wild type and the strain carrying Phe261Ser mutation exhibits a strong decrease in respiration rate (more than 90%), similar to the null mutant. Despite the slight defect in oxidative growth, the strain carrying *slm5*^{Met461Val} mutated allele shows, instead, a more severe reduction in respiration activity (~40%) (Figure 3.13 B). Phenotypical analyses revealed that both mutations induced an OXPHOS defect, thus confirming the impairment of mitochondrial functions.

3.3.2. Evaluation of Slm5 steady-state level

To investigate on the impact of the mutations on the level/stability of Slm5 protein, Western Blot was performed (Figure 3.14).

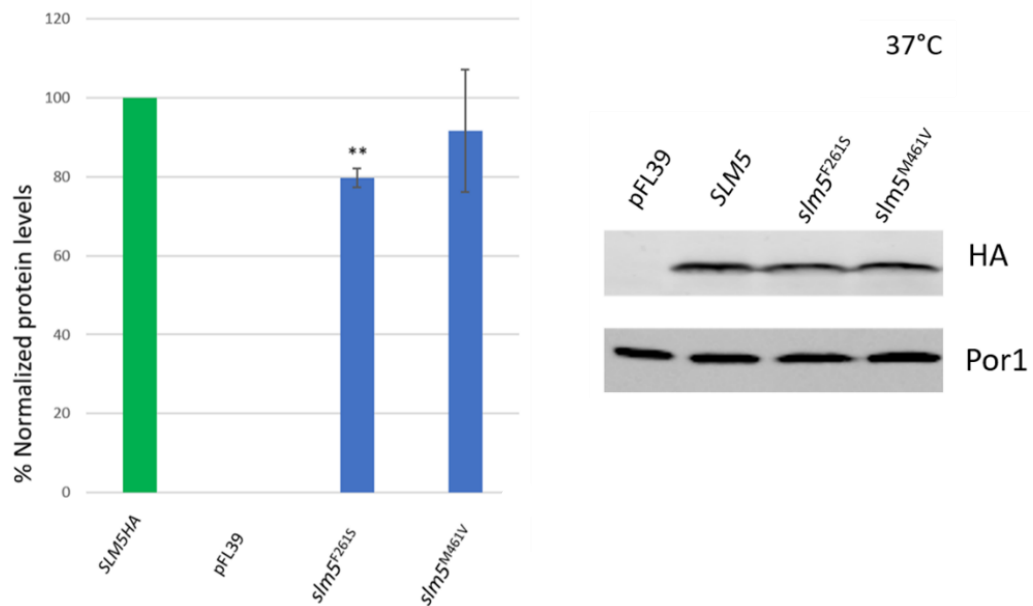


Figure 3.14 Slm5 steady-state level. Western Blot performed to detect Slm5 protein in the strains *slm5Δ* carrying mutated, wild type alleles or empty vector. Primary antibodies α-HA (1:1000 diluted) and α-Por1 (1:10000 diluted) were used. Secondary antibodies Dylight650 (1:2000) and StarBright520 (1:10000) were employed to recognize tagged Slm5 and Por1, respectively. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (** $p < 0,01$).

The results obtained in this analysis showed that only Phe261Ser mutation affects Slm5 stability causing a ~20% reduction in the steady-state level thus possibly explaining, at least in part, the OXPHOS defect observed. No protein level alteration was observed for the strain carrying Met461Val mutation.

3.3.3. Analysis of Cox2 steady-state level and *in vivo* mitochondrial protein synthesis

To analyze to which extent the mutations influence mitochondrial translation, Cox2 steady-state level and *in vivo* mitochondrial protein synthesis were carried out (Figure 3.15).

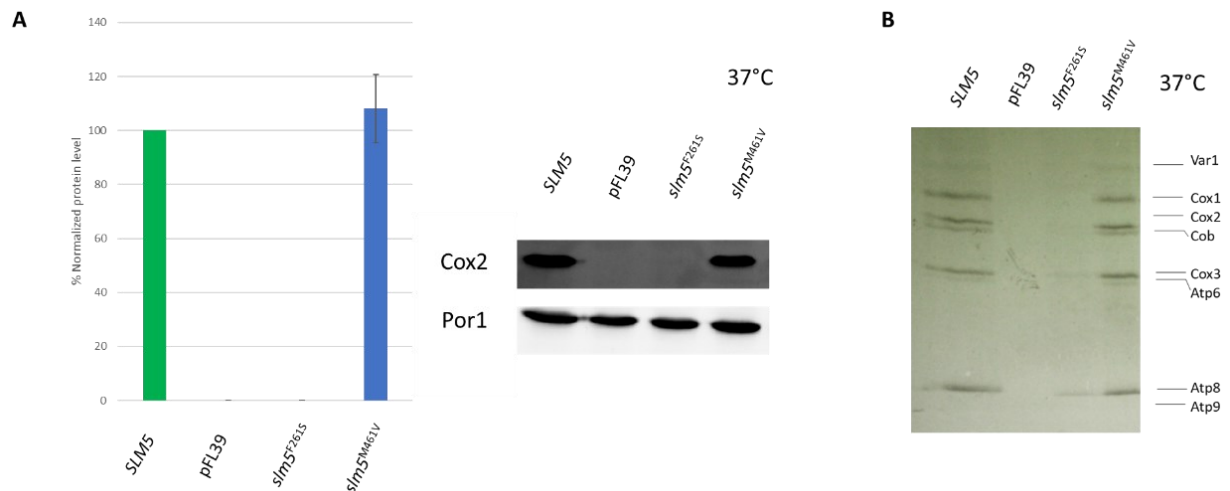


Figure 3.15 Cox2 steady-state level and *in vivo* mitochondrial protein synthesis. Western Blot performed to detect Cox2 protein in the strains *slm5Δ* carrying mutated, wild type alleles or empty vector. Primary antibodies α-Cox2 (1:2000 diluted) and α-Por1 (1:10000 diluted) were used. Secondary antibodies StarBright520 (1:10000) was employed to recognize both marked proteins. The statistical analysis applied is One-Way ANOVA with Bonferroni correction only for the strain carrying Met461Val mutation but no significant results were obtained (**A**). *In vivo* mitochondrial protein synthesis performed in the strains *slm5Δ* carrying mutated, wild type alleles or empty vector. Mitochondrial genes products were labeled with [³⁵S]-methionine in whole cells in the presence of cycloheximide for 10 minutes at 37°C (**B**).

The results obtained from the Western Blot analysis confirmed the pathogenicity induced by Phe261Ser mutation, since the strain expressing this mutant allele totally lacked for Cox2 protein, while the strain carrying *slm5^{Met461Val}* did not show any alteration in the steady-state level of the protein (Figure 3.15 **A**). In agreement, the results obtained in the *in vivo* mitochondrial protein synthesis parallelized with the ones described above: in fact, the strain harboring *slm5^{Phe261Ser}* allele revealed a strong impairment of all mitochondrial proteins while the strain carrying *slm5^{Met461Val}* shows a pattern similar to that of the wild type strain (Figure 3.15 **B**).

3.4. Study of missense variants in *RARS2/MSR1* encoding mitochondrial arginyl-tRNA synthetase

RARS2 is the gene encoding for mitochondrial arginyl-tRNA synthetase, the enzyme charging the arginine on its cognate tRNA. Mutations in this gene are associated with pontocerebellar hypoplasia,

type 6 (OMIM number: [611523](#)). Thanks to the use of NGS, 5 patients were identified with mutations in this gene at Carlo Besta Institute, in Milan. These mutations affected mitochondrial targeting signal (Met1Val/Lys and Gln12Arg), catalytic domain (Arg245Gln and His201Asp) and anticodon binding domain (Arg469His, Ser443Pro, Asp515Gly) of Rars2 protein (Figure 3.16).

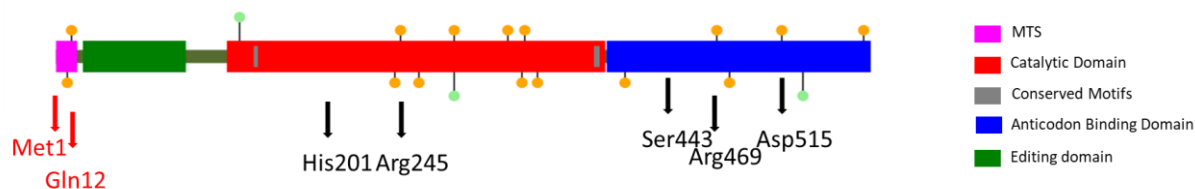


Figure 3.16 Rars2 domains affected by mutations (<http://misynpat.org/>). Dots represents mutations positions already found in patients; green and orange colours indicate homozygous and compound heterozygous missense mutations, respectively.

The corresponding ortholog gene in yeast, in which mutations were inserted and characterized, is *MSR1*. From the alignment between human and yeast aminoacidic sequences, 3 residues out of 7 were conserved (Arg245, Arg469 and His201, corresponding in yeast Msr1 to Arg306, Arg531 and His255, respectively), 2 were not conserved (Ser443, Asp515, corresponding in yeast Msr1 to Asn505 and Thr580); for the conserved residues, only the pathological alleles were constructed while for the not conserved residues humanized and pathological alleles were constructed. 2 mutations (Met1Val/Lys and Gln12Arg) were not studied because since the mutated amino acid is localized in the mitochondrial targeting signal (MTS), usually poorly conserved between human and yeast (Table 3.5) (Figure 3.17). 5 out of 7 of the mutations identified were then analyzed.

Patient	RARS2 variant	Aminoacid change	Yeast position (Msr1)	
1	chr6:88299675 c.1A>G	p.Met1Val	Ile43	Not analyzed
	chr6:88228436 c.1327T>C	p.Ser443Pro	Asn505	Not conserved
2	chr6 88226566 (hetero) T>C c.1544A>G	p.Asp515Gly	Thr580	Not conserved
	chr6 88299675 (hetero) T>A c.1A>T	p.Met1Lys	Ile43	Not analyzed
3	6:88240539 NM_020320.3:c.734G>A NP_064716.2	p.Arg245Gln	Arg306	Conserved
	6:88228357 NM_020320.3:c.1406G>A NP_064716.2	p.Arg469His	Arg531	Conserved
4	chr6:88251647 G>C	p.His201Asp	His255	Conserved
	chr6:88299641 T>C rs147391618	p.Gln12Arg	Thr54	Not analyzed

Table 3.5 Aminoacidic corresponding positions between human and yeast proteins.

RARS2	-----ACGFRRRAIACLSRVLN	18
MSR1	MFGIVYLKNSRLCKNPFSSYPYGFMPSTDTQFSNQFRKLEINIGRKRYSSKTLNTHYT	60
	: * * * : * . .	
RARS2	LPPENLITISISAVPISQKEEVADF-----QLSVDSLLEKDNHSDRPD-----IQV---	63
MSR1	DQPEGPIYPLDLRLDISKALHDSIDHSLIIN-ALESTNSMDRGDLLLLPLPKIKVADP	119
	** . * : . . : . : * : . * : * . * . * * : *	
RARS2	--QAKRLAEKLRCDTVVSEISTGQRTVNFKINRELLTKTVLQQVIEDGSKYGLKSELFSG	121
MSR1	VAVANRWAIELSTHGCIGKVCAGPFLQFFLDQRYLIQSTVFNILLQKGYGQKK----S	175
	* : * * : * . : . : . : : * : * : . : : : : . * * * * .	
RARS2	LPQKKIVVEFSSPNVAKKPHVGHRLSTIIGNFIANLKEALGHQVIRINYLGDWGMQFGLL	181
MSR1	RHQKKVVVEFSSPNIAKPFHAGHLRSTIIGGFLSNLYEAMGWSVTRMNYLGDWGRQFGLL	235
	:**:* * :*****:*:*** * * : * :***** *****	
RARS2	GTGFQLFGYEEKLQSNPLQLFEVYVQVNKEAADK-----SVAKAAQEFFQRLGLGD	234
MSR1	AVGFKRYGDEKTLQKQPIQLFDVYVKINMDLAKEEINGNSKCGISGEARSFFKNLENGD	295
	. : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
RARS2	VQALSLWQKEFLDSIEEYIRVYKRLGVYFDEYSGESFYREKSEQVLKLLSKGLLKTIK	294
MSR1	ENAIKIWNRFSLSIHHYIQTYSRLNINFDIFSGESQVSKESMNEALDIFRKNLNVKEID	355
	: * : : * : : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
RARS2	GTAVVDLSGNGDPSSICTVMRSDGTSVYATRDLAADRMKDYNFDTMIYVTDKQKQKH	354
MSR1	GALVIDLTQWSKRLGRVGVQKSDGTTLYLTDVGAIAERKKNLHFDKMYVVISSQQDLYM	415
	* : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
RARS2	QQVFQMLKIMGYDWAERCQHPFVGWQGMKTRRGDVTFLDVLNEIQLRMLQNMASIK-T	413
MSR1	SQFFMILKGMNFEWAKDLQHINFGMVQGMSTIRKGNVFLDTILDEARDKALQIMENNMK	475
	. * . * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
RARS2	TKELKNPQETAERVGLAALIIQDFKGLLSDYKFSWDRVFQSRGDTGVFLQYTHALHSL	473
MSR1	ISQVDNPQRVADLIGVSAIIQDMKSKRINNYEFNWNRMLSFEGDTGPYLYQYTHSLRSL	535
	. : : * * . * : : * : * : * : * : * : * : * : * : * : * : * : * : *	
RARS2	EETF---GCGYLNDENTACLQEPQSVSILQHLLRFDEVLYKSSQFQPRHIVSYLLTLSH	530
MSR1	ERTSSDFTTDMLIHADFSNLNEPQLVELVRLLAQYFQVLRRAFEQEPATIVTYLFFKVCH	595
	* . * . : * : : * : * * * * : * : : * : * : : : * : * : * : * : *	
RARS2	LAVAHKTLQIKDSPPEVAGARLHLFKAVRSVLANGMKLLGITPVCRM- 578	
MSR1	QVSSCYKKIWSGKPADIAIPRLAVYSASRQVLHNAMSLGLVPVDRM* 643	
	. : . : * : . . . * : * * : . : * * * * * . * : * : * : * : *	

Figure 3.17 Alignment between human and yeast mt-ArgRS. The result of the alignment between Rars2 and Msr1 by ClustalΩ. Marked in green the conserved residues, marked in yellow the not conserved residues, marked in red the not analyzed residues.

3.4.1. Mitochondrial function characterization: spot assay analysis and oxygen consumption rate

To investigate the ability to grow on oxidative carbon source, *msr1Δ* strains carrying mutated, humanized, wild type alleles or the empty vector were spotted with serial dilution on YNB DO-W with glucose as fermentable carbon source or glycerol as oxidative carbon source (Figure 3.18).

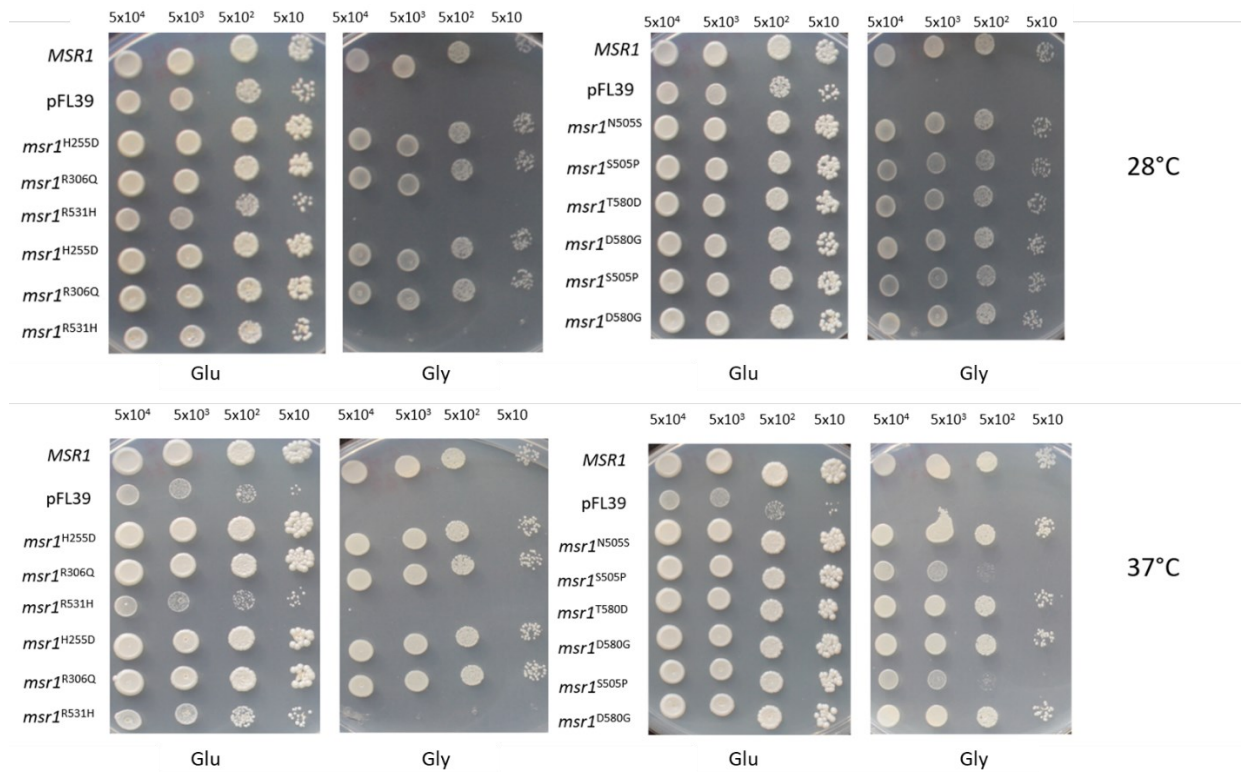


Figure 3.18 Ten-fold dilution spots for each strain, starting from 10^5 cells, were spotted on solid media added with glucose or glycerol incubated at 28°C or 37°C. Results scored after 72 hours.

All the humanized alleles, *msr1*^{Asn505Ser} and *msr1*^{Thr580Asp}, were able to complement *msr1* Δ strain, both at 28°C and 37°C. Incubation at 28°C revealed for the strain carrying *msr1*^{Arg531His} allele the total inability to grow on glycerol, indicating that this change leads to the production of an unfunctional protein; this mutation was previously characterized in yeast by Cassandrini et al., 2012 [77]. The other mutated strains did not show any oxidative growth defect at this temperature. Incubation at 37°C confirmed the phenotype observed at 28°C for the strain harboring *msr1*^{Arg531His} mutant allele, unable to grow on glycerol, and revealed for the strain carrying *msr1*^{Ser505Pro} a significant defect in oxidative growth, indicating that this mutation affects the ability to grow on non-fermentable carbon sources in a thermosensitive manner. The other three mutations, His255Asp, Arg306Gln and Asp580Gly, did not affect the oxidative growth (Figure 3.18).

To further investigate on the OXPHOS phenotype of these strains, OCR analysis was performed. As showed in Figure 3.19, the respiration activity agreed with the results obtained in the spot assay: in fact, the strain carrying the humanized alleles, *msr1*^{Asn505Ser} and *msr1*^{Thr580Asp}, exhibited a oxygen consumption rate similar to the wild type one at both temperatures; the respiration activity of the strain carrying *msr1*^{Arg531His} is strongly reduced at the same level of the null mutant at both temperatures; while the strain harboring *msr1*^{Ser505Pro} mutant allele showed a ~80% decrease in respiration activity only at 37°C. At the latest temperature, also the strain

carrying *msr1*^{Arg306Asn} mutant allele, that didn't show oxidative growth defect in the previous analysis, revealed a ~20% reduction in respiration rate (Figure 3.19). From these phenotypical analyses, only three mutations resulted to affect the mitochondrial functionality, Arg306Gln, Ser505Pro and Arg531His, with the latest two more severely than the first.

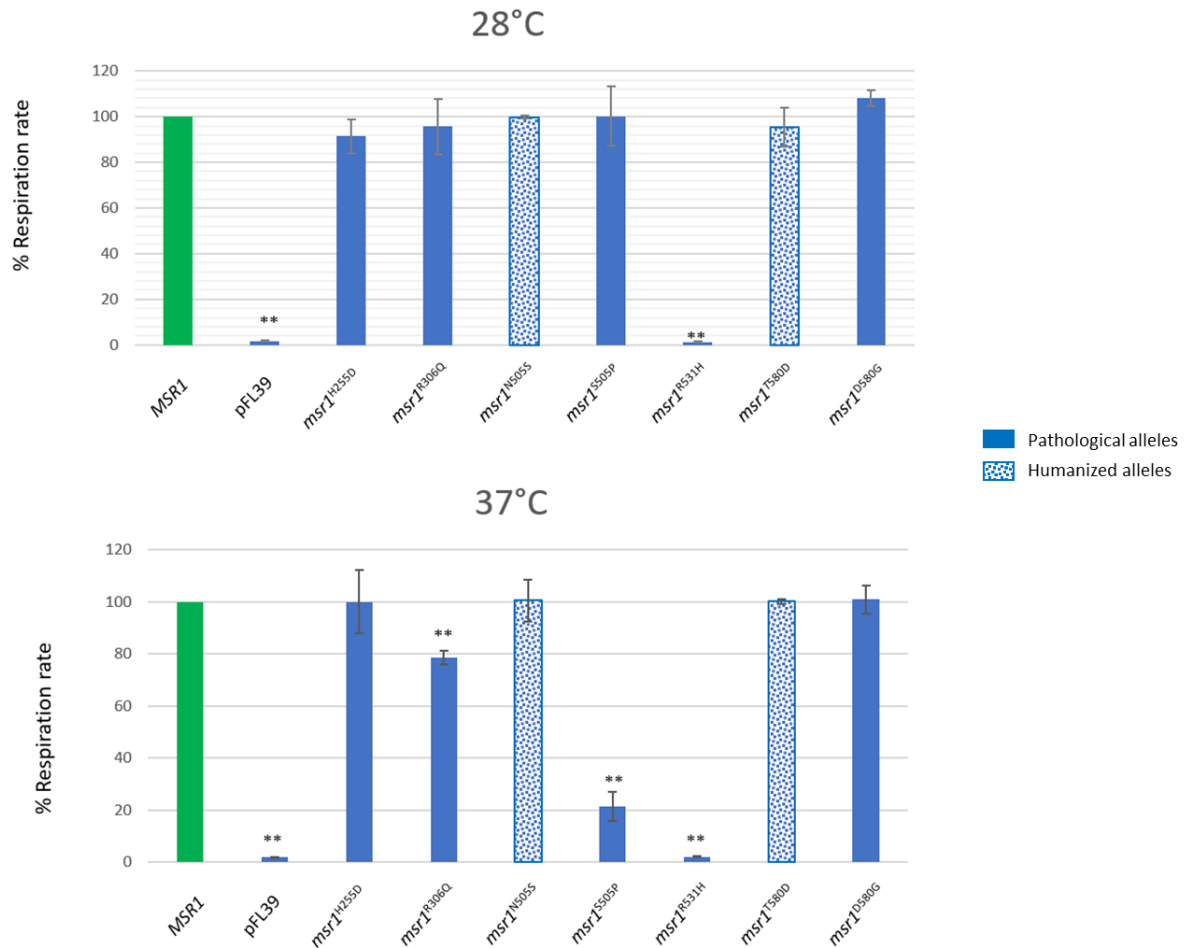


Figure 3.19 Oxygen consumption rate (OCR). Respiration activity measurements for *msr1Δ* harboring wild type, mutated, humanized alleles or empty vector grown in 0,6% glucose medium. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (* $p < 0,05$, ** $p < 0,01$).

