Universidade de Aveiro Departamento de Química
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Gil Daniel Bregieiro Carraco

Factores interactores como supressores de tRNA mitocondriais mutados

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia no ramo de Biotecnologia Molecular, realizada sob a orientação científica da Doutora Silvia Francisci, Professora do Departamento Biologia e Biotecnologia Charles Darwin da Universidade de "La Sapienza" e do Doutor António Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho aos meus pais que me proporcionaram a oportunidade de estudar e que sempre me apoiaram em todas as minhas decisões, aos meus amigos, família e em especial à Soraia que estiveram sempre ao meu lado nesta jornada.

o júri

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agradecimentos

À professora Silvia Francisci por me ter oferecido a oportunidade de trabalhar na sua unidade de investigação, pela orientação e pelo conhecimento proporcionado.

Aos meus colegas de laboratório Adriano Presciutti, Mario D'Orsi e Arianna Montanari pelo apoio no laboratório e pela amizade.

palavras-chave

Doenças mitocondriais, mutações em tRNAs, *Saccharomyces cerevisiae*, leucil-tRNA sintetase, EF-Tu, contexto nuclear.

resumo

Neste trabalho avaliou-se o efeito de mutações em tRNA mitocondrial humano usando a levedura *Saccharomyces cerevisiae* como modelo. Em termos genéricos *S. cerevisiae* é um microrganismo muito estudado e conhecido, sendo possível modificar o seu genoma de forma rápida e precisa. Como bom modelo permite estudar este tipo de doenças através da mutação dos seus genes que codificam para tRNAs através de um procedimento biobalístico. Com este procedimento é possível introduzir mutações nos genes que codificam para tRNAs mitocondriais da levedura em qualquer posição, incluindo mutações equivalentes às que são patogénicas em humanos. *S. cerevisiae* é também um bom modelo pois os seus mutantes que exibem deficiências respiratórias, complexas ou muitos graves, têm a capacidade de crescer pois continuam a realizar fermentação em glucose, permitindo assim efectuar estudos funcionais moleculares. Para além do referido, o uso de diferentes estirpes de *S. cerevisiae* com diferentes mutações permite estudar genes nucleares isolados capazes de suprimir o fenótipo anormal dos mutantes, neste caso o crescimento num meio respirável.

Na primeira parte deste projecto caracterizaram-se três estirpes diferentes de *S. cerevisiae wild-type*, MCC123, D273-10B/1A e W303-1B e subsequentemente o efeito de substituições nos tRNAs mitocondriais com o propósito de definir uma relação entre a eficiência mitocondrial e a presença de diferentes contextos nucleares. Concluiu-se que as estirpes MCC123 e W303-1B são mais apropriadas para o estudo de mutações severas, em contraste a D273-10B/1A é mais adequada para estudar mutações mais leves.

Na segunda parte estudou-se a actividade supressora da sobre-expressão dos genes que codificam para leucil-tRNA sintetase, o seu domínio C-terminal e algumas pequenas sequências em diferentes mutantes nos tRNAs incapazes de crescer em meios contendo glicerol (GlnC6T, AspC61T, GlyG30A e HisG51A). A tRNA sintetase inteira (genes de levedura e de humano) e o C-terminal têm alguma capacidade de suprimir o fenótipo anormal, mas o melhor resultado foi obtido sobre-expressando as sequências mais pequenas que codificam para péptidos de quinze aminoácidos do domínio C-terminal (β30-31 e β32-33). Outras sequências testadas não tiveram qualquer efeito supressivo.

Também foi caracterizada a capacidade supressora dos factores de elongação da síntese proteica mitocondrial de levedura e humana em células carregando a mutação AspC61T.

Os nossos resultados, sobre-expressando tRNA sintetases e factor EF-Tu, mostram que a capacidade catalítica não é necessária para a supressão e sugerimos que esteja envolvida uma catividade tipo *chaperone*.

keywords

Mitochondrial diseases, tRNA mutations, *Saccharomyces cerevisiae*, leucyl-tRNA synthetase, EF-Tu, nuclear background.

abstract

In this work we evaluate the effect of mitochondrial tRNA mutations using the yeast *Saccharomyces cerevisiae* as model. This simple organism is good model since it is possible to transform the mitochondria by a biolistic procedure. This makes possible to introduce mutations at any desired position in yeast mt tRNA genes, including mutations equivalent to those that are pathogenic in humans.

Yeast offers a unique possibility in that mutants exhibiting complete or very severe respiratory deficiencies can grow by fermentative metabolism on glucose and are therefore amenable to molecular functional studies. Moreover using the yeast strains with the different mutations is possible to isolate nuclear genes able to suppress the defective phenotype of the mutants, in this case to allow the growth on respirable medium (containing glycerol as unique carbon source).