For these strains was not possible to perform Western Blot for detecting Msr1 protein since *MSR1* allele with the insertion of HA tag is not able to complement *msr1Δ* strain.

3.4.2. Analysis of Cox2 steady-state level and *in vivo* mitochondrial protein synthesis

Also for *msr1* mutant strains, analysis of Cox2 level and *in vivo* mitochondrial protein synthesis were performed. Western Blot analysis conducted at 37°C reflected the OXPHOS defects observed since the strain carrying *msr1*^{Arg531His} allele lacks the Cox2 signal, while the strain harboring *msr1*^{Ser505Pro} mutant allele showed a ~70% reduction in Cox2 steady-state level, thus

confirming the impairment of mitochondrial translation caused by these mutations. Cox2 steady-state level is similar to that of wild type in all the other mutated and humanized strains (Figure 3.20 A).

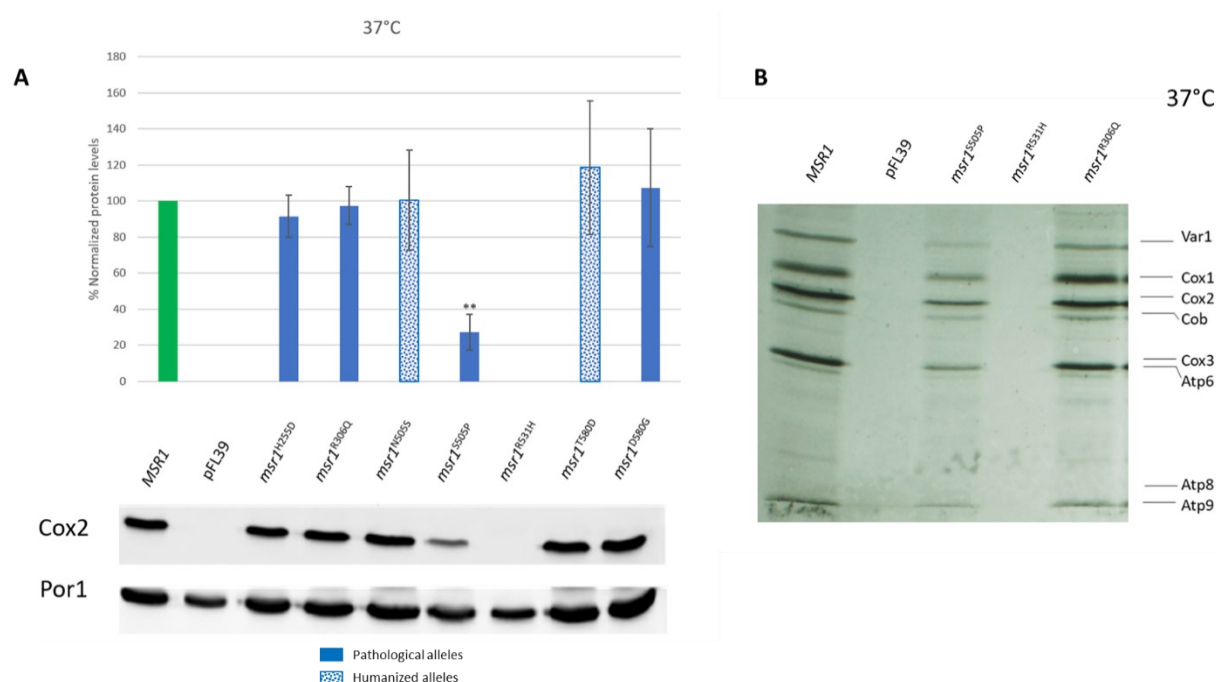


Figure 3.20 Cox2 steady-state level and in vivo mitochondrial protein synthesis. Western Blot performed to detect Cox2 protein in the strains *msr1Δ* carrying mutated, wild type alleles or empty vector. Primary antibodies α -Cox2 (1:2000 diluted) and α -Por1 (1:10000 diluted) were used. Secondary antibodies StarBright520 (1:10000) was employed to recognize both marked proteins. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (** $p < 0,01$) (A). *In vivo* mitochondrial protein synthesis performed in the strains *msr1Δ* carrying mutated, wild type alleles or empty vector. Mitochondrial genes products were labeled with [^{35} S]-methionine in whole cells in the presence of cycloheximide for 10 minutes at 37°C (B).

in vivo mitochondrial protein synthesis performed in mutant strains that presented OXPHOS defects showed that the strain carrying *msr1*^{Arg531His} mutant allele did not show any signal of mitochondrial proteins, confirming the null phenotype observed; while regarding the strain carrying *msr1*^{Ser505Pro} mutated allele, the mitochondrial translation resulted decreased, parallelizing, also in this case, the OXPHOS defect seen before. The third mutant strain analyzed, that showed only a slight decrease in respiration rate (carrying Arg306Gln mutation), did not exhibit mitochondrial translation defect (Figure 3.20 B).

3.5. Study of missense variants in *WARS2/MSW1* encoding mitochondrial tryptophanyl-tRNA synthetase

WARS2 is the gene encoding for mitochondrial tryptophanyl-tRNA synthetase in human, the enzyme charging tryptophan on its cognate tRNA. Mutations in this gene are associated with

Neurodevelopmental disorder, mitochondrial, with abnormal movements and lactic acidosis, with or without seizures (OMIM: [617710](#)) and Parkinsonism-dystonia 3, childhood-onset (OMIM: [619738](#)). In this gene three mutations were identified in two patients, thanks to the use of NGS: Trp13Gly, Gly54Glu and Met227Val, with Trp13Gly in compound heterozygosity with Gly54Glu in one patient and with Met227Val in the second patient. Mutations affect mitochondrial targeting signal (Trp13Gly), catalytic domain (Gly54Glu) and one of the conserved motifs of the protein (Met227Val) (Figure 3.21).

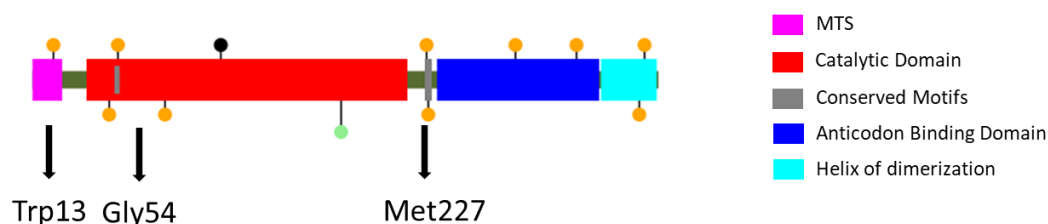


Figure 3.21 WARS2 domains affected by mutations (<http://misynpat.org/>). Dots represents mutations positions already found in patients; green, orange and black colours indicate homozygous, compound heterozygous missense mutations and nonsense mutations, respectively.

The analyses were performed introducing the mutations in the yeast ortholog gene, *MSW1* (Table 3.5).

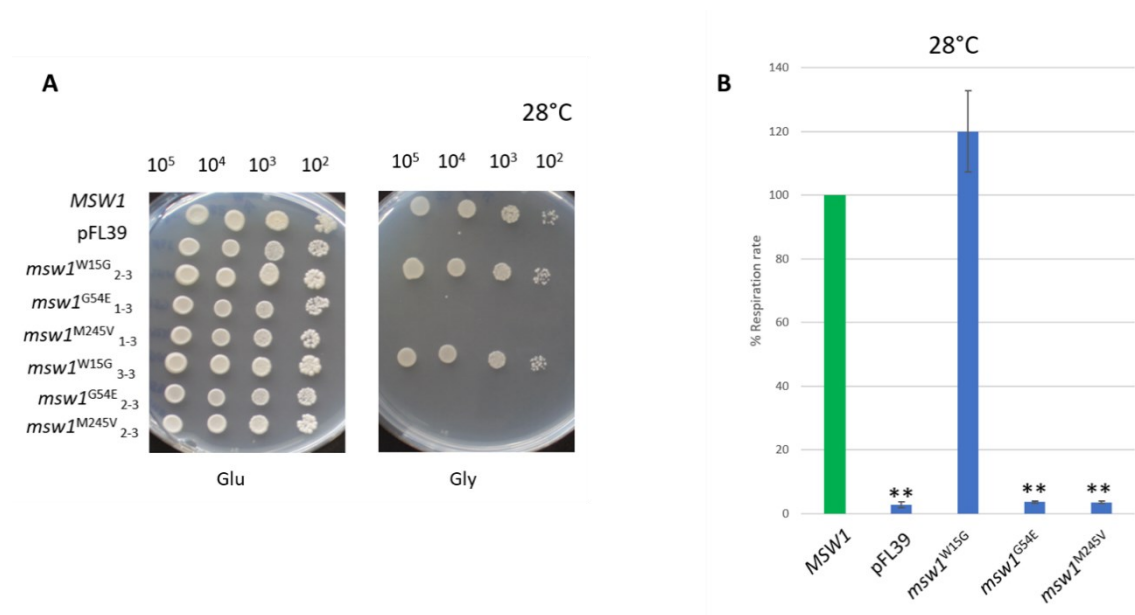
Patient	WARS2 variant	Aminoacid change	Yeast position (Msw1)	
1	chr1:119683231 A>C hetero c.37T>G	p.Trp13Gly	Trp15	Conserved
	chr1:119619160 C>T hetero c.161G>A	p.Gly54Glu	Gly54	Conserved
2	c.37T > G	p.Trp13Gly	Trp15	Conserved
	c.679A > G	p.Met227Val	Met245	Conserved

Table 3.5 Aminoacidic corresponding positions between human and yeast proteins.

All the mutated residues were conserved in the yeast aminoacidic sequence, Trp13 corresponds in yeast Msw1 to Trp15, Glu54 corresponds to Glu54 in yeast protein also and Met227 corresponds to Met245. Only pathological alleles were constructed (Figure 3.22). Despite Trp13 falls in the MTS, it was decided anyway to analyze it because this residue is conserved between human and yeast.

[illegible]

3.5.1. Mitochondrial function characterization: Spot assay analysis and Oxygen Consumption Rate



different yeast colonies obtained from the transformation (the second) (A). Oxygen Consumption Rate carried out on the same strains grown in liquid YNB DO-W with 0,6% of glucose added (B).

The analysis, conducted at 28°C and 37°C (results at 28°C are shown), revealed at both temperatures that the strain harboring *msw1*^{Trp15Gly} is able to grow on glycerol as well as the strain carrying the wild type allele, while the strains harboring *msw1*^{Gly54Glu} and *msw1*^{Met227Val} exhibited the total inability to grow on non-fermentable carbon source as the null mutant (Figure 3.23 A). The OCR analysis is in agreement with the spot assay: in fact, the strain carrying the mutation Trp15Gly had an elevated respiration activity, similar to the wild type one, while the strains carrying Gly54Glu and Met227Val mutations showed a severely decreased respiration activity at the same level of the null mutant, confirming that both changes lead to the production of an unfunctional protein (Figure 3.23 B).

3.5.2. Evaluation of Msw1 steady-state level

To evaluate if mutations could affect the steady state of Msw1 protein, Western Blot was performed.

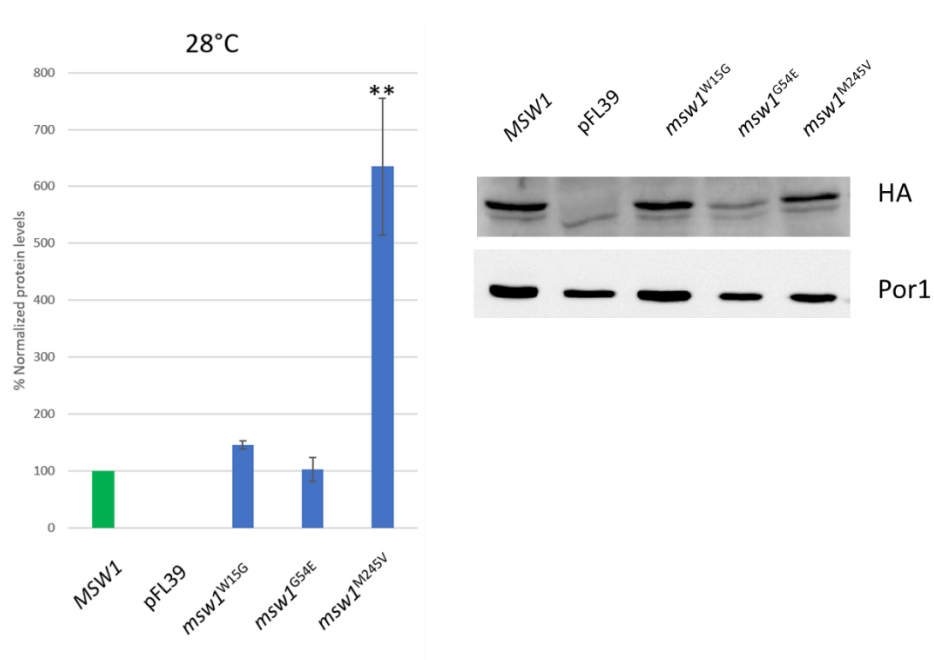


Figure 3.24 Msw1 steady-state level. Western Blot performed to detect Msw1 protein in the strains *msw1Δ* carrying mutated, wild type alleles or empty vector. Primary antibodies α -HA (1:1000 diluted) and α -Por1 (1:10000 diluted) were used. Secondary antibodies Dylight650 (1:2000) and StarBright520 (1:10000) were employed to recognize tagged Msw1 and Por1, respectively. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (** $p < 0,01$).

As shown in Figure 3.24, in the strain carrying the mutation Trp15Gly the steady-state level of Msw1 protein is not changed, in agreement with the wild type growth phenotype also the

protein level is not compromised. The mutated Msw1^{Gly54Glu} protein did not show alterations in steady-state level, suggesting that respiratory defective phenotype could be due to a defective enzymatic activity. Mutation Met245Val caused, instead, a strong increase of the steady-state level of Msw1 protein (~6 times higher than wild type), indicating a more stable protein structure. The OXPHOS defects observed in the last two described mutant strains are not due to altered protein level, thus indicating enzymatic activity impairment as cause of defective mitochondrial functionality (Figure 3.24).

3.5.3. Analysis of Cox2 steady-state level and *in vivo* mitochondrial protein synthesis

To investigate the impact of *msw1* mutations on mitochondrial translation, Cox2 steady-state level and *in vivo* mitochondrial protein synthesis were performed. In the strain carrying the *msw1*^{Trp15Gly} allele, the results obtained by both analyses are in agreement with the OXPHOS phenotype observed; in fact, the Cox2 level is normal as well as the mitochondrial protein pattern. Regarding the two others mutant strains, Cox2 is undetectable and mitochondrial proteins are absent in the strain harboring Gly54Glu mutation and strongly reduced in the strain carrying Met245Val variant (Figure 3.25).

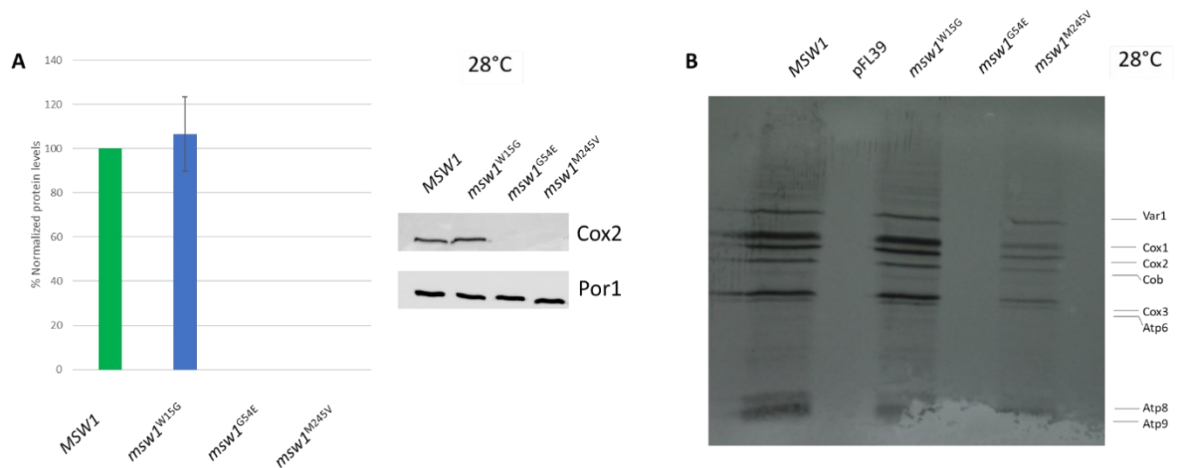


Figure 3.25 Cox2 steady-state level and *in vivo* mitochondrial protein synthesis. Western Blot performed to detect Cox2 protein in the strains *msw1Δ* carrying mutated and wild type alleles. Primary antibodies α -Cox2 (1:2000 diluted) and α -Por1 (1:10000 diluted) were used. Secondary antibodies StarBright520 (1:10000) was employed to recognize both marked proteins. The statistical analysis One-Way ANOVA with Bonferroni correction was applied for the mutant strain carrying Trp15Gly mutation, but no significant results were detected (**A**). *In vivo* mitochondrial protein synthesis performed in the strains *msw1Δ* carrying mutated, wild type alleles or empty vector. Mitochondrial genes products were labeled with [³⁵S]-methionine in whole cells in the presence of cycloheximide for 10 minutes at 28°C (**B**).

3.5.4. Cellular localization of Msw1^{Trp15Gly} protein

Trp15 residue, as said before, is located in the mitochondria targeting signal and mutations affecting this residue could lead to protein mislocalization. Since in HEK293T cells was demonstrated that Wars2^{Trp15Gly} mutated protein was significantly reduced in mitochondria respect to its wild type counterpart [95], to investigate if this amino acid substitution could affect the import in mitochondria also in yeast, localization studies were performed. To this purpose, intact mitochondria were extracted, as described in material and methods, from the strains harboring the *msw1*^{Trp15Gly} mutant allele or the wild type allele. Subsequently, equal amounts of mitochondrial pellet (M) and post-mitochondrial supernatant (PMS) fractions were subjected to Western Blot analysis (Figure 3.26).

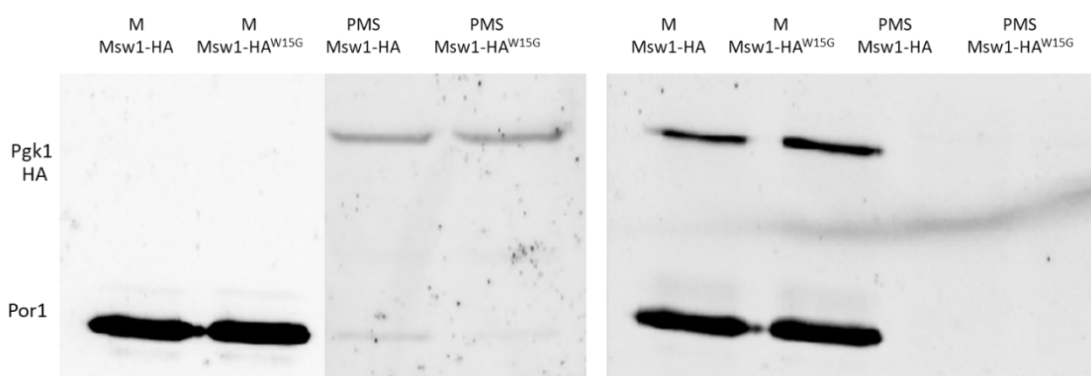


Figure 3.26 Msw1^{Trp15Gly} localization. Immunoblotting signal obtained by equal amounts (~20 mg) of the mitochondrial fraction (M) and post-mitochondrial supernatant (PMS) obtained from *msw1Δ* strain harboring wild type allele or *msw1*^{Trp15Gly} mutant allele. The nitrocellulose membrane was incubated with α-HA, to detect the protein of interest, α-Pgk1 and α-Por1 were used to detect the identically named proteins used as cytosolic and mitochondrial outer membrane marker, respectively. The first filter was hybridated with α-Pgk1 and α-Por1 primary antibodies, two times of exposure were used to detect both signal. The second filter was hybridated with α-HA primary antibody.

As shown in figure 3.26, Msw1^{Trp15Gly}-HA mutated protein localized in the mitochondrial fraction with the mitochondrial membrane protein porin (Por1), thus excluding mislocalization problem. This result is not surprising considering that the aminoacidic sequence of MTS is poorly conserved between human and yeast protein.

3.6. Study of missense variant in *VARS2/VAS1* encoding mitochondrial valyl-tRNA synthetase

VARS2 is the gene encoding for mitochondrial valyl-tRNA synthetase and mutations in this gene are associated with combined oxidative phosphorylation deficiency type 20 (COXPD20) (OMOM number: [615917](#)). Thanks to the use of Next Generation Sequencing, two mutations were found in compound heterozygosity in one patient, Thr367Ile and Asp384Asn, both are localized in the

catalytic domain. Thr367Ile mutation was previously described and validated [30], so only Asp384Asn was analyzed (Figure 3.27).

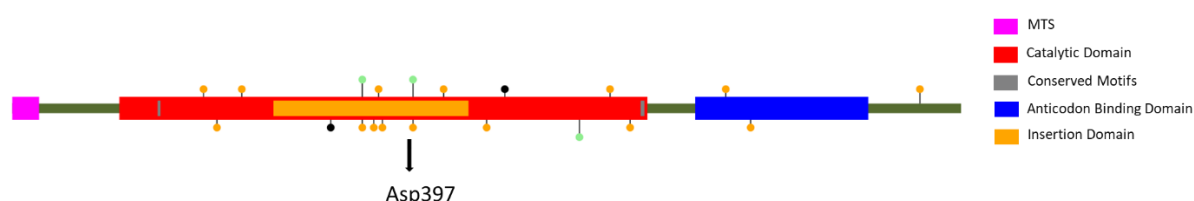


Figure 3.27 Vars2 domains affected by mutations (<http://misynpat.org/>). Dots represents mutations positions already found in patients; green, orange and black colours indicate homozygous, compound heterozygous missense mutations and nonsense mutations, respectively.

The corresponding ortholog gene in yeast is *VAS1*, that belongs to the “Two to One” category [54], therefore *VAS1* encodes for both the cytoplasmic and the mitochondrial valyl-tRNA synthetase. For this reason, the yeast model was constructed disrupting *VAS1* at the chromosomal locus and re-expressing cytosolic *VAS1*, obtained modifying the first aminoacid of the *Vas1* sequence. The strain derived was viable but OXPHOS incompetent since it lacked for the mitochondrial isoform. [30] (Table 3.6).

Patient	<i>VAR2</i> variant	Aminoacid change	Yeast position (<i>Vas1</i>)	
1	chr6:30886628 c.1100C>T	p.Thr367Ile	Thr380	Conserved (Diodato et al., 2014)
	chr6:30886678 c.1150G>A	p.Asp384Asn	Asp397	Conserved

Table 3.6 Aminoacidic corresponding positions between human and yeast proteins.

3.6.1. Mitochondrial function characterization: Spot assay analysis and Oxygen Consumption Rate

The strain described above was transformed with *vas1*^{Asp384Asn} mutant allele and spot assay and OCR analyses were performed at both temperature 28°C and 37°C to investigate if this mutation affects OXPHOS. As shown in Figure 3.28, both OXPHOS growth and OCR were not affected. This mutation in yeast is not pathological and no further analysis were carried out.

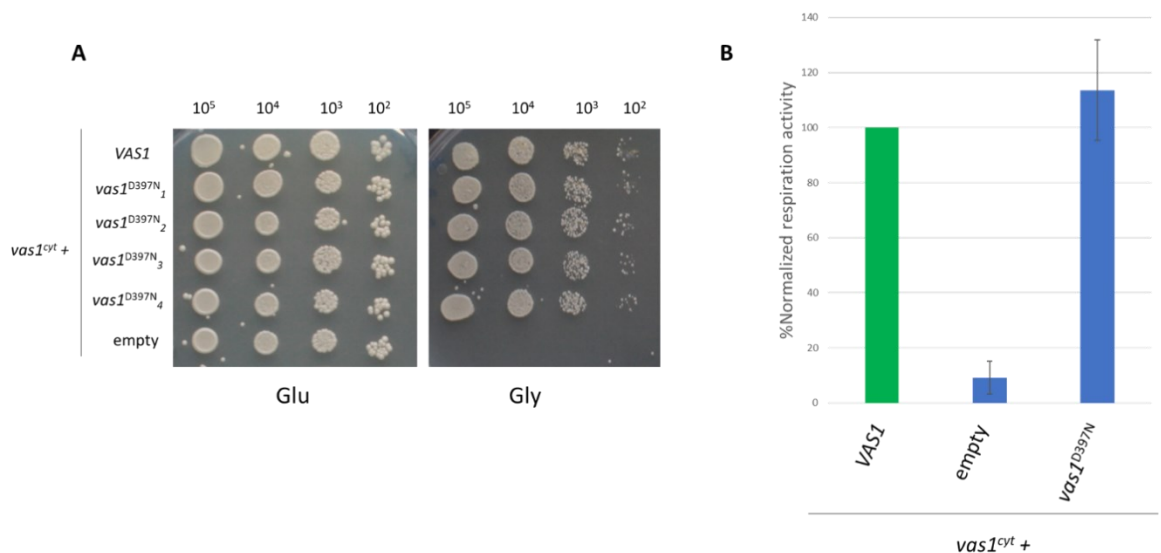


Figure 3.28 Ten-fold dilution spots for each strain, starting from 10⁵ cells, were spotted on solid media added with glucose or glycerol. Results scored after 72 hours. Numbers are referred to different yeast transformants clones (A). Oxygen Consumption Rate carried out on the same strains grown in liquid YNB DO-W-L with 0,6% of glucose added (B).

3.7. Study of missense variants in *AARS1/ALA1* encoding alanyl-tRNA synthetase

AARS1 gene encodes for cytosolic isoform of alanyl-tRNA synthetase, the enzyme charging the alanine on its cognate tRNA. Mutations in this gene are associated with leukoencephalopathy, developmental and epileptic encephalopathy, Charcot-Marie-Tooth disease and trichothiodystrophy (OMIM numbers: [619661](#), [616339](#), [613287](#), [619691](#)). Thanks to the collaboration with Bambino Gesù Hospital, in Rome, through NGS one missense mutation Val665Ala was identified in a patient in compound heterozygosity with a mutation leading to exon deletion [96]. Val666 is localized in the editing domain of the protein and the residue is conserved in yeast corresponding to Val662 in the yeast gene *ALA1* (Table 3.7) encoding for both isoforms of alanyl-tRNA synthetase thus belonging to the “Two to One” category [54].

Since the absence of *ALA1* is lethal, a strain deleted at *ALA1* locus and carrying a wild type copy of *ALA1* gene on a *URA3* bearing plasmid was utilized [70]. The *ala1Δ/ALA1* strain was then transformed with a *TRP1* bearing plasmid carrying wild type *ALA1*, *ala1*^{Val662Ala} or no insert. The growth on solid media containing 0.1% 5-fluororotic acid (5-FOA) was analyzed to evaluate yeast viability. Four independent clones from two independent transformation were analyzed. Each colony was cultured in SC medium without uracil and tryptophan and 5 µl were then transferred in a medium lacking only tryptophan, to induce the loss of the plasmid carrying the wild type *ALA1*, and grown for 24 h. 5 µl of undiluted, 1:10, 1:100 and 1:1000 dilution were spotted on 5-FOA solid medium and plates were incubated at 28°C and growth was assessed after 48h and 72h. As shown in Figure 3.29 the

expression of the mutant allele Val662Ala reduced the ability to rescue yeast growth respect to wild type supporting pathogenicity of this missense variant.

Patient	AARS2 variant	Aminoacid change	Yeast position (Ala1)	
1	16-70292116-A-G c.1997T>C	p.Val666Ala	Val662	Conserved
	Exon 1-4 Deletion	-----	-----	-----

Table 3.7 Aminoacidic corresponding positions between human and yeast proteins.

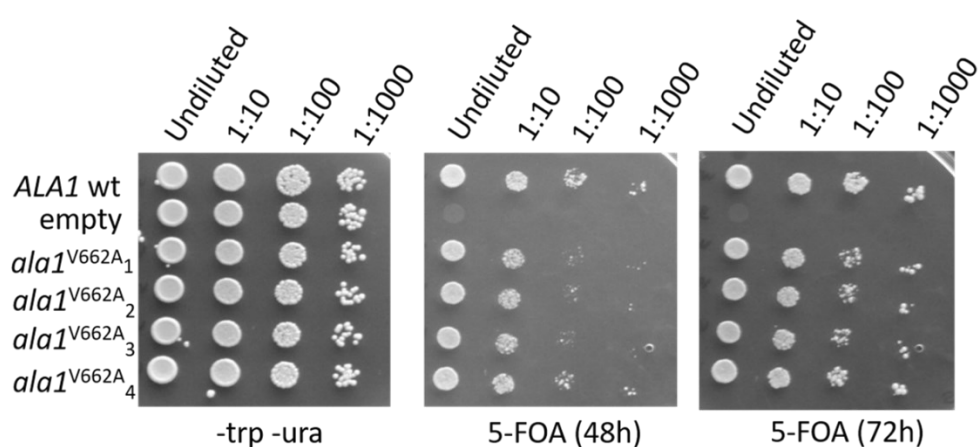


Figure 3.29 Viability analysis. Spot assay conducted on *ala1Δ*/pFL38ALA1 strain carrying wild type, humanized, mutated alleles or empty vector incubated at 28°C. Results were scored after 48 and 72 hours. Numbers are referred to the different yeast transformant clones [96].

These results are available in the paper entitled “Expanded phenotype of AARS1-related white matter disease” [96]

3.8. Therapeutical approaches: aminoacid supplementation and Drug drop test

3.8.1. Is it possible to improve the activity of the mutant mtARSs by amino acid supplementation?

The knowledge on the pathogenic basis of MD is still incomplete, and prevents the development of rational treatments, actually missing for large part of MD. This is true also for the group of MD caused by mutations in genes encoding mtARSs.

Vitamins, cofactors and antioxidant supplementation resulted in some beneficial, however partial, effects in specific mitochondrial syndromes [29]. In the hypothesis that increased availability of the amino acid substrate specific to a given mtARS could ameliorate the mutant

enzyme efficiency, it was evaluated if supplementation of the amino acid substrate of the defective mtARS succeeded in rescue yeast pathological phenotype associated to the different mutations in mtARS genes considered in this work.

To this end, once the mutations were validated, spot assay analysis was repeated for all the mutant strains that showed oxidative growth defect on media lacking or supplemented with different concentrations of the specific amino acid. The results obtained indicated that specific amino acid supplementation can improve the ability to grow on oxidative carbon source of two strains expressing specific mtARS mutant alleles.

Asparagine supplementation was able to partially rescue the OXPHOS defective growth of *slm5Δ/pFL39slm5^{Phe261Ser}* (Figure 3.30 A).

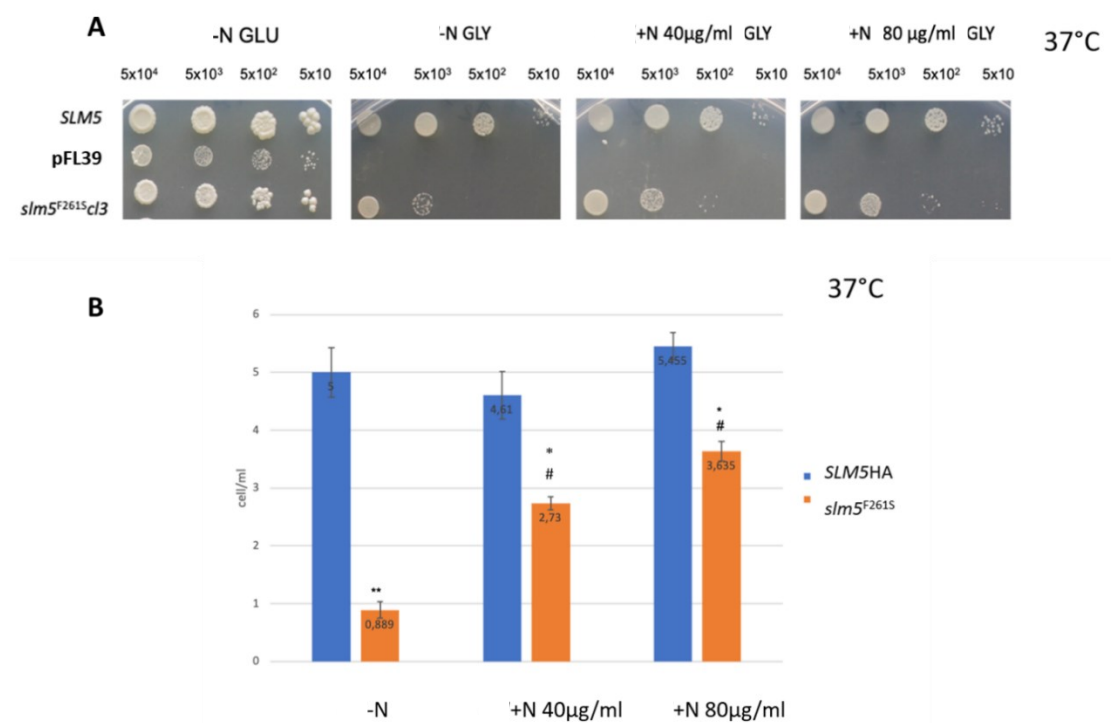


Figure 3.30 Spot assay and cell yield with asparagine supplementation. Strains *slm5Δ* carrying wild type, *slm5^{Phe261Ser}* alleles or empty vector were spotted in ten-fold dilution, starting from 5*10⁴ cells/spot, on solid media YNB DO-W with glucose or glycerol and lacking or supplemented with 40 μg/ml or 80 μg/ml asparagine. Plates were incubated at 37°C and results were scored after 72 hours (A). 5*10⁵ cells/ml of the strains mentioned above were inoculated in liquid media YNB DO-W with glycerol and lacking or supplemented with 40 μg/ml or 80 μg/ml asparagine and incubated in shaking at 37°C. Cellular concentration was scored after 72 hours. The statistical analysis applied is One-Way ANOVA with Bonferroni correction (* *p* < 0,05, ** *p* < 0,01 compared to wild type; # *p* < 0,05 compared to mutant strain grown in absence of asparagine) (B).

To further evaluate the beneficial effect asparagine supplementation, cell yield in liquid media supplemented with different concentration of asparagine was measured. As seen in first

analysis, the mutant strain exhibited an increased cell yield increasing asparagine concentration in the medium (Figure 3.30 B).

The mutated strain *msr1Δ*/pFL39*msr1*^{Ser505Phe} also showed an improved oxidative growth when the medium was supplemented with different concentrations of arginine; the amino acid supplementation also increased the respiration activity of the same strain, that was significantly higher than that of the mutated strain grown in absence of arginine (Figure 3.31).

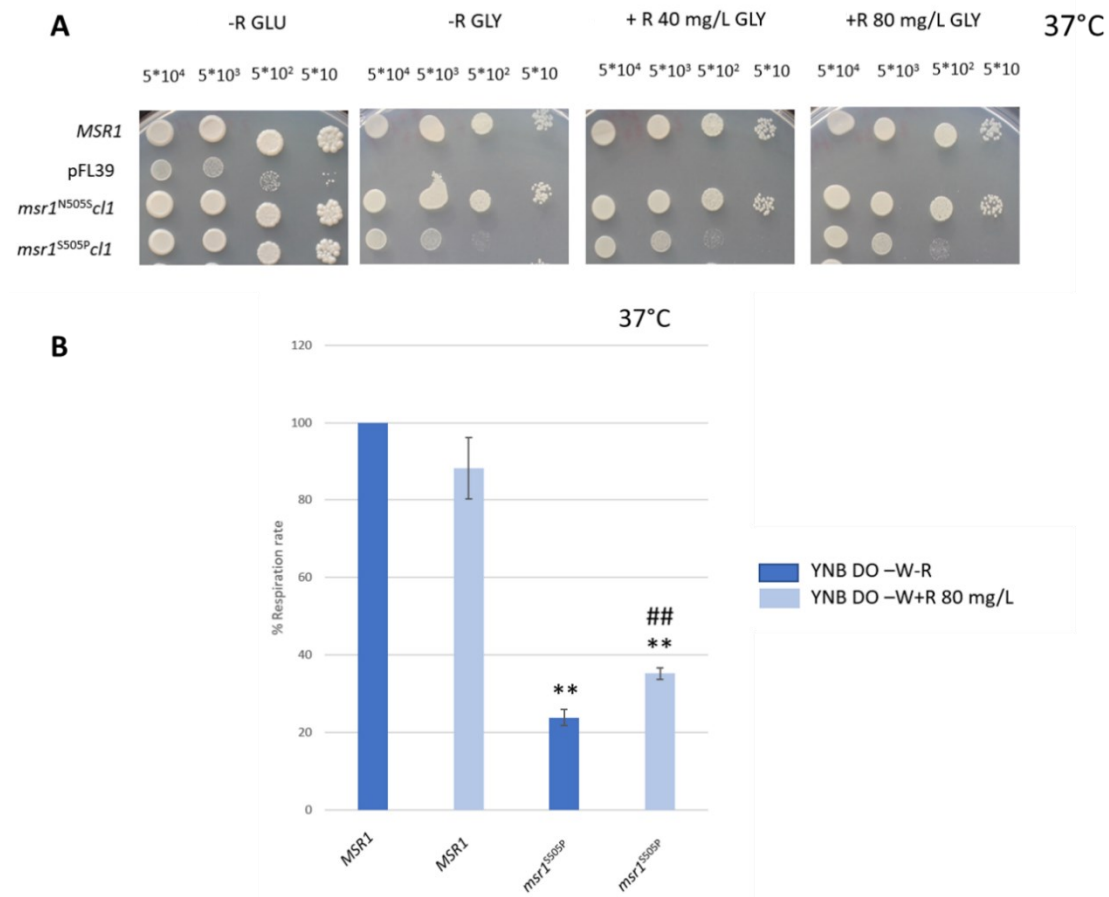


Figure 1.31 Spot assay and OCR with arginine supplementation. Strains *msr1Δ* carrying wild type, *msr1*^{Ser505Phe} alleles or empty vector were spotted in ten-fold dilution, starting from 5*10⁴ cells/spot, on solid media YNB DO-W with glucose or glycerol and lacking or supplemented with 40 mg/L or 80 mg/L asparagine. Plates were incubated at 37°C and results were scored after 72 hours (A). Oxygen consumption rate measured in *msr1Δ* carrying wild type, *msr1*^{Ser505Phe} alleles grown in liquid media with 0,6% glucose and lacking or supplemented with 80 mg/L arginine (B).

These results indicated that the beneficial effect of amino acid supplementation was gene specific and restricted to some mutation.

3.9.2. Drug drop test

In order to contribute to the identification of molecules with therapeutic activity, able to restore or ameliorate the pathological OXPHOS phenotype showed by mtARS mutant strains, the assay called “Drug drop test” was used to screen a library of chemical compounds FDA-approved and already utilized for other diseases (Drug repurposing approach).

The test consists in spotting molecules on filters located on plates seeded with mutant cells and, after incubation, the potential active compounds are identified by a halo of improved growth around the filter. The analysis was performed on *msd1* mutants, starting from the choice of the idoneous mutant and of the conditions for the test. The screening was performed on YP solid media since it is known that synthetic media could make molecules less bioavailable. *msd1* mutant strains were tested for their growth on different oxidative carbon sources at different concentrations and temperatures (data not shown), in order to find the optimal number of cells, carbon source and temperature to utilize for the screening experiment. *msd1Δ/pFL39msd1^{Ser617Gly}* was the strain chosen, ethanol the oxidative carbon source, 1×10^5 cells/plate the number of cells to seed in the plate and 30°C the incubation temperature. The library of molecules tested for the screening was the SCREEN-WELL® FDA approved drug library by Enzo Life Sciences. This library contains ~800 molecules structurally different and FDA approved for their use in cardiology, neuropsychiatry, immunology, oncology and other fields. A primary screening was performed spotting 2,5 µl of each compound on the filters (30 filters for each plate), one additional filter was used as negative control, spotting 2,5 µl of DMSO and in the center of the plate was spotted wild type strain to use as positive control. Six molecules looked to have a beneficial effect and were further analyzed in a secondary screening that was performed spotting the molecules on plates containing only 3 filters, to better analyze the results. Only one molecule, haloperidol confirmed the beneficial effect showed in the previous experiment (here the image of the primary screening plate is showed) (Figure 3.32).

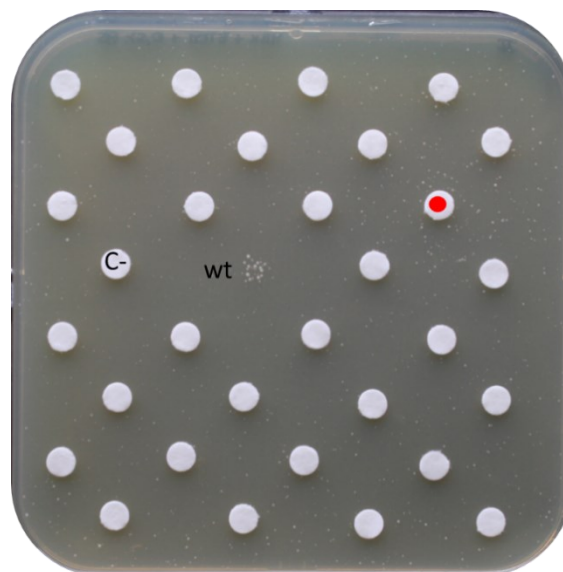


Figure 3.32 Identification of Haloperidol in the screening of molecules. Plate of YP solid media with ethanol 2%. 1×10^5 cells of *msd1Δ/pFL39msd1^{Ser617Gly}* mutant strains were seeded and molecules spotted on filters for discovering their impact on the oxidative growth of the mutant. Marked in red the Haloperidol, potential beneficial molecule found during the screening. Marked with labels wild type spot and negative control (DMSO).

Haloperidol is utilized in pharmacology as antipsychotic for schizophrenia treatment and interacts with dopamine and serotonin receptors in human [97]. In *Saccharomyces cerevisiae* it was demonstrated that haloperidol is involved in ergosterol biosynthesis, the major sterol synthesized by yeast cells that has a determinant role in the regulation of membrane permeability and fluidity [98]. Haloperidol, in fact, inhibits sterol D8,7 isomerase and C-14 reductase activities, which are catalytically performed by Erg2 and Erg24 proteins, respectively. Moreover, haloperidol binds Erg2 protein, resulting in a decrease in ergosterol levels leading to a more permeable membrane [99,100]. Haloperidol was also seen to be involved in amino acid biosynthesis in yeast [101].

To better investigate on the beneficial effect of haloperidol, there was the need to know the concentrations of the compound allowing yeast growth. For this purpose, the minimum inhibitory concentration (MIC) was determined growing the wild type strain in liquid YP medium added with ethanol and supplemented with different concentrations of haloperidol, starting from 256 μ M (data not shown). MIC was found at 128 μ M haloperidol. Successively, cell yield was measured growing wild type and *msd1Δ/pFL39msd1^{Ser617Gly}* mutant strain in the same liquid medium supplemented with different concentration of haloperidol, starting from 128 μ M. Cell growth was monitored for three days and, at haloperidol concentration 64 μ M and 32 μ M, a significant increase of the oxidative growth in the mutant strain was observed (Figure 3.33 A).

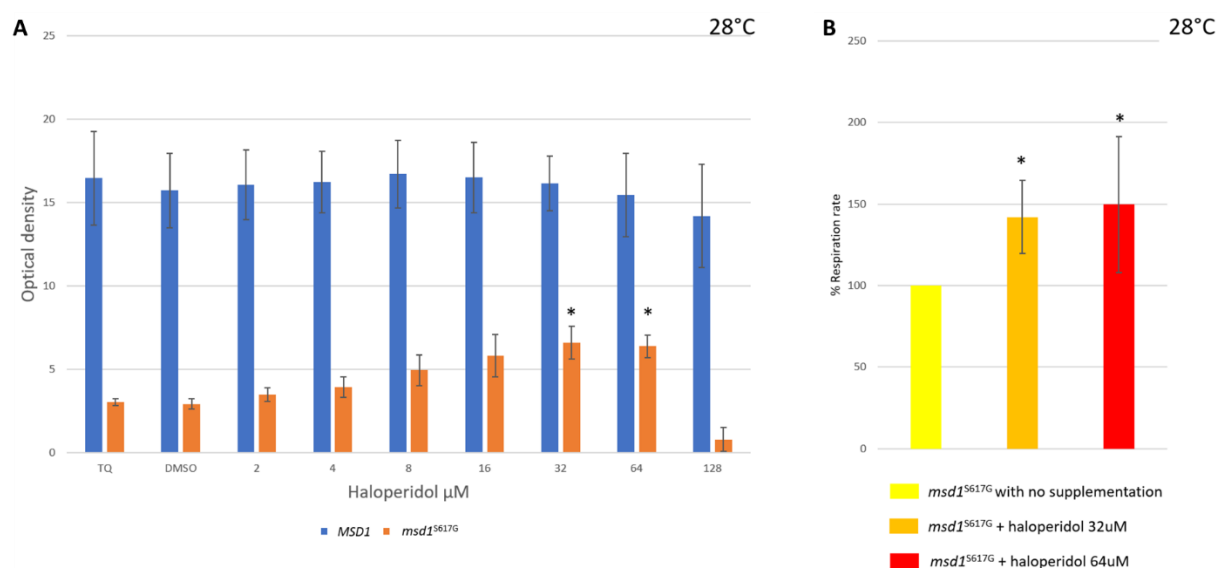


Figure 3.33 Cell yield and OCR with haloperidol. 1×10^5 cells/ml of wild type or *msd1* Δ /pFL39*msd1*^{Ser617Gly} were inoculated in YP media added with ethanol and supplemented with different concentrations of haloperidol starting from 128 μ M or no haloperidol or DMSO as negative control. Results scored after 72 hours (A). Oxygen consumption rate measured in the same strains grown in YP media added with 0,6% glucose and supplemented with the haloperidol concentrations that improved the oxidative cell growth (B).

The OCR investigated on the mutant strain harboring *msd1*^{Ser617Gly} allele showed that the haloperidol concentrations found beneficial in the cell growth analysis succeeded also in significantly improving the respiration activity (Figure 3.33 B).

Since only one positive molecule was found in the screening of the SCREEN-WELL molecule library, a second screening was very recently performed, changing the library of chemical compounds and the *mtars* mutant strain. In particular, the strain carrying the previously validated [75] Arg205Cys mutation in *KARS1* gene was utilized to screen the Compound Library Screening LOPAC^{®1280} Small Molecules. 1×10^5 cells/plate of the strain *msk1* Δ /*kars1*^{Arg205Cys} were seeded, glycerol 2% was the carbon source used and 28°C the incubation temperature. This molecules library is composed by 1280 pharmacologically active compounds, highly pure and pre-solubilized in DMSO. This high-throughput screening led to the identification of three positive molecules called KRM after K*A*R*S*1 Rescue Molecule, able to rescue the defective OXPHOS phenotype of the mutant strain. (Figure 3.34).

Further investigations on these molecules and on their molecular effects are under investigation.

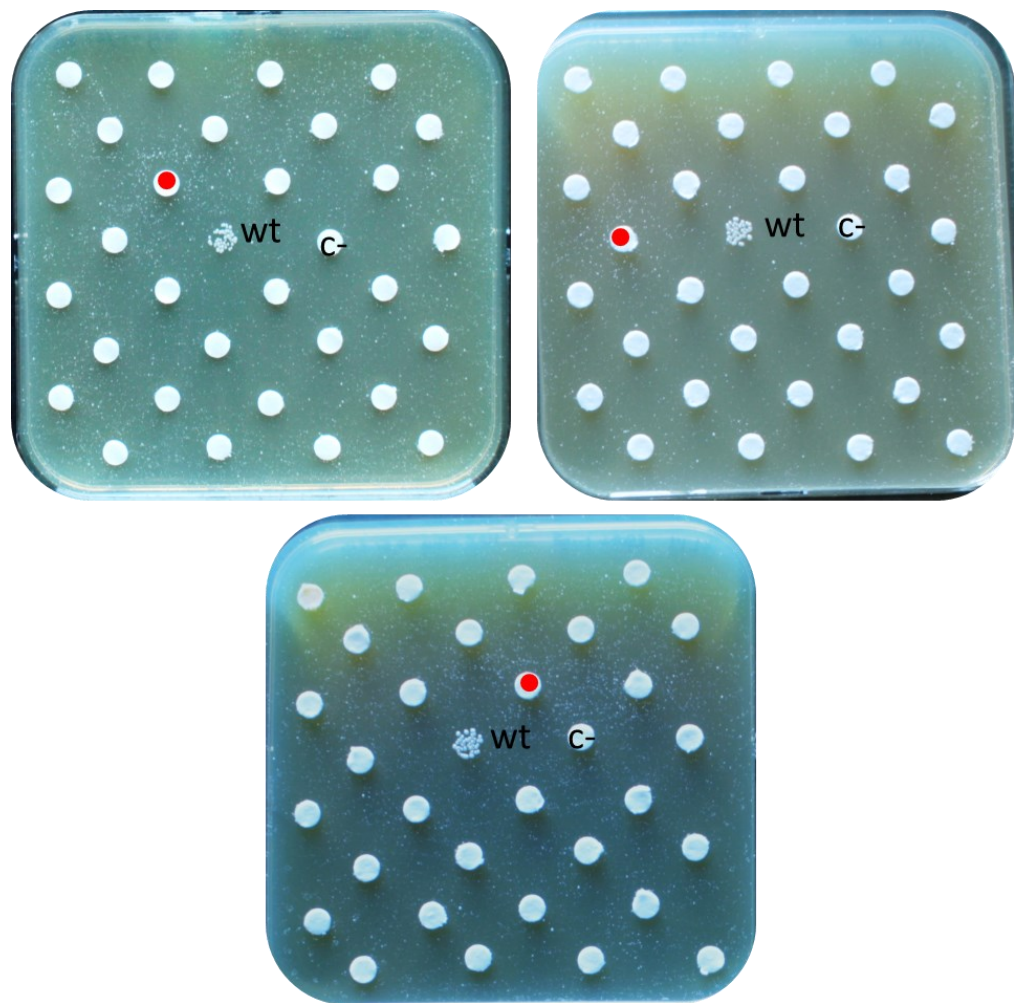


Figure 3.34 KRM (KARS1 Rescue Molecules) identification. Secondary high-throughput screening performed on *msk1Δ*/pFL39-TEToffKARS1^{Arg205Cys}. 1×10^5 cells/plate were seeded and molecules spotted on filters for discovering their impact on the oxidative growth of the mutant. Marked in red the KRMs. Marked with labels wild type spot and negative control (DMSO).

4. Discussion

Mitochondrial aminoacyl-tRNA synthetase (mtARSs) are key components of the mitochondrial translation apparatus and mutations in the genes encoding for mtARSs are associated in human with a wide spectrum of clinical manifestations linked to mitochondrial dysfunctions. Being mitochondrial protein synthesis essential for life, mtARSs mutations identified in MD patients are not complete loss of function but have to maintain partial activity of the enzyme.

Thanks to extensive use of Next Generation Sequencing, an increasing number of variants have been identified in mtARS genes in recent years and a major challenge is functional validation to confirm pathogenicity and an in-depth analysis to assess the mechanisms through which they are associated with mitochondrial disease. To this purpose, several approaches can be adopted, such as to perform the analysis in patients fibroblasts, but in most of cases this approach does not give any answer because of lack of a clear phenotype in these cells [102]. Model systems (yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, zebrafish, mouse) have proved to be useful to validate the pathogenicity of variants. In this context the unicellular and simple eukaryote *Saccharomyces cerevisiae* thanks to its peculiar features, is a particularly suitable model to study mitochondrial disease and contributed to assess the pathogenicity of alleged pathological mutations including those found in mtARSs genes (see Introduction, section 1.5), taking advantage of the high degree of conservation of mtARS proteins during the evolution [53,54].

During the PhD period, a large number of patients was collected from our collaborators of Istituto Neurologico Besta and Ospedale Bambin Gesù and cases with a biochemical defect or an MRI pattern similar to those described in patients with mutations in mtARS genes were selected. For the search of the genetic cause responsible for the pathologies the innovative sequencing technologies (NGS), associated with appropriate bioinformatics analyses, were exploited using customized gene panels containing all genes coding for mtARS.

After the identification of candidate mtARS genes, this research work was aimed to functionally validate the pathogenic role of the identified variants, through studies in yeast models. In particular, novel variants found in *DARS2*, *NARS2*, *RARS2*, *WARS2*, *VARS2* human genes, encoding for mitochondrial aspartyl-, asparaginyl-, arginyl-, tryptophanyl- and valyl-tRNA synthetases, respectively, and one mutation in human cytosolic *AARS1*, encoding for alanyl-tRNA synthetase were deeply studied. Thanks to the presence of ortholog genes in yeast for each gene under analysis, *MSD1*, *SLM5*, *MSR1*, *MSW1*, *VAS1* and *ALA1*, respectively, it was possible to construct *ad hoc* yeast models by introducing mutated alleles, in which the corresponding wild type aminoacidic residue in yeast protein was replaced by the mutated one found in patients, in a yeast strain lacking for the endogenous ortholog gene.

An important part of the experimental work regarded the study of mutations identified in *DARS2* gene. The variants are present in compound heterozygosity and were found in 15 patients at Emma Children

Hospital, in Amsterdam. The role of yeast was to assess the impact of missense mutations on Dars2 functionality analyzing their effect on yeast mitochondrial functionality and to look for a possible molecular correlation between the effect observed in yeast and patients phenotype. Nine missense mutations were analyzed in this gene: Leu250Val, Phe484Leu, Ala262Val, Ser597Gly, Lys606Met, Arg179Cys, Val481Met, Leu588Val, Arg58Gly with correspond respectively to residues Leu232, Phe487, Ala244, Ser617, Lys626, Arg157, Ile484, Met608 and Lys42 in yeast protein. After construction, the mutated strains were first analyzed for their ability to grow on oxidative carbon sources and for their respiratory activity. The results obtained highlighted an OXPHOS defect for all the mutations studied, with some of them that induce severe impairment of mitochondrial functionality (Leu232Val, Phe487Leu, Ala244Val, Ser617Gly, Arg157Cys, Val484Met, Leu608Val) and some other that led to a less severe phenotype (Lys626Met and Arg42Gly). The severity degree observed in yeast almost always correlate with the bioinformatic predictions and the severity of human clinical phenotype. The only two exceptions are Leu588Val variant that in yeast shows a severe phenotype while predicted effects on protein structure and the phenotype of the patients suggest a less serious defect, and Arg58Gly variant whose impact in yeast is less severe than that of the same variant in human. These differences could be explained by a dissimilar amino acid composition and protein structure between human and yeast AspRS [55]. The decreased protein level showed by strains expressing Phe487Leu and Leu608Val mutations suggest that in these cases the lower amount of the mutated protein or its instability contributes to mitochondrial defect and to a pathological phenotype. Proteins carrying Arg157Cys, Ser617Gly and Lys626Met substitution showed an increased amount/stability; all the other amino acid substitution in Msd1 protein do not affect protein level suggesting that the mitochondrial defect and consequently the pathogenetic mechanism were mainly caused by deficiency of enzyme activity rather than reduced protein levels. Analysis of Cox2 protein, encoded by mtDNA, revealed that all the mutation under study reduced, although at different extent, the protein level suggesting a defect in mitochondrial protein synthesis. Mutations Arg42Gly and Lys626Met do not have any effect on Cox 2 amount. Despite the defects showed in the previous analyses, the *in vivo* mitochondrial protein synthesis was not affected for almost all the mutated strains. The latest result could be due to misaminoacylation, thus the charger could load the wrong amino acid possibly not influencing the protein production but only its functionality. Alternatively, the normal mitochondrial synthesis could be assigned to the residual activity of the mutated protein that, when it is at least 5%, can fully sustain protein synthesis [103]. The fact that only the strain mutated in Arg157 position showed a basically null mitochondrial protein synthesis suggests that only the mutation Arg157Cys could lead to a less than 5% reduction of Msd1 activity.

Two more mutations were studied in *DARS2* gene, Arg125His and Gln184Lys, corresponding to yeast positions Lys107 and Gln184, respectively. The expression of these two mutations in yeast led to a quite severe OXPHOS phenotype, and both mutations showed an increased steady-state level of Msd1 protein,

indicating that both aminoacidic changes could stabilize protein structure and the phenotype observed could be associated to enzyme dysfunction. The *in vivo* mitochondrial protein synthesis resulted not affected by both mutations, suggesting a residual activity of the charger higher than 5% or misaminoacylation, as discussed above.

In *NARS2* gene two mutations were identified and therefore analyzed in the present study. The two variants, Met447Val and Phe265Ser, were in compound heterozygosity and correspond in yeast Slm5 protein to Leu461 and Phe261 residues. Characterization of the strains expressing these variants showed that Phe261Ser caused a severe OXPHOS phenotype as well as a significantly decreased Slm5 protein level, that could explain in part the OXPHOS defect. According with the phenotype observed, the *in vivo* mitochondrial protein synthesis is strongly impaired as confirmed also by the absence of Cox2 in the mutant strain affected by Phe261Ser mutation suggesting that the latest impacts negatively on mitochondrial translation machinery. Met461Val mutation led to a less severe OXPHOS phenotype as evidenced by a mild reduction in oxidative growth and a significative impairment of oxygen consumption rate. In this case this phenotype is not associated with altered protein levels, since in the mutant strain the Slm5 steady-state level was similar to that of the wild type protein, indicating that mutation affects the enzymatic activity. However, the defect caused by this mutation did not generate impairment in mitochondrial translation as demonstrated by *in vivo* mitochondrial protein synthesis and Cox2 detection experiments.

Five mutations were analyzed in *RARS2* gene, Arg245Gln, Arg469His, His201Asp, Ser443Pro and Asp515Gly, corresponding in yeast Msr1 protein to Arg306, Arg531, His255, Asn505 and Thr580 residues. Incubation at the permissive temperature 28°C showed a total impairment of oxidative growth and respiration rate for the strain carrying Arg531His mutation, thus indicating the production of an unfunctional Msr1 protein, as already described [77]. Experiments performed to analyze the *in vivo* mitochondrial protein synthesis as well as the steady-state level of Cox2 protein confirmed that this mutation totally compromise mitochondrial translation. Incubation at the restrictive temperature 37°C showed a severe OXPHOS defect in the strain carrying Ser505Pro and a slight impairment of the respiration activity in the strain harboring Arg306Gln, indicating that these changes affect mitochondrial activity in a thermosensitive manner. Further studies on the mitochondrial translation confirmed a strong decrease in mitochondrial protein synthesis as well as a reduction in Cox2 protein level while no defects were observed in the strain expressing Arg306Gln mutation. Regarding Ser505Pro, it falls in the anticodon binding domain and, as mentioned before, *RARS2* yeast model with mutations that fall in this domain not always can reflect the real patients' phenotype because of differences in the mitochondrial genetic code between human and yeast; however, if a detrimental effect is observed in a mutant yeast strain, as evidenced in the case of Ser505Pro, it is highly probable that the mutation is also pathogenic in humans [54]. The other two mutations analyzed (human His201Asp and Asp515Gly) did not have impact

on yeast mitochondrial functionality; in particular, Asp515 human residue is not conserved in yeast and it falls in a not conserved stretch, so it can not be excluded that this change is pathogenic in human. Human His201 is instead conserved in yeast and falls in a conserved stretch; however, the mutation affecting this residue is in compound with Gln12Arg that affect the mitochondrial targeting signal (MTS), so possibly this second mutation could prevent the translocation of the protein in the mitochondria and, subsequently could be the cause of pathology.

Three mutations were analyzed in *WARS2*, Trp13Gly, Gly54Glu and Met227Val, corresponding in yeast Msw1 protein to Trp15, Gly54 and Met245 residues. Gly54Glu and Met227Val showed a total impairment of mitochondrial activity, thus producing a non-functional protein. The steady-state level of Msw1 resulted normal for the strain carrying Gly54Glu and 6 times increased than wild type for the one harboring Met227Val, suggesting that the defective OXPHOS phenotype observed could be due to an unfunctional enzyme more than to a decreased quantity/stability of the protein, that results, in fact, even more stable when affected by Met245Val mutation. Confirming the mitochondrial impairment caused by these mutations, both Cox2 Western blot and *in vivo* mitochondrial protein synthesis showed that Cox2 protein in both strains is absent as well as mitochondrial protein pattern was undetectable in the strain carrying Gly54Glu mutation or showed a strong impairment in the strain carrying Met245Val. Trp15Gly mutation, on the contrary, did not affect in any way mitochondrial functionality. This mutation affects the MTS and, even if this residue is conserved between the two proteins, the amino acid sequence of the MTS domain is poorly conserved; moreover, studies on the localization of Msw1^{Trp15Gly} confirmed that it localizes entirely in the mitochondria, differently from what seen by Martinelli et al. and Musante et al., that demonstrated in patients fibroblasts [104] and in cellular lines [95], respectively, a substantial reduction of *WARS2* level in mitochondria. In this case, yeast could not be the best model to analyze this kind of mutations.

The only mutation analyzed in *VAR2* gene, Asp384Asn, did not affect mitochondrial functionality when introduced in yeast *VAS1*, despite the residue is conserved between human and yeast and the stretch is also quite conserved. In this case, the major cause of the pathogenesis could be attributed to the mutation found in compound heterozygosity, Thr367Ile, already validated by Diodato et al., 2014 [30].

The last ARS analyzed in this research work is the only cytosolic ARS studied, encoded by *AARS1*. One mutation, Val662Ala, was investigated for its ability to rescue yeast growth when expressed in yeast ortholog gene *ALA1*, since it was known that the lack of *ALA1* lead to an unviable strain. The mutation caused a reduction of the strain growth compared to wild type indicating that the mutation partially affects protein functionality [96].

Another aspect of the project was aimed to the search of possible therapeutical approaches, since effective therapy for pathologies caused by mutations in *ARSs* genes and in general for MD is still missing and the therapeutic intervention is limited to symptomatic treatment. Supplementation of vitamins,

cofactors, antioxidant agents etc. have obtained some beneficial, however partial, effects in specific mitochondrial syndromes [29]. In the hypothesis that increased availability of the amino acid substrate specific to a given mtARS could ameliorate the efficiency of the mutant enzyme, it was evaluated if supplementation of the amino acid substrate of the defective mtARS succeeded in rescue yeast pathological phenotype associated to the different mutations in mtARS genes considered in this work. The results indicated that the beneficial effect of amino acid supplementation was gene specific and restricted to some mutation.

A partial rescue of the oxidative growth both in liquid and solid media was found when asparagine was supplemented to the strain *slm5Δ/pFL39slm5^{Phe261Ser}* but not to *slm5Δ/pFL39slm5^{Met447Val}*. Phe261 residue falls in the catalytic domain, thus it is possibly involved in the binding of the amino acid; in this case it is possible to hypothesize that an increased concentration of asparagine could shift the enzymatic reaction towards the binding of the amino acid and the following aminoacylation, partially reversing the pathological phenotype.

Another mutant strain that showed an improved oxidative growth and an increased respiration rate when arginine was supplemented was the strain *msr1Δ/pFL39msr1^{Ser505Phe}* suggesting that arginine levels are a limiting factor for the activity of this mutant allele although in this case, it is more difficult to explain the possible mechanism underlying the rescue since the mutated residue falls in the anticodon binding domain. Interestingly, preliminary results obtained in a trial still in progress at Ospedale Bambin Gesù (Rome) and at Istituto Neurologico Besta (Milano), aimed to test the beneficial effects of amino acid supplementations in patients with mtARS mutations, are in line with what was observed in the yeast model. Table 4.1 summarizes the results obtained from yeast characterization.

Always with the aim of contributing to the identification of molecules with therapeutic activity, able to restore or ameliorate the pathological phenotype showed by mtARS mutant strains, the assay called “Drug drop test” was used to screen a library of chemical compounds FDA-approved and already utilized for other diseases (Drug repurposing approach). From one of the libraries tested on *msd1Δ/pFL39msd1^{Ser617Gly}* mutant strain, one molecule succeeded in slightly restoring the defective oxidative growth, haloperidol.

As mentioned before, haloperidol in yeast is involved in ergosterol biosynthesis and amino acid biosynthesis but it is not exactly known how haloperidol influences the amino acid biosynthesis. In the hypothesis that haloperidol increases amino acid biosynthesis, the slightly improved phenotype could be attributed to a coupled effect derived from a more permeable membrane and a major concentration of aminoacid, although in this mutant aspartate supplementation did not show any beneficial effect.

Very recently another library of molecules was screened using a mutant strain in a different mtARS gene: *msk1Δ/pFL39-TEToffKARS1^{Arg205Cys}*. The secondary screening performed allowed the identification of

three molecules that we called KRS (*KARS1* Rescue Molecules) that revealed a potential beneficial effect; these molecules and their molecular effect need to be further investigated in the future.

In conclusion the generation and characterization of several yeast models expressing alleged pathological mutations identified in *mtARS* genes through NGS of patients without genetic diagnosis allowed to confirm the pathogenicity of almost all these novel identified variants and to give some insight on the pathogenetic mechanism.

Regarding the therapeutic approach using yeast as a first tool, the results obtained, together with those obtained by clinicians, support the possible use of amino acid supplementations in patients with *mtARS* mutations at least to alleviate symptoms or delay/arrest disease progression.

Although very preliminary the Drug drop test allowed the identification of new compounds that after further investigations and studies in other cellular and animal models could be employed in the treatment of *mtARS* patients.

Gene	Human variant	Yeast residue	Yeast characterization				
			Effect on OXPHOS phenotype	Mutant protein level	Cox2 protein level	<i>in vivo</i> mitochondrial protein synthesis	Effect of amino acid supplementation on oxidative growth
<i>DARS2/MSD1</i>	Arg58Gly	Lys42	mild	unchanged	unchanged	unaffected	none
	Arg125His	Lys107	severe	increased	np	unaffected	none
	Arg179Cys	Arg157	severe	increased	decreased	absent	none
	Gln184Lys	Gln162	severe	increased	np	unaffected	none
	Leu250Val	Leu232	severe	unchanged	decreased	unaffected	none
	Ala262Val	Ala244	moderately severe	unchanged	decreased	unaffected	none
	Val481Met	Ile484	severe	unchanged	decreased	unaffected	none
	Phe484Leu	Phe487	severe	decreased	decreased	unaffected	none
	Leu588Val	Met608	severe	decreased	decreased	unaffected	none
<i>NARS2/SLM5</i>	Ser597Gly	Ser617	severe	increased	decreased	unaffected	none
	Lys606Met	Lys626	mild	increased	unchanged	unaffected	none
	Phe265Ser	Phe261	severe	decreased	absent	reduced	positive
<i>RARS2/MSR1</i>	Met447Val	Leu461	moderately severe	unchanged	unchanged	unaffected	none
	His201Asp	His255	none	np	unchanged	unaffected	np
	Arg245Gln	Arg306	mild	np	unchanged	unaffected	none
	Ser443Pro	Asn505	severe	np	decreased	reduced	positive
	Arg469His	Arg531	severe	np	absent	absent	none
<i>WARS2/MSW1</i>	Asp515Gly	Thr580	none	np	unchanged	unaffected	np
	Trp13Gly	Trp15	none	unchanged	unchanged	unaffected	np
	Gly54Glu	Gly54	severe	unchanged	absent	absent	none
<i>VAR2/VAS1</i>	Met227Val	Met245	severe	increased	absent	reduced	none
	Asp384Asn	Asp397	none	np	np	np	np

Table 4.1 *mtARS* considered mutations and effects on yeast models. np: not performed.

PART 2

5. Introduction

5.1. Coenzyme A (CoA)

Coenzyme A (CoA) is a key molecule involved in the metabolism of fatty acids, carbohydrates, amino acids and ketone bodies [105–109], that was identified in 1953 by Baddiley et al. [110]. CoA is a molecule that plays a central role in metabolism; in fact, it is involved in more than 100 different reactions [111], being used as obligate cofactor by 9% of known enzymes [112]. Structurally, CoA is composed by 3'-phosphoadenosine linked through the 5' position of the ribose to pantothenic acid via pyrophosphate linkage. Through a peptidic bond, the carboxyl end of pantothenic acid is linked to 2-mercaptoethanol amine and possess a thiolic group (-SH) that forms thioesters acting as carrier for acyl groups [113].

In human, the pathway leading to the biosynthesis of CoA starts with the phosphorylation of pantothenate by pantothenate kinase enzymes (PankKs), with the formation of 4'-phosphopantothenate, that is subsequently condensated with cysteine by 4'-phosphopantothenoylcysteine synthase (PPCS). The new compound is then decarboxylated by 4'-phosphopantothenoylcysteine decarboxylase (PPCDC), followed by the addition of AMP (from ATP) catalyzed by 4'-phosphopantetheine adenylyltransferase (PPAT) to form dephospho-CoA. Eventually, CoA is formed by a phosphorylation reaction catalyzed by dephospho-CoA kinase (DPCK) enzyme [114].

In the yeast *Saccharomyces cerevisiae*, five essential genes are involved in the CoA pathway: *CAB1*, *CAB2*, *CAB3*, *CAB4* (PPAT activity) and *CAB5* (DPCK activity). The proteins encoded by *CAB2*, *CAB3*, *CAB4* and *CAB5* seem to form a complex called CoA-SPC, that however is not essential for the activity of the single enzymes, since was observed that when each enzyme is substituted by its bacterial ortholog (that cannot form complexes) the biosynthesis of CoA is not compromised [115].

5.2. Pank isoforms

In human, four isozymes with pantothenate kinase activity are present: Pank1 α , Pank1 β , Pank2 and Pank3, encoded by the three human genes *PANK1*, *PANK2* and *PANK3*. The four enzymes are differently compartmentalized. In particular, in studies performed on human and mice cell lines, Pank1 α was demonstrated to localize only in the nucleus, with a preferential association in the nucleolus; while Pank1 β was found into clathrin-coated vesicles and inside endosomes in both human and mice. Regarding hPank2, was discovered that it is distributed both in nucleus and mitochondria. Specifically, hPank2 translocates from nucleus to intermembrane space of mitochondria and not *vice versa*, since the presence of the Nuclear Localization Signal (NLS) was

detected only in the full-length protein and not in the cleaved one present in mitochondria [116]. Heterologous complementation in yeast had previously demonstrated that hPank2 was localized in the nucleus [117], but the NLS sequence predicted in that study was not functional. In human cell lines hPank2 was detected in both compartments in each phase of cell cycle, except for phases G2/M in which the protein localizes only in mitochondria. Mouse Pank2 was, instead, found exclusively in the cytoplasm as well as human and mouse Pank3 [116] (Figure 5.1).

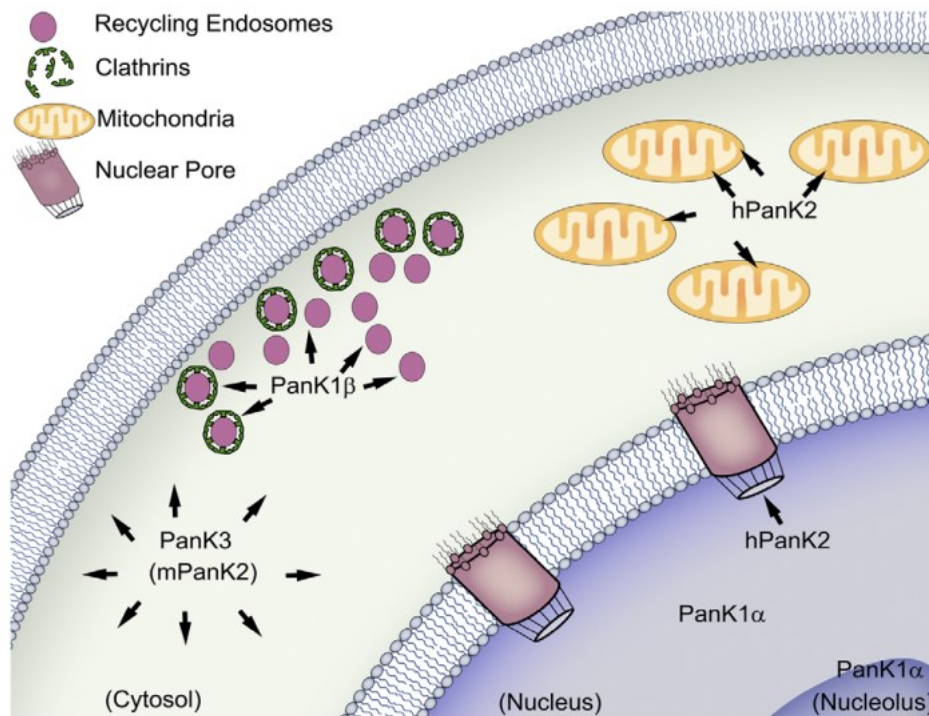


Figure 5.1 Subcellular localization of human PanKs [116].

A fourth genes exist in human, *PANK4*, that encodes for a pseudo-enzyme that lacks for two particular residues essential for the pantothenate kinase activity, Glu138 and Arg207 (PanK3 numbering); it was demonstrate, in fact, that PanK4 protein expresses in the same position Glu138Val and Arg207Trp substitution, that inactivate pantothenate activity [118].

5.3. PKAN (Pantothenate Kinase-Associated Neurodegeneration)

Pantothenate kinase-associated neurodegeneration (PKAN) is as disorder associated with mutation in *PANK2* gene. PKAN is part of a bigger group of diseases called NBIA (Neurodegeneration with brain iron accumulation) disorders and is characterized by accumulation of iron in basal ganglia [119]. Between manifestations of PKAN are included pigmented retinopathy, loss of movement control, severe dystonia and young-onset Parkinsonism [120].

PKAN can be classified into two groups on the basis of the age of onset:

- early onset, when the symptoms start before 10 years old and it is also known as “classic onset”; this kind of patients usually show a rapid pathogenesis progression with loss of ambulation and tendency to develop pigmentary retinopathy.
- late onset, when symptoms start after 10 years old, known also as “atypical onset”; the pathogenesis progression is slower in these patients, with tendency to have speech disorders and psychiatric features [121].

All patients have in common a characteristic sign on brain MRI, called eye-of-the-tiger [122].

Deficiency of CoA biosynthesis due to mutations in *PANK2* could lead to a wide range of symptoms since this molecule is involved in several pathways as tricarboxylic acid cycle, fatty acid oxidation and synthesis and amino acid metabolism [112] and besides iron accumulation in the brain, as previously reported, also mitochondrial and lipid metabolism [123] and oxidative status [124–126] resulted altered.

5.4. *Saccharomyces cerevisiae* as model to study PKAN disease

To date, there is not cure for PKAN disease and only therapeutical approaches aimed to treat symptoms are available [127]. Therefore, there is the need to understand the insight of the pathology using model organism. Different disease models have been created: *Drosophila melanogaster fumble* [128], Zebrafish [129], *Pank2* KO mouse model [130], patient fibroblast but no one succeeded in recapitulate PKAN condition or, in some case, showed only isolated features [86]. More recently neurons derived from iPSCs (induced pluripotent stem cells) carrying pathological mutation are a more representative model of this kind of neurodegeneration [131–135], *Saccharomyces cerevisiae* has been extensively used in modeling human disorders and proved to be a good model in the study of neurodegenerative diseases [136] and iron-related disorders [137], despite the lack of a neuronal system.

Ceccatelli Berti et al. [86] characterized several *PANK2* mutations found in PKAN patients, introducing them in the only yeast gene encoding pantothenate kinase activity, *CAB1*. All the strains deleted in the endogenous *CAB1* and expressing *cab1* mutations, recapitulate all the main phenotypes found in patients thus making yeast a useful tool to investigate on the pathogenic mechanisms of PKAN disease.

All the yeast PKAN models showed, indeed, mitochondrial dysfunction (reduced oxygen consumption, oxidative growth and activity of respiratory chain complexes), as well as increased iron levels and altered lipid metabolism. Indirectly, PKAN yeast models demonstrated to have decreased CoA levels through the analysis of lipoic acid levels that resulted reduced thus suggesting impairment of mitochondrial iron-sulfur cluster biogenesis.

All these findings established yeast as an appropriate model for helping in the understanding the molecular events hidden by this pathology.

6. Aim of the research

This research is part of a collaboration between the Laboratory of Molecular Genetics and Biotechnology of SCVSA Department (University of Parma) and Yale University School of Medicine, in New Haven (Connecticut), in which part of the experiments were performed. The aim of this research is to study the biological activity of human PANK isoforms using *Saccharomyces cerevisiae* as a model.

PANK genes encode pantothenate kinases, which are enzymes that catalyze the first step of CoA biosynthesis, a key molecule involved in the metabolism of fatty acids, carbohydrates, amino acids and ketone bodies. In humans, 4 different genes encode PanK isoforms: *PANK1*, *PANK2*, *PANK3* and *PANK4*. The first three enzymes share the same function, that is the phosphorylation of pantothenate to obtain 4'-P-pantothenate but are differently compartmentalized; in particular, Pank1 and Pank3 are mostly in the cytosol and nucleus [116,123,138], while Pank2 is localized in the mitochondrial inner membrane [116,117,123,138]. Pank4 is probably not active as kinase since it lacks an essential residue for the enzyme activity [118,139]. Mutations in *PANK2* gene have been linked to a neurodegenerative disease called pantothenate kinase-associated neurodegeneration (PKAN), characterized by iron accumulation, oxidative stress, mitochondrial dysfunction and altered lipid metabolism [116,117,140–142].

The biochemical properties of PanK enzymes have been well-characterized *in vitro*, so there was the need to express them in model organisms such as yeast to investigate on their function under cellular conditions; in fact, the principal aim of this research is to evaluate the functional conservation of PanK function in yeast evaluating the ability of complementation of these isoforms in yeast. In this organism, pantothenate kinase is encoded by an essential single copy gene, called *CAB1*. Since the human isoforms are not able to complement a strain totally lacking for the endogenous copy of *CAB1* [86], three yeast strains deleted in the genomic *CAB1* and carrying a plasmid containing missense mutations in *CAB1* were employed in this study. The mutated strains recapitulate all the major features of PKAN disease and were transformed with human PanK isoforms as described in Results section.

As mentioned above, mutations in *PANK2* gene are associated with (PKAN) disease and, in order to perform analyses of the effect of mutations affecting *PANK2*, the new model constructed could be useful since it will be possible to introduce mutations directly in the human gene.

7. Results

7.1. Strains construction

In order to investigate on the complementation of human PanK isoforms in yeast, strains construction was performed. It was already known that full length human *PANKs* failed in complementing W303-1B yeast strain lacking the only yeast gene encoding for PanK function, *CAB1* (data not shown). Consequently, in this study the human genes encoding for the different hPANKs were transformed in strains lacking the endogenous *CAB1* and carrying a *cab1* mutated alleles causing a reduction but not the total loss of Cab1 activity. The mutated strains chosen for the analyses were *cab1Δ/pFL39cab1^{G351S}*, *cab1Δ/pFL39cab1^{N290I}* and *cab1Δ/pFL39cab1^{I291T}*, all obtained from W303-1B strains. Mutation G351S was isolated in a research for mutants presenting altered fatty acid synthase activity and showed a thermosensitive phenotype [105]. Further studies also showed defective oxidative growth, respiration and mitochondrial function [86] and susceptibility to ergosterol inhibitors [143]. Moreover, analysis on purified Cab1^{G351S} highlighted a ~10% residual pantothenate kinase activity compared to its wild type counterpart [109].

The other 2 substitutions were instead pathological mutations found in PKAN patients; in particular, N290I correspond to N500I in human PanK2 while I291T correspond to I501T in human PanK2. Both mutations induced a thermosensitive phenotype in yeast strains and reproduced all the major phenotypes observed in PKAN patients, like iron susceptibility, mitochondrial dysfunction, oxidative damage and defective lipid metabolism [86].

Full length human *PANK2* failed in complementing also the strains carrying hypomorphic alleles *cab1Δ/cab1HA^{G351S}* and *cab1Δ/cab1HA^{N290I}* (Figure 7.1), probably because of differences in localization; in fact, Cab1 is localized in cytoplasm while PanK2 in mitochondria [86]; for this reason, was used a *PANK2* gene encoding for an enzyme lacking for the mitochondrial targeting signal.

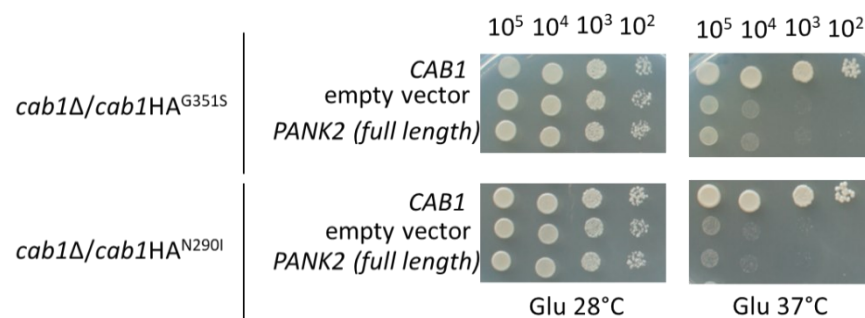


Figure 7.1 Full length PanK2 complementation assay. *cab1Δ/cab1HA^{G351S}* and *cab1Δ/cab1HA^{N290I}* mutant strains transformed with pFL38*CAB1*, pYEX-BX empty vector and pYEX-BXPANK2 (full length) spotted in ten-fold serial dilutions, starting from 10⁵ cells/spot, on SC solid media with glucose 2%. Results scored after 72 hours.

Human isoforms, full length PanK1 and PanK3 and PanK2 lacking the mitochondrial targeting signal (PanK2^{mtmΔ}), were codon optimized and fused with c-Myc tag in the N-terminal. Successively, were

cloned in pESC vector under *GAL1* promoter, that allows the expression of PanKs isoforms only in presence of galactose and used to transform the strains previously described. The resulting strains were *cab1Δ*/pFL39*cab1*^{G351S}/pESCPANK1-3-2^{mtmΔ}, *cab1Δ*/pFL39*cab1*^{N290I}/pESCPANK1-3-2^{mtmΔ} and *cab1Δ*/pFL39*cab1*^{I291T}/pESCPANK1-3-2^{mtmΔ}.

7.2. Complementation studies on solid and liquid media

The first analysis performed to investigate on the complementation of human isoforms was a spot assay: equal amount of each strain was spotted in serial dilutions on glucose and galactose. Together with the strains transformed with hPANKs, were also analyzed for each genetic background a strain transformed with pFL38*CAB1*, to use as control of the endogenous protein activity, and a strain transformed with pESC empty vector, to compare the known effect of the mutation with the effect produced by human isoforms (Figure 7.2).

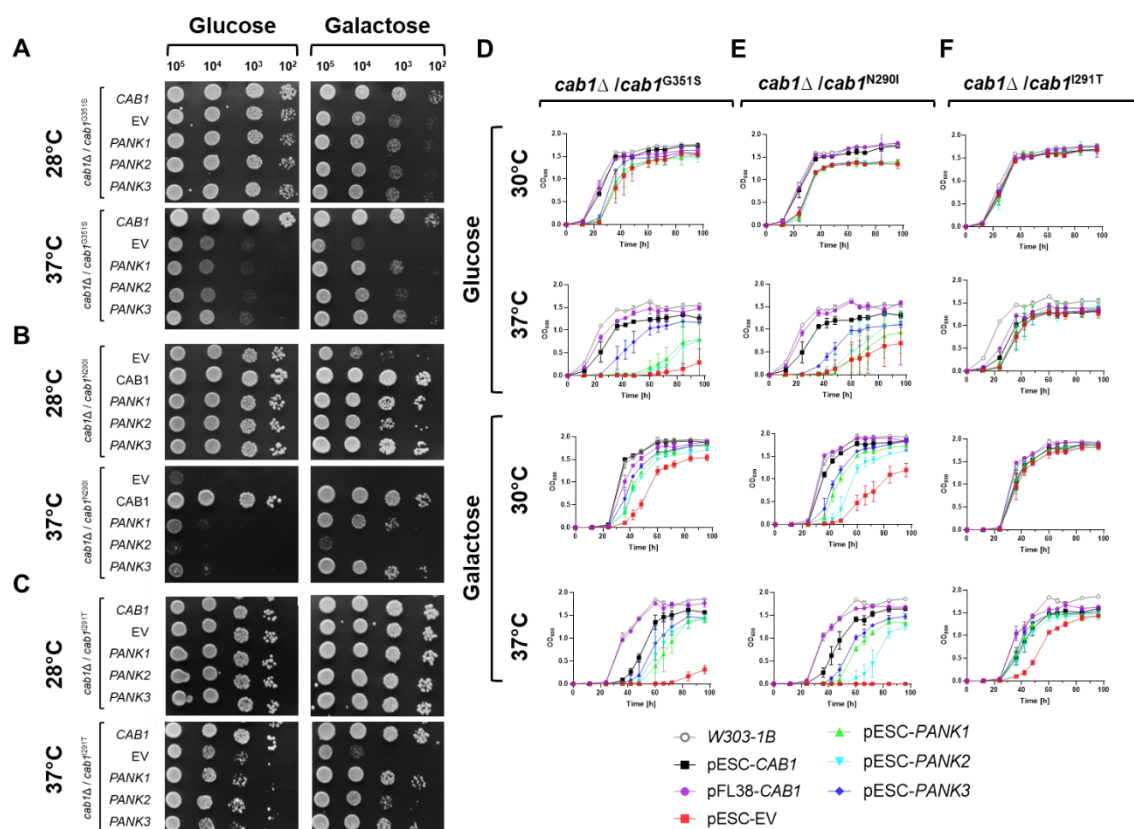


Figure 7.2 Human PanKs complementation on solid and liquid media. Analysis on solid SC-URA-TRP with glucose or galactose (A-C). Same amount of cells of *cab1Δ*/pFL39*cab1*^{G351S} (A), *cab1Δ*/pFL39*cab1*^{N290I} (B) and *cab1Δ*/pFL39*cab1*^{I291T} (C) carrying pFL38*CAB1* or pESC strains carrying human isoforms or empty vector were spotted on the solid media described above and plates were incubated at 28°C or 37°C. Results scored after 48 or 72 hours. Analysis in liquid media SC-URA-TRP with glucose or galactose (D-F). 1×10^5 cells/ml for *cab1Δ*/pFL39*cab1*^{G351S} (D), *cab1Δ*/pFL39*cab1*^{N290I} (E) and *cab1Δ*/pFL39*cab1*^{I291T} (F) carrying pFL38*CAB1* or pESC with human isoforms or empty vector were inoculated in liquid media and growth rates were monitored at precise time points for 96 hours [144].

For all the genetic background, strain carrying the empty vector showed the same phenotype already observed [86], that is a thermosensitive phenotype, thus showing a reduced growth on glucose and galactose at 37°C with an effect more emphasized for the strains harboring G531S and N290I mutations; also on galactose at 28°C the strains carrying G351S and N290I mutations exhibited a reduced growth (Figure 7.2 **A** and **B**); the strain carrying I291T exhibited instead a mild thermosensitive phenotype on glucose that is more visible when it is grown on galactose (Figure 7.2 **C**). Regarding human isoforms, all of them were able to increase the strains growth when they were expressed on galactose in all the genetic backgrounds, although at different extent, with optimal complementation level reached by PanK3. PanK2 isoform was capable to only partially complement the defective phenotype induced by hypomorphic mutant alleles (Figure 7.2 **A-C**).

To better quantify the complementation degree of human isoforms, the same strains were grown in liquid media and monitored for 96 hours. The results were in agreement with what observed on solid media: in fact, all the strains carrying the hPankKs had a more elevated speed growth than the ones carrying the empty vector and confirmed the different extent of complementation seen in the previous analysis (Figure 7.2 **D-F**).

7.3. Complementation analysis: Oxygen consumption rate (OCR)

Since it is known that the expression of *cab1*^{G351S}, *cab1*^{N290I} and *cab1*^{I291T} mutated alleles lead to a decreased respiratory activity [86], the ability of human PanKs to rescue the mitochondrial dysfunction phenotype of the *cab1* mutants was also investigated. As showed in Figure 7.3, hPankKs are able to significantly increase the respiration activity in all the genetic backgrounds, particularly in those strains harboring PanK1 and PanK3 isoforms that reached respiration activity levels similar to those showed by the strains carrying the wild type allele. PanK2 isoform succeeded in improving the respiratory rate less than the other isoforms but still in a significant manner (Figure 7.3)

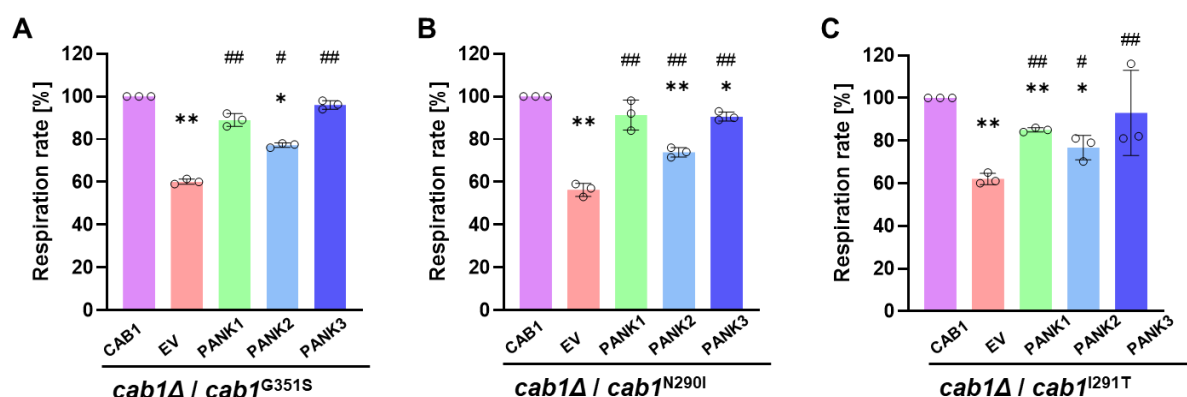


Figure 7.3 OCR analysis. Oxygen consumption rate measured in *cab1Δ*/pFL39*cab1*^{G351S} (**A**), *cab1Δ*/pFL39*cab1*^{N290I} (**B**) and *cab1Δ*/pFL39*cab1*^{I291T} (**C**) mutated strains carrying pFL38CAB1 or pESC with human isoforms or empty vector grown at 28°C or 37°C in SC-URA-TRP liquid media with galactose 2%. OCR was normalized to the corresponding mutant strain transformed with the CAB1 wild type allele. The statistical analysis applied is ONE-WAY ANOVA with Bonferroni

correction (*: $p < 0.05$, **: $p < 0.01$ relative to mutant strain transformed with *CAB1* allele and #: $p < 0.05$, ##: $p < 0.01$ relative to mutant strain transformed with the empty vector) [144].

7.4. Complementation analysis: iron sensitivity

One of the feature characterizing PKAN disease is iron dyshomeostasis [86,138,145,146] and, as demonstrated, G351S and N290I mutations caused iron sensitivity and accumulation in yeast strains [86]. For investigating on human PanKs capability to decrease iron sensitivity of mutant strains, growth assay on solid and liquid media with galactose and lacking or supplemented with 7 mM FeSO_4 was performed (Figure 7.4).

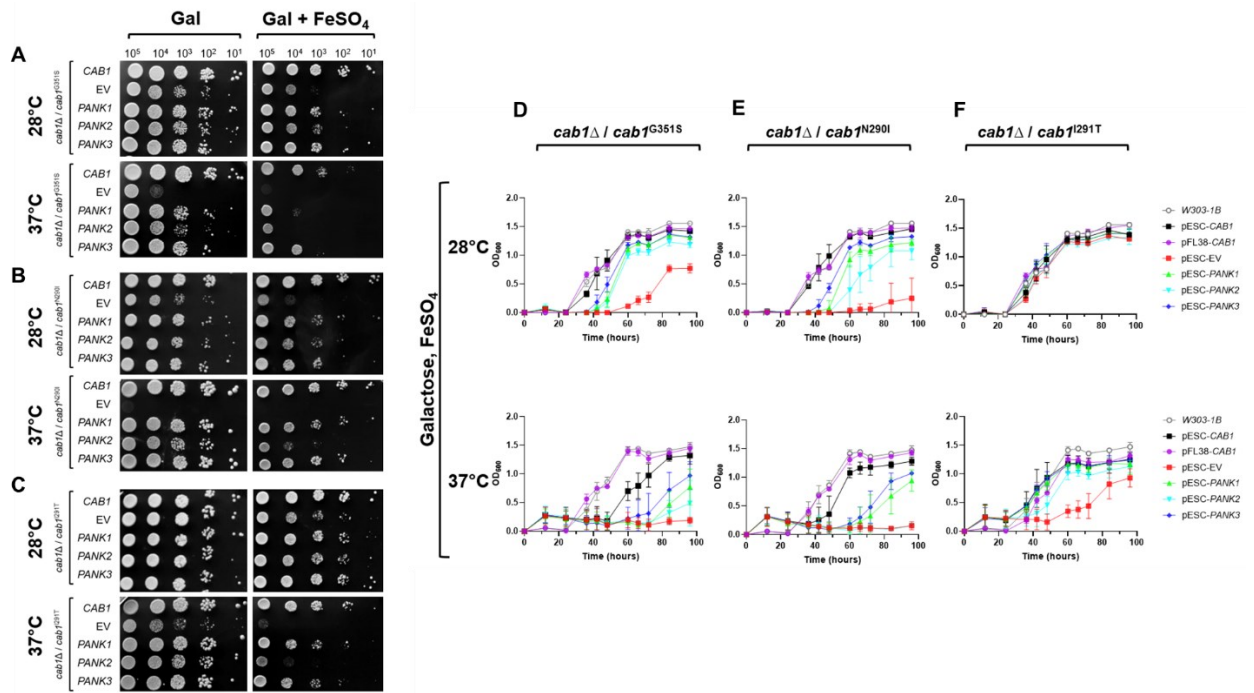


Figure 7.4 Solid and liquid growth assay with iron supplementation. Analysis on solid SC-URA-TRP with galactose lacking or supplemented with 7 mM FeSO_4 (A-C). Same amount of cells of *cab1Δ*/pFL39*cab1*^{G351S} (A), *cab1Δ*/pFL39*cab1*^{N290I} (B) and *cab1Δ*/pFL39*cab1*^{I291T} (C) carrying pFL38CAB1 or pESC strains carrying human isoforms or empty vector were spotted on the solid media described above and plates were incubated at 28°C or 37°C. Results scored after 48 or 72 hours. Analysis in liquid media SC-URA-TRP with galactose supplemented with 7 mM FeSO_4 (D-F). 1×10^5 cells/ml for *cab1Δ*/pFL39*cab1*^{G351S} (D), *cab1Δ*/pFL39*cab1*^{N290I} (E) and *cab1Δ*/pFL39*cab1*^{I291T} (F) carrying pFL38CAB1 or pESC with human isoforms or empty vector were inoculated in liquid media and growth rates were monitored at precise time points for 96 hours [144].

The strains carrying the three mutated alleles showed a decreased growth on solid galactose-based media when supplemented with 7 mM FeSO_4 than the same strains grown on galactose without supplementation. hPanK isoforms were able to rescue this defective phenotype, achieving a full complementation with PanK1 and PanK3 and a partial complementation with PanK2 (Figure 7.4 A-C). The liquid assay carried out in liquid media with galactose supplemented with FeSO_4 revealed

results that parallelized with the on-solid findings: in this analysis also, all the strains carrying hPankS exhibited a higher growth speed than the corresponding mutated strain (Figure 7.4 D-F).

7.5. Complementation analysis: Reactive Oxygen Species (ROS) measurement

In PKAN disease iron accumulation is often coupled with alterations in the oxidative status [124–126] and, in agreement with this feature, the strains harboring *cab1*^{G351S} and *cab1*^{N290I} showed 3.5 times increased Reactive Oxygen Species (ROS) than the strain carrying wild type allele [86]. For this reason, was tested if hPankS could potentially restore the normal ROS levels or, at least, lead to more reduced ROS levels than the mutated strains (Figure 7.5).

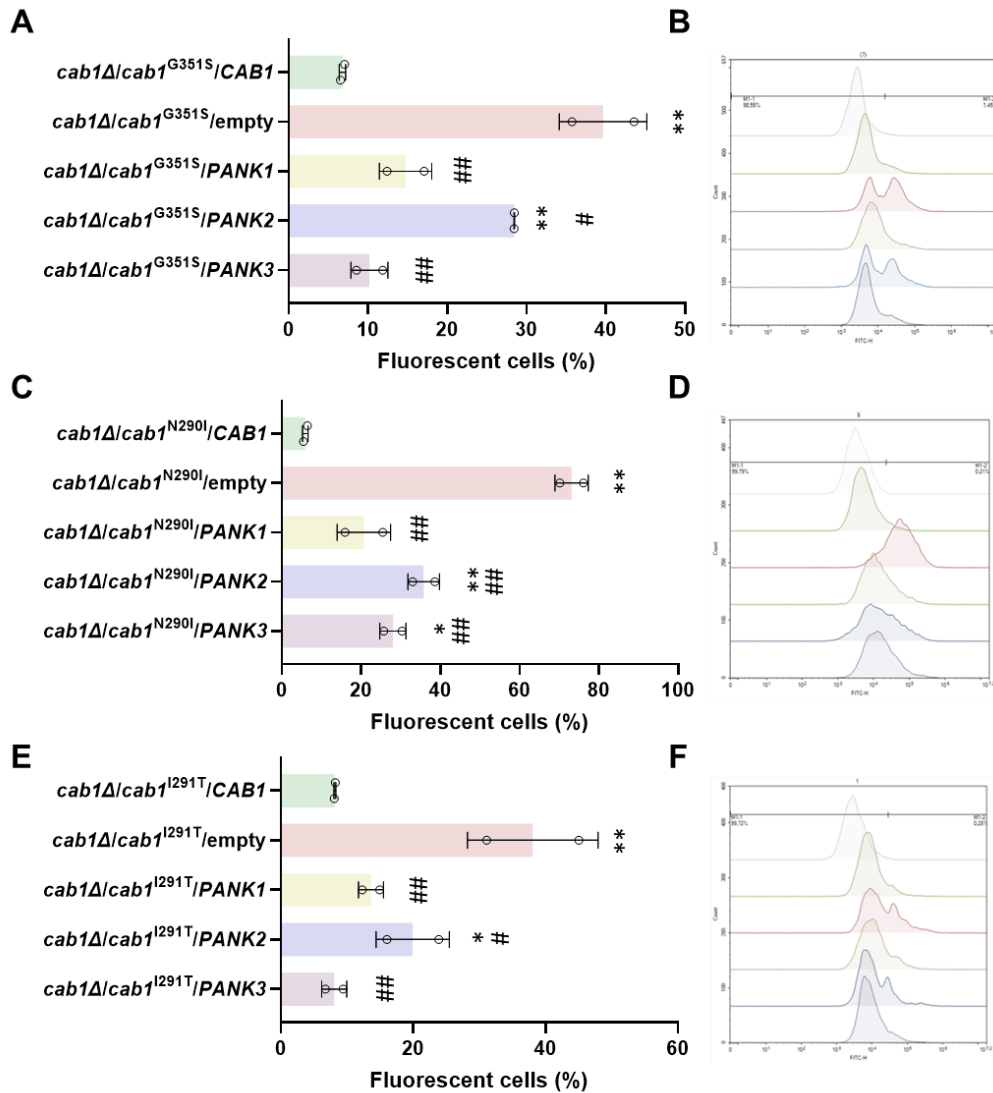


Figure 7.5 ROS analysis. *cab1Δ/pFL39cab1*^{G351S} (A), *cab1Δ/pFL39cab1*^{N290I} (C) and *cab1Δ/pFL39cab1*^{I291T} (E) mutated strains carrying pFL38*CAB1* or pESC with human isoforms or empty vector were grown in YP media with galactose at 28°C. ROS production was calculated as percentage of fluorescent cells using dihydrorodhamine 123 (DHR123) probe. The statistical analysis applied is ONE-WAY ANOVA with Bonferroni correction (*: p<0.05, **: p<0.01 relative to mutant strain transformed with *CAB1* allele and #: p<0.05, ##: p<0.01 relative to mutant strain transformed with the empty vector). Cytofluorometry analysis of the *cab1Δ/cab1*^{G351S} (B), *cab1Δ/cab1*^{N290I} (D), and *cab1Δ/cab1*^{I291T} (F) yeast strains

transformed with either pFL38/*CAB1*, empty vector or empty vector containing different human PanK isoforms following staining with DHR123 probe. Each curve represents the distribution of the measured events (counts) according to their fluorescence intensity expressed in a log unit. The grey spectrum represents background autofluorescence ([144] modified).

ROS measurement was performed by cytofluorimetric analysis using, as ROS indicator, the fluorescent molecule dihydrorhodamine 123 (DHR123). As expected, all the strains carrying the empty vector showed the highest ROS levels, confirming the negative effect of mutations on the oxidative status of yeast cells, while the strains carrying the wild type allele showed the lowest ROS levels. All hPanKs in all mutant backgrounds were able to decrease ROS content at different extent, with a more improved oxidative status reached by mutated strains carrying *PANK1* and *PANK3* alleles and a partial restoration of the normal ROS content achieved by strains harboring *PANK2* allele, confirming the ability of human isoforms to rescue ROS defective phenotype also (Figure 7.5).

7.6. Complementation analysis: correlation between PanKs steady-state and PanKs activity

To evaluate if the degrees of complementation observed in the previous analyses were linked to the expression level of each hPanK isoform, Western Blot was performed thanks to the presence of c-Myc tag fused with *PANK* genes; thus, an anti-Myc monoclonal antibody was used to bind the protein of interest.

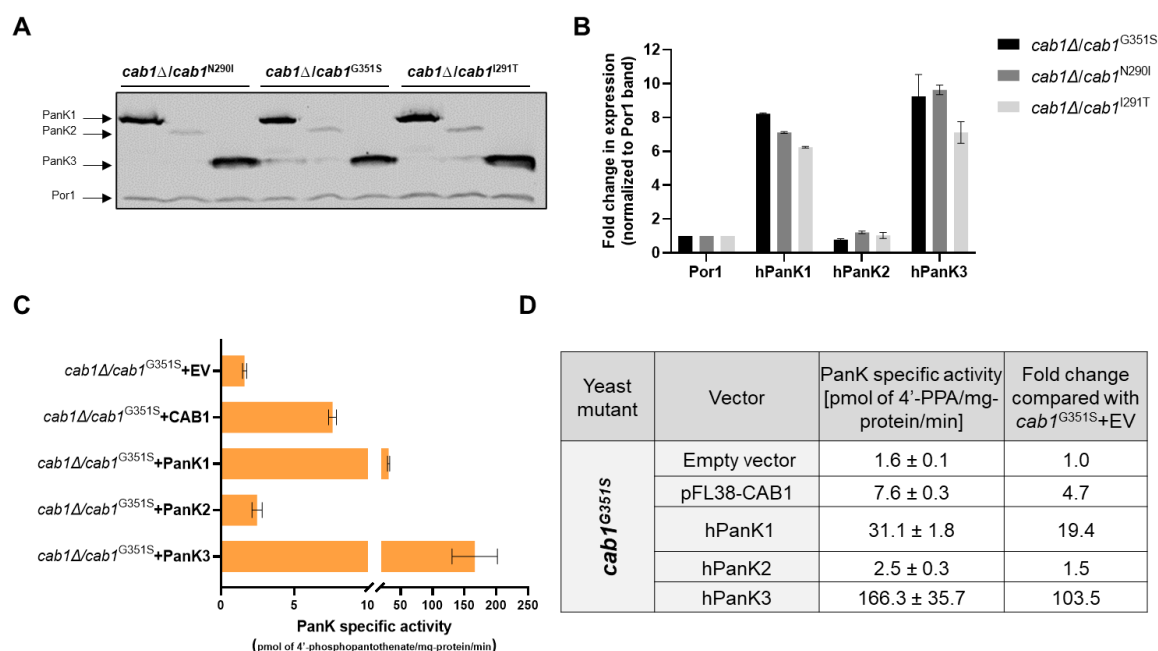


Figure 7.6 hPanKs steady-state level and activity evaluation. Western Blot conducted on *cab1Δ/cab1* mutant strains transformed with *PANK1*, *PANK2* and *PANK3* grown on SC-URA-TRP liquid media with galactose: membrane shot (**A**) and bar graph with quantified bands intensities detected with Image Lab software and normalized with Por1 signal (**B**). Pantothenate kinase specific activity was measured from fresh cell extracts obtained from *cab1Δ/cab1*^{G351S} yeast cells

harboring the empty vector or *CAB1* allele or the hPANKs alleles grown in SC-URA-TRP media with 2% galactose at 30°C.

The activity assay measures the phosphorylation of D-[1-¹⁴C] pantothenate (**C, D**) [144].

As shown in Figure 7.6 **A** and **B**, hPanK1 and hPanK3 are strongly expressed in all genetic backgrounds, while hPanK2 is weakly expressed; this result could possibly explain the differences in complementation ability.

Consistently, endogenous PanK activity measured in *cab1Δ/cab1^{G351S}* carrying *CAB1* allele or hPANKs alleles or empty vector using D-[1-¹⁴C] pantothenic acid showed that the strain carrying the empty vector had the lowest PanK activity, while the expression of PanK1 and PanK3 led to a ~20 times and ~100 times increased specific activity compared to the strain expressing Cab1. The expression of PanK2 revealed instead only a moderate improving of the specific activity, ~1.5 times increased. This result could also relate with the different extent of complementation associated with hPanK isoforms (Figure 7.6 **C, D**).

These results are available in the paper, attached at the end of this manuscript, entitled “Evidence for a Conserved Function of Eukaryotic Pantothenate Kinases in the Regulation of Mitochondrial Homeostasis and Oxidative Stress” [144].

8. Discussion

The results obtained in this research provided the first proof of the functional conservation of pantothenate kinase function between human and yeast, demonstrating that human *PANK1*, *PANK2* and *PANK3* (that show pantothenate kinase activity) are able to rescue cellular growth and mitochondrial defect induced by mutated alleles *cab1*^{G351S}, *cab1*^{N290I} and *cab1*^{I291T}. These mutations were found in PKAN patient in *PANK2* gene and were introduced into the only yeast gene encoding for PanK activity, *CAB1*. Regarding *PANK2*, encoding the mitochondrial isoform of PanK a first attempt to complement the null mutant strain *cab1*, or mutant strains expressing G351S and N290I mutations with the full-length gene, comprehensive of its MTS, failed. The reasons for this lack of complementation by the full-length *PANK2* could be ascribed to a different localization of human PanK2 and yeast Cab1 protein: it was already known that PanK2 is the only pantothenate kinase localized in mitochondria, while it was recently demonstrated that Cab1 localizes in cytoplasm [86]. For this reason, a *PANK2* gene lacking MTS sequence was used in this study. Finding a reliable model as yeast to express *PANK2* mutations, associated with PKAN disease, is important for expressing and analyzing individual mutant alleles, since the molecular mechanism implied in PKAN is poorly understood. Moreover, yeast mutants used in this study previously proved to recapitulate many PKAN features, making yeast a good model for deeper understanding this pathology [86].

Data provided in this work demonstrated that a good level of complementation was achieved by *PANK1* and *PANK3* and only partial by *PANK2*. These differences in complementation could be due to dissimilarities in the protein expression: in fact, immunoblot analysis showed a dramatically lower PanK2 expression than PanK1 and PanK3. One hypothesis for the lower expression of PanK2 is the possibility that its overexpression in yeast cells is detrimental through the modification of unknown metabolites, thus suggesting that this enzyme could play a role in other pathways (not yet discovered). This hypothesis could be also supported by the complex molecular and metabolic profile showed by PKAN disease.

Besides the ability to improve the cellular growth of the mutant strains, all human isoforms demonstrated to be also able to rescue the PKAN phenotypes analyzed, although at different extent: they were able to improve the respiration activity and to decrease iron sensitivity and reactive oxygen species (ROS) level [144].

Since there is not an effective cure for PKAN disease, different therapeutical approaches have been used to treat symptoms, like the activation of the cytosolic PanKs (PanK3 in particular) to compensate the alteration of PanK2 activity [147] or supplementation with intermediates of the CoA pathway [141] or the use of iron chelators [148]; but no one of these approaches gave efficacy results, thus there is the need to understand the molecular mechanism of the pathology and yeast could contribute to the discovery of an effective treatment for this disorder.

In conclusion, this study demonstrated the conservation of pantothenate kinase function between human and yeast, thus confirming the conservation of these enzymes through the evolution. This finding gives the chance to use yeast as model to probe the effect of PanK2 mutations on cellular metabolism and mitochondrial activity and to evaluate new therapeutical strategies that could rescue the altered phenotype due to the above-mentioned mutations.

9. Materials and Methods

9.1. Strains used

All *S. cerevisiae* strains used in this work are listed below:

STRAIN	GENOTYPE	REFERENCE
W303-1B	<i>Mata, ade2-1, leu2-3, ura3-1, trp1-1, his3-11, can1-100</i>	[149]
BY4741 <i>msd1Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msd1::kanMX4</i>	Euroscarf collection; [150]
BY4741 <i>slm5Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 slm5::kanMX4</i>	Euroscarf collection; [150]
BY4741 <i>msr1Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msr1::kanMX4</i>	Euroscarf collection; [150]
W303-1B <i>msd1Δ</i> pFL39MSD1-HA	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Arg42Gly}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Lys42Arg}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Leu232Val}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Phe487Leu}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Ala244Val}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Arg157Cys}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Ser617Gly}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Ile484Val}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Val484Met}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Met608Leu}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39msd1-HA ^{Leu608Val}	<i>Mata, ade2-1, leu2-3, ura3-1, his3-11,</i>	This work

	<i>msd1::kanMX4</i>	
W303-1B <i>msd1Δ</i> pFL39 <i>msd1</i> -HA ^{Phe136Leu}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39 <i>msd1</i> -HA ^{Leu136Ile}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>msd1Δ</i> pFL39 <i>msd1</i> -HA ^{Lys606Met}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msd1::kanMX4</i>	This work
W303-1B <i>slm5Δ</i> pFL39 <i>SLM5</i> -HA	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, slm5::kanMX4</i>	This work
W303-1B <i>slm5Δ</i> pFL39 <i>slm5</i> -HA ^{Phe261Ser}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, slm5::kanMX4</i>	This work
W303-1B <i>slm5Δ</i> pFL39 <i>slm5</i> -HA ^{Leu461Met}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, slm5::kanMX4</i>	This work
W303-1B <i>slm5Δ</i> pFL39 <i>slm5</i> -HA ^{Met462Val}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, slm5::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>MSR1</i>	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>msr1</i> ^{His255Asp}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>msr1</i> ^{Arg306Gln}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>msr1</i> ^{Arg531His}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>msr1</i> ^{Asn505Ser}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>msr1</i> ^{Ser505Pro}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>msr1</i> ^{Thr580Asp}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msr1Δ</i> pFL39 <i>msr1</i> ^{Asp580Gly}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msr1::kanMX4</i>	This work
W303-1B <i>msw1Δ</i> pFL39 <i>MSW1</i>	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msw1::kanMX4</i>	This work
W303-1B <i>msw1Δ</i> pFL39 <i>msw1</i> -HA ^{Trp15Gly}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11,</i>	This work

	<i>msw1::kanMX4</i>	
W303-1B <i>msw1Δ</i> pFL39 <i>msw1</i> -HA ^{Gly54Glu}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msw1::kanMX4</i>	This work
W303-1B <i>msw1Δ</i> pFL39 <i>msw1</i> -HA ^{Met245Val}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msw1::kanMX4</i>	This work
W303-1B <i>ala1Δ</i> pFL39 <i>ALA1</i>	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, ala1::kanMX4</i>	[70]
W303-1B <i>ala1Δ</i> pFL39 <i>ala1</i> ^{Val662Ala}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, ala1::kanMX4</i>	This work
W303-1B <i>vas1Δ</i> pFL36 <i>vas1</i> ^{Met1Ala} pFL39 <i>VAS1</i>	<i>Mataα, ade2-1, ura3-1, his3-11, vas1::kanMX4</i>	[30]
W303-1B <i>vas1Δ</i> pFL36 <i>vas1</i> ^{Met1Ala} pFL39 <i>vas1</i> ^{Asp397Asn}	<i>Mataα, ade2-1, ura3-1, his3-11, vas1::kanMX4</i>	This work
W303-1B <i>msk1Δ</i> /pFL39-TEToff <i>KARS1</i> ^{Arg205Cys}	<i>Mataα, ade2-1, leu2-3, ura3-1, his3-11, msk1::kanMX4</i>	[75]
W303-1B <i>cab1Δ</i> pFL39 <i>cab1</i> ^{Gly351Ser} pFL38 <i>CAB1</i>	<i>Mataα, ade2-1, leu2-3, his3-11, cab1::kanMX4</i>	[86]
W303-1B <i>cab1Δ</i> pFL39 <i>cab1</i> ^{Asn290Ile} pFL38 <i>CAB1</i>	<i>Mataα, ade2-1, leu2-3, his3-11, cab1::kanMX4</i>	[86]
W303-1B <i>cab1Δ</i> pFL39 <i>cab1</i> ^{Ile291Thr} pFL38 <i>CAB1</i>	<i>Mataα, ade2-1, leu2-3, his3-11, cab1::kanMX4</i>	[86]
W303-1B <i>cab1Δ</i> pFL39 <i>cab1</i> ^{Gly351Ser} p <i>ESCPANK1-2-3</i>	<i>Mataα, ade2-1, leu2-3, his3-11, cab1::kanMX4</i>	This work
W303-1B <i>cab1Δ</i> pFL39 <i>cab1</i> ^{Asn290Ile} p <i>ESCPANK1-2-3</i>	<i>Mataα, ade2-1, leu2-3, his3-11, cab1::kanMX4</i>	This work
W303-1B <i>cab1Δ</i> pFL39 <i>cab1</i> ^{Ile291Thr} p <i>ESCPANK1-2-3</i>	<i>Mataα, ade2-1, leu2-3, his3-11, cab1::kanMX4</i>	This work

The bacteria strain used in this work is listed below:

STRAIN	GENOTYPE
DH10B	<i>F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80d DlacZ ΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galKλ-rpsL hupG</i>

9.2. Media and growth conditions

For yeast the following media were used:

- YP (1% peptone, 0.5% yeast extract)
- YPA (2% peptone, 1% yeast extract, 75mg/ml adenine)
- YNB (YNB ForMedium™ w/o aminoacids w/o NH₄SO₄ 1,9 g/L, NH₄SO₄ 5 g/L). Minimum media was enriched with drop-out powder (Kaiser et al. 1994).
- 5-FOA YNB: YNB ForMedium™ with 1 g/l 5-Fluoroorotic Acid (Melford), 50 mg/l uracile with aminoacids necessary to complement the auxotrophies (Boeke et al., 1984).

If necessary, single amino acids could be excluded from complete drop-out to maintain selective pressure. Agar ForMedium™ 2% was added as solidifying agent. Carbon sources were added at final concentration of 2% if not specified differently. The following sources were used: Glucose (D), Ethanol (E), Glycerol (G) and Galactose (Gal). In liquid media, *S. cerevisiae* was cultured at 28°C, in constant shaking 120 rpm.

For *E. coli* LB media was used (1% bacto tryptone Difco™, 0.5% yeast extract Difco™, 0.5% NaCl, pH 7.2-7.5). Agar 2% and ampicillin (Sigma-Aldrich®) 100mg/ml were added if needed. For α-complementation selection 80μl of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Xgal) 2% (dissolved in dimethylformamide) and 40μl isopropyl-beta-D-thiogalactopyranoside (IPTG) 23.8 mg/ml were added. Cultures were incubated at 37°C in constant shaking if necessary.

9.3. Vectors

PLASMID	MARKER	TYPE	REFERENCE	FIGURE
pFL39	<i>TRP1</i>	CENTROMERIC	[151]	9.1 A
pFL38	<i>URA3</i>	CENTROMERIC	[151]	9.1 B
pFL36	<i>LEU2</i>	CENTROMERIC	[151]	9.1 C
pFL39TEToff	<i>TRP1</i>	CENTROMERIC	[152]	No maps
pESC	<i>URA3</i>	MULTICOPY	GenScript Inc., Piscataway, NJ, United States	9.1 D

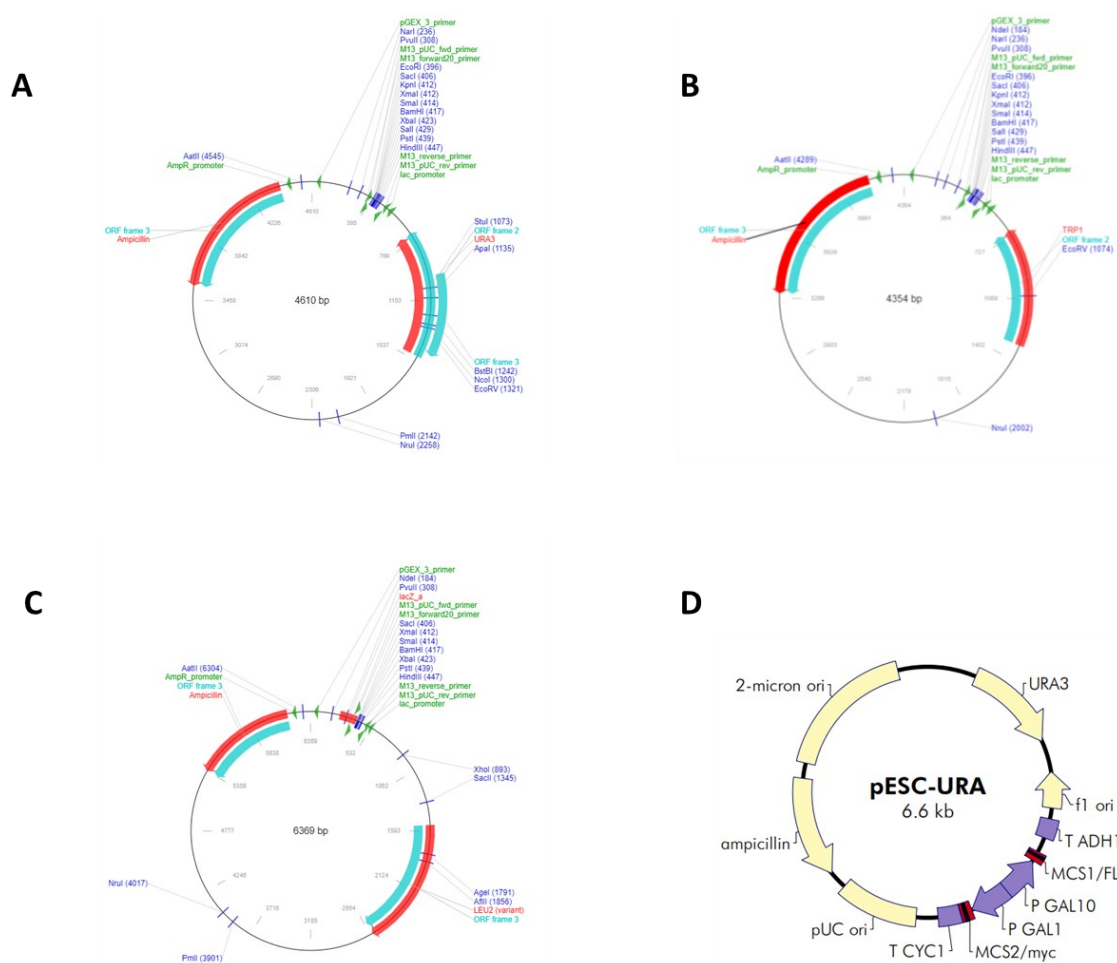


Figure 9.1 Vectors representation.

9.4. Polymerase Chain Reaction (PCR)

Manufacturer indications were followed to perform PCR reactions. Two types of DNA polymerase were utilized, GoTaq® DNA polymerase (Promega) for analytical purposes, and KOD Hot Start HiFi DNA polymerase (Novagen®) for preparative PCR, a more accurate enzyme used to introduce site-specific mutations or for the gene amplification in the cloning process. All the reactions were performed using “Applied Biosystem 2720 Thermal Cycler”.

9.4.1. Overlap PCR

This kind of PCR protocol is divided into two parts and includes three different PCR reaction. The first part of the protocol consists in amplifying two contiguous portions of the gene of interest, with an overlap zone, in which the mutation to introduce lies. The subsequent reaction leads to the formation of the complete fragment bearing the mutation of interest. In fact, a mixture of the amplicons produced during the first two reactions was used as a template. The first 10 reaction cycles were performed without primers, to allow the 3'OH ends recognition by

polymerase, synthesizing the missing part of the gene. At the end of the 10th cycle, the external primers to each reaction to amplify the fragment carrying the mutation. The amplification conditions of the extension phase (10 cycles without primers) and of the subsequent elongation phase (after adding the external primers) were reported below.

Extension phase

- 2' at 95°C
- 10 cycles:
- 20'' at 95°C
- 30'' at 60°C
- 1'10''/Kb at 70°C

Elongation phase

25 cycles:

- 20'' at 95°C
- 18'' at 54°C
- 1'10''/Kb at 70°C
- 3' at 70°C
- ∞ at 10°C

9.4.2. Quick-Change PCR

DNA used as a template normally was a vector containing the gene of interest, isolated from a methylating microorganism (dam+); the two primers utilized in this reaction are oligonucleotides, containing the substitution to introduce in the wild type gene, complementary to the vector region: these primers, once annealed to the template, were extended by KOD DNA polymerase, until the amplification of the entire plasmid was done. Annealing and extension of the mutagenic primers lead to the obtaining of a large amount of mutant plasmids. The PCR reaction details are reported below:

- 2' at 95°C
- 18 cycles:
 - 30'' at 95°C
 - 50'' at annealing temperature
 - 45''/Kb at 68°C
- 5' at 68°C
- ∞ at 10°C

At the end of the reaction, three types of plasmids were present:

- Parental plasmids: containing both parental strands (without mutation)
- Mixed plasmids: consisting of a newly synthesized strand (containing the mutation) and a wild type parental strand
- Mutated plasmids: containing the mutation on both strands.

In order to obtain only the latter, it was necessary to carry out digestion using the enzyme *DpnI*. This enzyme recognized the 5'-Gm6ATC-3' sequence and was able to digest methylated

and hemi-methylated DNA. Since the template of the reaction was derived from a methylating *E. coli* (dam+) and the amplification reaction was carried out *in vitro*, without enzymes able to methylate the DNA, after the *DpnI* treatment only the mutated plasmid should be obtained. After digestion, the mix containing the plasmid of interest was precipitated overnight through sodium acetate and ethanol at -20 °C. Subsequently the mix, after wash steps with ethanol, was utilized to transform *E. coli*, which allows amplifying the mutant plasmid of interest.

9.4.3. Primers

Name	Sequence 5'->3'
Fw <i>MSD1</i> SacI	5' GGGCCGTCGACCAATCTTGTGCAAGCTGTGGG 3'
Rv <i>MSD1</i> Sall	5' GGGGGAGCTCGGAGATTGTACTCACTGAAC 3'
Fw <i>msd1</i> ^{Arg157Cys}	5' CCTGAGTTCGCTATTTGCAATTGTGTAATCCCAAATATCAAG 3'
Rv <i>msd1</i> ^{Arg157Cys}	5'CTTGATATTTGGGATTACACAATTGCAAATAGCGGAACCTCAGG 3'
Fw <i>msd1</i> ^{Leu232Val}	5' CCTCAACAATACAAGCAACTCGTTATGGCTAGTGGTGTCAAC 3'
Rv <i>msd1</i> ^{Leu232Val}	5' GTTGACACCACTAGCCATAACGAGTTGCTTGTATTGTTGAGG 3'
Fw <i>msd1</i> ^{Leu608Val}	5' GCTATTGGTTTTGATCGTGTGCGCTATGATCTGTGAAAC 3'
Rv <i>msd1</i> ^{Leu608Val}	5'GTTTCACAGATCATAGCGCAAACACGATCAAAACCAATAGC 3'
Fw <i>msd1</i> ^{Ser617Gly}	5' GCTATGATCTGTGAAACTGAGGGTATAAGGGACGTAATCGCCTTCC 3'
Rv <i>msd1</i> ^{Ser617Gly}	5' GGAAGGCGATTACGTCCCTTATACCCTCAGTTTCACAGATCATAGC 3'
Fw <i>msd1</i> ^{Lys42Arg}	5' CCTCGACCATCAAACAGTTAAGAGGACTGTCTAGCGGCCAG 3'
Rv <i>msd1</i> ^{Lys42Arg}	5' CTGGCCGCTAGACAGTCCTCTTAAGTGTGTTGATGGTCGAGG 3'
Fw <i>msd1</i> ^{Arg42Gly}	5' CCTCGACCATCAAACAGTTAGGTGGACTGTCTAGCGGCCAG 3'
Rv <i>msd1</i> ^{Arg42Gly}	5' CTGGCCGCTAGACAGTCCACCTAACTGTTTGATGGTCGAGG 3'
Fw <i>msd1</i> ^{Lys107Arg}	5' GCCGTAGGTATACTCTCTTTGAGAAGGAAGCTATCAAATGAGG 3'
Rv <i>msd1</i> ^{Lys107Arg}	5' CCTCATTTGATAGCTTCCTCTCAAAGAGAGTATACCTACGGC 3'
Fw <i>msd1</i> ^{Arg107His}	5' GCCGTAGGTATACTCTCTTTGCATAGGAAGCTATCAAATGAGG 3'
Rv <i>msd1</i> ^{Arg107His}	5' CCTCATTTGATAGCTTCCTATGCAAAGAGAGTATACCTACGGC 3'
Fw <i>msd1</i> ^{Ala136Phe}	5' GCATCTAATAAAAAACCATTTCAAATGCAGGATTTTAAATTG 3'
Rv <i>msd1</i> ^{Ala136Phe}	5' CAATTTAAAATCCTGCATTTGAAATGGTTTTTTATTAGATGC 3'
Fw <i>msd1</i> ^{Phe136Ile}	5' GCATCTAATAAAAAACCAATTCAAATGCAGGATTTTAAATTG 3'
Rv <i>msd1</i> ^{Phe136Ile}	5' CAATTTAAAATCCTGCATTTGAATTGGTTTTTTATTAGATGC 3'
Fw <i>msd1</i> ^{Gln162Lys}	5' GCAATTGAGAAATCCCAAATATAAAGATTTTTTAAAGAAGAGG 3'
Rv <i>msd1</i> ^{Gln162Lys}	5' CCTCTTCTTTAAAAAATCTTTATATTTGGGATTCTCAATTGC 3'
Fw <i>msd1</i> ^{Ala244Val}	5' CAACAAATATTATCAAATGGTTAGGTGCTTTAGAGATGAAG 3'
Rv <i>msd1</i> ^{Ala244Val}	5' CTTCTCTCTAAAGCACCTAACCATTTGATAATATTTGTTG 3'
Fw <i>msd1</i> ^{Ile484Val}	5' GGATGTTGCCTCATGGGTTGTGGATTTCCTGTTATTTCTCC 3'
Rv <i>msd1</i> ^{Ile484Val}	5' GGAGAAAATAACGGGAAATCCACAACCCATGAGGCAACATCC 3'
Fw <i>msd1</i> ^{Val484Met}	5' GGATGTTGCCTCATGGATGGTGGATTTCCTGTTATTTCTCC 3'
Rv <i>msd1</i> ^{Val484Met}	5' GGAGAAAATAACGGGAAATCCACCATCCATGAGGCAACATCC 3'
Fw <i>msd1</i> ^{Phe487Leu}	5' GTTGCTCATGGATTGTGGATTGCGGTTATTTCTCCCG 3'
Rv <i>msd1</i> ^{Phe487Leu}	5' CGGGAGAAAATAACGGCAAATCCACAATCCATGAGGCAAC 3'
Fw <i>msd1</i> ^{Met608Leu}	5' GCTATTGGTTTTGATCGTCTATGCGCTATGATCTGTGAAAC 3'
Rv <i>msd1</i> ^{Met608Leu}	5' GTTTCACAGATCATAGCGCATAGACGATCAAAACCAATAGC 3'
Fw <i>msd1</i> ^{Lys626Met}	5' GGGACGTAATCGCCTTCCCAATGAGTATTACGGGGGCTG 3'

Rv <i>msd1</i> ^{Lys626Met}	5' CAGCCCCGGTAATACTCATTGGGAAGGCGATTACGTCCC 3'
Fw <i>SLM5BamHI</i>	5' GCGCGGATCCCTTACAGAGATTAGGCCGTC 3'
Rv <i>SLM5SalI</i>	5' GCGCGTCGACCCGAGATTTCCAATGAGCGG 3'
Fw <i>SLM5HA</i>	5' <u>GAAACAAATGGAATATCAAGCGTAATCTGGAACATCGTATGGGTAAAAATCGATGGATTCTGCAG3'</u>
Rv <i>SLM5HA</i>	5' <u>GAATCCATCGATTTTTACCCATACGATGTTCCAGATTACGCTTGATATTCCATTTGTTTCTTATC 3'</u>
Fw <i>slm5</i> ^{Leu461Met}	5' CCACACGGAGGCTTTGGTATGGGGTTTGAGAGATTTATCTC 3'
Rv <i>slm5</i> ^{Leu461Met}	5' GAGATAAATCTCTCAAACCCCATACCAAAGCCTCCGTGTGG 3'
Fw <i>slm5</i> ^{Leu461Val}	5' CCACACGGAGGCTTTGGTGTGGGGTTTGAGAGATTTATCTC 3'
Rv <i>slm5</i> ^{Leu461Val}	5' GAGATAAATCTCTCAAACCCAAACACCAAAGCCTCCGTGTGG 3'
Fw <i>slm5</i> ^{Phe261Ser}	5' GATGCTTGAAGTGAAATGTGCTCGGTAAATAGCGTCAAC 3'
Rv <i>slm5</i> ^{Phe261Ser}	5' GTTGACGCTATTAACCGAGCACATTTCCACTTCAAGCATC 3'
Fw <i>MSR1SacI</i>	5' GGGGGAGCTCGAGAGGTACTGACTACAACGC 3'
Rv <i>MSR1BamHI</i>	5' GCGCGGATCCCTAACGGTAGGTGGGTTATTC 3'
Fw <i>MSR1HA</i>	5' <u>CCGGTTGATAGAATGTACCCATACGATGTTCCAGATTACGCTTAATTATATGAATAAGCTAGC 3'</u>
Rv <i>MSR1HA</i>	5' <u>CTTATTCATATAATTAAGCGTAATCTGGAACATCGTATGGGTACATTCTATCAACCGGGAC 3'</u>
Fw <i>msr1</i> ^{His255Asp}	5' CGTTACAAAAGCAGCCAATTCAGGACTTATTCGACGTCTACG 3'
Rv <i>msr1</i> ^{His255Asp}	5' CGTAGACGTCGAATAAGTCCTGAATTGGCTGCTTTTGTAAACG 3'
Fw <i>msr1</i> ^{Arg306Gln}	5' GCCATTAAGATATGGAATAGGTTTCAGTCGTTATCCATCCACCAC 3'
Rv <i>msr1</i> ^{Arg306Gln}	5' GTGGTGATGGATAACGACTGAAACCTATTCCATATCTTAATGGC 3'
Fw <i>msr1</i> ^{Asn505Ser}	5' GATATGAAATCAAAGCGGATATCTAACTATGAATTCAACTGGAAC 3'
Rv <i>msr1</i> ^{Asn505Ser}	5' GTTCCAGTTGAATTCATAGTTAGATATCCGCTTTGATTTTATATC 3'
Fw <i>msr1</i> ^{Asn505Pro}	5' GATATGAAATCAAAGCGTATCCCGAAGTATGAATTCAACTGGAAC 3'
Rv <i>msr1</i> ^{Asn505Pro}	5' GTTCCAGTTGAATTCATAGTTCCGGATACGCTTTGATTTTATATC 3'
Fw <i>msr1</i> ^{Arg531His}	5' CCCTATTTACAATACACACATTCCCACTTAGATCTTTAGAAAAGG 3'
Rv <i>msr1</i> ^{Arg531His}	5' CCTTTCTAAAGATCTAAGGTGGGAATGTGTGTATTGTAAATAGGG 3'
Fw <i>msr1</i> ^{Thr580Asp}	5' GATGTCCTTAGAAGAGCATTCTGAAGATCAAGAGCCGGCCACG 3'
Rv <i>msr1</i> ^{Thr580Asp}	5' CGTGGCCGGCTCTTGATCTTGAATGCTCTTCTAAGGACATC 3'
Fw <i>msr1</i> ^{Thr580Gly}	5' GATGTCCTTAGAAGAGCATTCTGAAGGTCAAGAGCCGGCCACG 3'
Rv <i>msr1</i> ^{Thr580Gly}	5' CGTGGCCGGCTCTTGACCTTGAATGCTCTTCTAAGGACATC 3'
Fw <i>MSW1SalI</i>	5' GGGGGTTCGACGCACATCAGAGTCAGATTCC 3'
Rv <i>MSW1BamHI</i>	5' GGGGGGATCCGACCTGTTTTCGAGCCAGAC 3'
Fw <i>MSW1HA</i>	5' CATAAAATAATGGGCTTCTACCCATACGATGTTCCAGATTACGCTTGA CTGTCCCCGGCTTTAGGCC 3'
Rv <i>MSW1HA</i>	5' TAAAGCCGGGGACAGTCAAGCGTAATCTGGAACATCGTATGGGTAGA AGCCCATTATTTTATG 3'
Fw <i>msw1</i> ^{Trp15Gly}	5' GAAGTTAATCAGTAAAAGGTATAAGCACAGTGCAACGTGCCG 3'
Rv <i>msw1</i> ^{Trp15Gly}	5' CGGCACGTTGCACTGTGCTTATACCCCTTTTACTGATTAACCTC 3'
Fw <i>msw1</i> ^{Gly54Glu}	5' CCACCTTGTAATTATCTAGAAGCTACACGTGTTTGGACAG 3'
Rv <i>msw1</i> ^{Gly54Glu}	5' CTGTCCAAACACGTGTAGCTTCTAGATAATTACCAAGGTGG 3'
Fw <i>msw1</i> ^{Met245Val}	5' GCACGCCTGAAAAGAAAGTTTCCAAGAGTGATCCGAATCACG 3'
Rv <i>msw1</i> ^{Met245Val}	5' CGTGATTCGGATCACTCTTGAAACTTTCTTTTCAGGCGTGC 3'
Fw <i>vas1</i> ^{Asp397Asn}	5' GCCGTTGCAGTTCATCCTGATAATGACCGTTACAAACACTTGC 3'
Rv <i>vas1</i> ^{Asp397Asn}	5' GCAAGTGTTTGTAAACGGTCATTATCAGGATGAACTGCAACGGC 3'
Fw <i>ala1</i> ^{Val662Ala}	5' GCAAATTAAGAAAACCTACAAGCTTTTTACAAGGAAATTCC 3'

9.5. Sequencing

Sequencing of all genes was performed with external services (©Eurofins-MWG).

9.6. Nucleid acid manipulation

All the manipulations were carried out with standard techniques [153]. Genomic DNA from *S. cerevisiae* was extracted as previously described [154,155]. Plasmid DNA was extracted from *E. coli* with Wizard® Plus SV Minipreps, Wizard® Plus Minipreps (Promega©) or following standard procedures [153]. DNA recovery from agarose gel and purification of PCR products were carried out with GenElute™ PCR-Clean Up kit (Sigma-Aldrich®) commercial kit. Enzymatic manipulations (restriction, ligation, dephosphorylation) were carried out following manufacturer indications (New England Biolabs® Inc. NEB, Invitrogen™).

9.7. Transformation protocols

9.7.1. *S. cerevisiae* transformation

Yeast transformation was performed following the short protocol described in Daniel Gietz and Woods, 2002 [156]. To reach greater efficiency, the long LiAc/SS carrier DNA/PEG method was used. This protocol was exploited for gene disruption, regenerating the cells in two ml of YPAD for 2h, before plating them on medium added with 200 µg/ml G418-sulphate.

9.7.2. *E. coli* transformation

E. coli transformation was achieved with electroporation. Competent cells were prepared as previously described [157]. Transformation was carried out with 1-3 µl of plasmid DNA or ligation product. 2 mm cuvettes were used, applying respectively a current of 2KV, 25 Mf 200 Ω. Alternatively, CaCl₂ competent cells were prepared and transformed with standard techniques [153].

9.8. Analyses in whole cell

9.8.1. Spot assay

Spot assay is a classical comparative phenotypic analysis used to test the oxidative growth of different strains in different conditions. 10-fold serial dilutions were prepared starting from 1x10⁷ cells/ml culture, performed three times to the final 1x10⁴ cells/ml suspension. From each dilution, 5 µl were spotted on agar plates and then incubated for several days at 28°C, the yeast growth physiological temperature, or 37°C to induce a heat-stress.

9.8.2. Liquid growth assay

For yeast growth assays, precultures of *S. cerevisiae* cells were grown overnight at 30 °C in SC-URA-TRP media (2% glucose). Cells were diluted to OD of 0.2 in the fresh media, grown 4 h to mid-log phase, and then harvested, washed, and adjusted in sterile water to $OD_{600nm} = 1$. Yeast cells were inoculated in polypropylene tubes at a final volume of 3.5 mL. The initial cell density was 10^4 cells/mL in SC-URA-TRP media (2% glucose or galactose). A total of 6 mM $FeSO_4$ was added to the media when needed. Cells were incubated at 30 °C and 37 °C and optical density (OD_{600}) measurements were taken at specific time points using a spectrophotometer.

9.8.3. Oxygen consumption rate

To measure the respiratory activity, strains were pre-grown in a medium supplemented with 2% glucose. Cells were inoculated at the optimized concentration, depending on the strains type, in a medium supplemented with 0.5% glucose to induce oxidative metabolism after fermentation and grown for 18 h. The evaluation of oxygen consumption rate was performed on whole cells at 30°C using a Clark-type oxygen electrode (Oxygraph System Hansatech Instruments England) after the total exhaustion of glucose by adding in the oxigraph chamber 50 µl of concentrated cells, 850 µl of air-saturated respiration buffer (0,1M phthalate-KOH, pH 5,0) and 100 µl 5% glucose. Oxygen consumption rate was expressed as nmol O₂ consumed in one minute for mg of dry cells (nmolO₂/min mg).

9.8.4. Plasmid shuffling

The plasmid shuffling technique was exploited to construct the strains, to avoid irreversible loss of mitochondrial DNA. Plasmid shuffling was used to promote the loss of plasmids pFL38, containing the *URA3* selection marker. In fact, in the presence of the gene product of the *URA3* gene, 5-FOA was converted into a toxic product that do not permit growth, allowing to counter-select all the cells that possessed a plasmid with *URA3* marker. Experimentally, the cells were cultured in a uracil-supplemented medium, to allow the loss plasmid harboring *URA3* gene. Subsequently, the cells were replica-plated twice on medium containing 5-FOA, allowing the growth only of cells that lost the plasmid (*URA3* marker). After this counterselection phase, cells were recovered in a selective medium for the maintenance of the plasmid of interest, thus ensuring that the strains were unable to grow on medium without uracil.

9.8.5. Measurement of ROS content

The measure of the ROS amount was performed using dichlorofluorescein diacetate (DCFDA) that inside the cells can be oxidized by ROS to dichlorofluorescein (DCF), fluorescent molecule, according to the modified version of Marchetti et al., 2006. 1×10^7 cells grown until stationary phase were incubated with DCFDA (20 μ M) (Sigma-Aldrich®) for 30 minutes at 28°C, washed twice with PBS, and resuspended in lysis buffer (20mM Tris HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100 and 2.5mM Na₄P₂O₇). The samples were mechanically broken with glass beads for 3-4 times for 30 sec and the protein precipitated for 10 min. 100 μ l of supernatant, whole-cell extract was measured by the “Tecan SPECTRAR Fluor Plus” system (excitation at 485 nm and emission at 535 nm) indicating indirectly an amount of ROS. The fold change in the ROS content of mutant strains is calculated by dividing its content by the wild-type content value, which yields the fold change compared with wild type, which now has a value of 1.

9.9. Western blot

9.9.1. TCA protein extraction

Cells were pre-inoculated for 24h on medium supplemented with 2% glucose and then were inoculated in medium added with 0.5% glucose for 18h. The TCA extraction protocol is reported below:

1. centrifugate the culture 5' 5000 rpm
2. Harvest the equivalent of 10 OD of cells through centrifugation 1' 6000-8000 rpm.
3. Wash the pellet with water and resuspend the cells with 75 μ l of Rodel mix (NaOH 1.85 M, β -mercaptoethanol 7.5%, PMSF 0.1 M)
4. Add 500 μ l of water
5. Add 575 μ l of TCA 50%
6. Incubate on ice for 30 minutes. Centrifuge 10' at 15000 rpm 4°C.
7. Wash the pellet with 1 ml of 0.5M Tris-base without resuspending, spin the tube and remove the supernatant
8. Wash pellet with 1 ml of water without resuspending, spin the tube and remove the supernatant
9. Resuspend in 1X Laemmli Buffer (Tris-HCl pH 6.8 33.5 mM, 5% glycerol, 5% β -mercaptoethanol, 2% SDS, Bromophenol blue) (150 μ l for 10 OD)
10. Add 1-5 μ l of Tris-base 0.5M if the color is yellow, until become blue

9.9.2. Protein separation with SDS-page

Protein separation with SDS-page was performed following the Laemmli protocol (Laemmli 1970). Running gels were prepared at 12% polyacrylamide, stacking gels at 6%. Gels were prepared using the following reagents

SOLUTION	RUNNING GEL 12% (TWO GELS)	STACKING GEL 6% (TWO GELS)
Tris HCL 1M pH 8.8	1.876 ml	-
Tris HCL 1M pH 6.8	-	750 µl
Poliacrilamide-bis (37.5-1)	4.5 ml	900 µl
SDS 20%	150 µl	60 µl
APS 10%	50 µl	30 µl
TEMED	12,5 µl	9 µl
H2O	8,4 µl	4,26 µl

Running Buffer 5X (1L): 15 g Tris-Base, 72 g glycine, H2O to final volume. Running buffer was used at the final concentration 1X and added with 0.5% SDS. Running was performed for 1h 30'-2h at 100 Volts.

9.9.3. Western Blotting and Ig-detection

The gels containing the separated proteins were transferred to nitrocellulose membranes through electro-blot for 1hr 40' at 2.5-3mA/cm² in PerfectBlue™ Semi-Dry ElectroBlotter (PeqLab). Transfer buffer, containing 200mM glycine, 25 mM Tris, 20% methanol, was used. After semi-dry protein transfer, membranes were incubated 1hr at room temperature with 5% non-fat dry milk prepared in washing buffer, containing TBS (TBS 10X: TRI-HCL 0,2 M, NaCl 1,37 M pH 7,6) 1%, tween 0.1%, and then incubated o/n with appropriate primary antibody (mono or polyclonal, 5% non-fat dry milk prepared in washing buffer) at 4°C. After the incubation with the antibody, the membranes were washed with 10' 3 times with washing buffer (TBS 1%, tween 0.1%,). The secondary antibodies were prepared in 5% non-fat dry milk dissolved in washing buffer (containing only TBS 1X, without tween). The membranes were incubated 2h at room temperature in dark boxes. To remove the excess of antibody, the membranes were washed three times with washing buffer containing TBS 1x tween 0,05%. Detection of the protein of interest was performed by ChemiDoc MP Imaging, able to detect the fluorophore conjugated with the secondary antibody, using the specific channel of fluorescence. List of primary and secondary antibodies used was reported below.

The bands quantification was performed using the ImageLab software (BioRad).

9.10. Mitochondrial protein synthesis

The ability to synthesize mitochondrial proteins in *mtARSs* strains was evaluated using the following protocol. Strains were pre-inoculated in SC-TRP medium supplemented with 2% glucose for 24h, and subsequently the strains were inoculated in the same medium supplemented with 2% galactose and 0.2% glucose. The cells were grown until $OD_{600}=0,6-2$.

1. Harvest the cell cultures, centrifuge 1' at RT 5000 rpm and discard the supernatant
2. Wash the pellet twice with 1ml of phosphate buffer (400 mM phosphate, 2% galactose), to completely remove the methionine.
3. Resuspend the cells in 1 ml of phosphate buffer and transfer the equivalent of 1.2 OD in a new eppendorf, adding 500 μ l water
4. Incubate 3' the cells at 28°C with 10 μ l of cycloheximide (8 mg/ml in water)
5. Add 2-5 μ l of mix 35S-L-methionine and 35S-L-cysteine purchased by Perkin Elmer (EasyTagTM EXPRE35S35S Protein. Mix Stabilized aqueous solution Shipped ambient. >1000Ci(37.0TBq)/mmo)) and incubate 10' at 28°C
6. Centrifuge 1' 13000 rpm and discard the supernatant
7. Resuspend pellet with 75 μ l of Rodel mix (NaOH 1.85 M, β -mercaptoethanol 7.5%, PMSF 0.1 M)
8. Add 500 μ l of water and then 575 μ l of TCA 50%
9. Chill on ice 30'
10. Centrifuge 10' 13000 rpm 4°C and discard the supernatant
11. Add 1 ml of Tris-base 0.5 M without resuspending and centrifuge 5' 13000 rpm 4°C
12. Wash the pellet with 1 ml of water without resuspending, centrifuge 2' 13000 rpm 4°C
13. Resuspend the pellet in 23 μ l of loading buffer (2% SDS; 10%Glycerol; 60 mM Tris-HCl pH 6,8; 2,5% β -mercaptoethanol; bromophenol blue)

The samples were loaded on the polyacrylamide gels. Run was performed at a maximum of 40 mA until the blue reach the bottom. Semidry transfer into nitrocellulose membrane for 90' at 200mA was then performed. The membranes containing the radioactive mitochondrial proteins were used to impress the autoradiography films.

9.11. Analysis in mitochondria

9.11.1. Preparation of yeast mitochondria with intact outer membrane

[158] Cells were pre-grown on YNB medium supplemented with 2% glucose were inoculated in the same

medium supplemented with 0.6% glucose until an $OD/ml = 4-5$ was reached.

The following protocol was used:

1. Harvest cells at 2000-3500 rpm for 7 minutes and wash once with sorbitol 1.2M

2. Resuspend washed cells in digestion buffer at concentration of 1gr of cells in 3ml

Digestion Buffer:

- 30ml 2M sorbitol
- 3ml 1M phosphate buffer, pH 7.5
- 0.1ml 0.5M EDTA pH 8
- 0.5ml β -mercaptoethanol
- 50mg of zymolyase 20000
- 16ml of water (Vf=50ml)

Incubate at 30°C or 37°C depending on the used growth conditions until most of the cells have been converted to spheroplasts.

3. Add cold Buffer A and immediately centrifuge 4000rpm for 10 minutes. Wash two additional times with Buffer A

Buffer A: 1.2M deionized sorbitol (BioRad® AG501-X8 resin)

20mM KPO4 pH 7.5

4. Suspend washed spheroplasts in 0.6M sorbitol, 20mM KMES pH 6, 0.5mM PMSF at concentration of 1gr/3ml. Homogenize with and glass/Teflon pestle and overhead stirrer (Wheaton Science Products)

5. Centrifuge 3000rpm for 5 minutes to pellet debris. Collect the supernatant and centrifuge 12000rpm for 10 minutes to sediment mitochondria. The supernatant is the post mitochondrial fraction (PMS) and represent the cytosolic component

6. Suspend mitochondria briefly with plastic sticks in 0.6M sorbitol, 20mM KMES pH 6 (1ml/g of starting cells wet weight) in eppendorf tubes. Centrifuge at 3000rpm for 5 minutes to pellet broken mitochondria

7. Collect supernatant and centrifuge at 12000rpm for 10 minutes. Suspend mitochondrial pellet in 0.6M sorbitol, 20mM KMES (100 μ l/g of starting cells wet weight) and dilute with 20ml of 0.6M sorbitol, 20mM HEPES pH 7.4 (1ml/g of starting cells wet weight). Centrifuge 12000rpm for 10 minutes.

8. Suspend mitochondrial pellet in 0.6M sorbitol, 20mM HEPES accordingly to the pellet dimension. Mitochondria can be stored at -80°C.

9.11.2. Quantification of proteins with Bradford method

Protein concentration was determined with Bradford method (Bradford, 1976 [159]). The “BioRad Protein Assay” commercial kit was utilized according to the manufacturer’s instructions.

9.12. High throughput screening: Drug drop test

(Couplan et al., 2011 [82])

Cells were inoculated in YP medium + 2% ethanol and incubated at 28°C in constant shaking. 12x12 plates were filled with 90 ml of YP solid medium supplemented with 2% oxidative carbon source chosen and 0.5X penicillin/streptomycin. After 2 days of growth a precise number of mutant cells, previously evaluated, were seeded onto the plates. After the seed, filters of 6 mm of diameter were put on the agar surface and each compound to test is spotted on them. As positive control, a central spot of wild type was used while DMSO was used as negative control. Plates were incubated at 28/30°C for 7 days.

9.13. PanK enzymatic activity in yeast

Pantothenate kinase activity assay was performed as previously described [105]. Briefly, the assay tracked the ATP-dependent conversion of D-[1-¹⁴C] pantothenate to phosphopantothenate and binding to the DEAE cellulose ion exchange filter. Cell-free extracts from yeast expressing human PanK proteins were obtained by homogenization using glass bead beating, followed by centrifugation at 1000 rpm for 5 min. The 40 µL enzyme reaction contained reaction buffer (100 mM Tris HCl, 2.5 mM MgCl₂, 2.5 mM ATP, pH 7.4), D-[1-¹⁴C] pantothenate (2 nmol, 0.1 µCi), and 72 µg cell-free extracts. Protein content of the cell-free extracts was determined using the Bradford assay. The reaction was done at 30 °C for 10 min following the addition of 4 µL of 10% acetic acid to stop the reaction. The reaction mixture was spotted on a DE-81 filter (0.6mm in diameter) placed within a spin column with a 2 mL collection tube. Following 5 min incubation, the spotted filters were centrifuged for 20 s at 1000 rpm, washed twice with 1% acetic acid in ethanol, and collected for liquid scintillation spectrometry.

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