In the first part of this project we characterized three different *S. cerevisiae wild-type* strains, MCC123, D273-10B/A1 and W303-1B and subsequently the effect of mt tRNA substitutions in order to define a relationship between the mitochondrial efficiency and the presence of different nuclear backgrounds. We concluded that MCC123 and W303-1B are more appropriate to study severe mutations whereas the D273-10B/A1 is more suitable to study milder mutations.

In the second part of ours experiments we studied the suppression activity overexpressing the mt leucyl-tRNA synthetases genes, it C-terminal domain and some shorter sequences in different tRNA mutants unable to grow in glycerol containing media (GlnC6T, AspC61T, GlyG30A and HisG51A). The entire tRNA synthetases (yeast and human genes) and the C-terminal have some capability of suppress the defective phenotype, but the best result was obtained overexpressing two shorter sequences coding for fifteen aminoacids from its C-terminal domain (β30-31 and β32-33). Other sequences tested did not have any supress effect. I also report a further characterization of the functionality of mt yeast and human mt protein synthesis elongation factors in their suppressing activities in cells bearing the AspC61T mutation. Our results, overexpressing either the tRNA synthetases or the EF-Tu factor, show that the catalytic function is not necessary for suppression and we suggest that a chaperone like activity is involved.

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Abbreviations list

aaRS - Aminoacyl-tRNA synthetase

aa-tRNA – Aminoacyl-tRNA

ATP - Adenosine-5'-triphosphate

CAP^r - Chloramphenicol resistance

CPEO - Chronic progressive external ophthalmoplegia

Cyt - Cytosolic

DNA - Deoxyribonucleic acid

EF-Tu - Elongation factor Tu

EGTA - Ethylene glycol tetraacetic acid

LB - Lysogeny broth media

MELAS - Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes

MIDD - Maternally inherited diabetes and deafness

MM - Mitochondrial myopathy

mRNA - Messenger RNA

mt - Mitochondrial

mtDNA - Mitochondrial DNA

NADH - Nicotinamide adenine dinucleotide

Ori - Replication origin

OXPHOS - Oxidative phosphorylation

RNA - Ribonucleic acid

rRNA - Ribosomal RNA

tRNA - Transfer RNA

W0 - Minimum media

WT - *Wild-type*

YP - Yeast extract peptone

Introduction

Mitochondria are the organelles responsible for the production of 95% of all the ATP that is needed for the human cells. Nowadays there are estimated that one in five thousand people suffers of a mitochondrial disease and one in two hundred of living born has a mutation in the cord blood that has the potential to lead to a mitochondrial disease. It is important to refer that there is no cure or available treatments for mitochondrial diseases. One of the most frequent causes of mitochondrial diseases are point mutations in the mitochondrial tRNA genes, which could promote severe neurodegenerative diseases like MELAS, CPEO and MIDD among others.

The fact that is difficult to create a superior animal model for this kind of diseases makes the yeast model one useful model to study the molecular mechanism of working of point mutations in tRNA genes and allows the research for treatments.

This thesis has three main aims; the first one is to compere three different *Saccharomyces cerevisiae* strains MCC123, D273-10B/A1 and W303-1B, in order to understand the involvement of the nuclear background in the mitochondrial phenotype of this yeast and how the different nuclear backgrounds may influence the same mitochondrial point mutation. The second objective is to understand the suppression mechanism obtained overexpressing the leucyl-tRNA synthetase gene, their C-terminal domain or some shorter sequences from the C-terminal domain in three mutated strains (GlnC6T, AspC61T and GlyG30A). The third part is to study the suppressive mechanism of TUF1, TUFM (genes that encode for mitochondrial protein elongation factor on yeast and human respectively), and some derivatives of the defective phenotype due to AspC61T mutation.

Bibliographic Review

1.1 Mitochondria emergence and evolution

Mitochondria are organelles responsible for energy production and are present in almost all eukaryotic organisms except in a small filo of Protists, Metamonada, which lost this organelle in the course of the evolution [1].

This remarkable organelle is responsible not only for the production of 95% of all ATP trough oxidative reactions is also involved in intermediate metabolism, such as steroids [2], aminoacids and iron-sulphur (Fe-S) clusters [3], heme group biosynthesis and regulation of calcium [4, 5] and also involved in the apoptotic death [5].

The endosimbiotic hypothesis is the one with the best acceptance in the scientific society, in that the mitochondria are the descendants of an ancient endosymbiotic bacteria, an α-protobacteria (called proto-mitochondrion), that are related with intracellular parasites such as *Rickettsia*, *Anaplasma*, and *Ehrlichia* [6], the host cell was a *Thermoplasma* like archaebacter [7].

Between species the mitochondrial DNA (mtDNA) have variations in conformation (linear or circular), in size and in gene content [8]. These variations can be explained by the different evolutionary paths of the different organisms.

The proto-mitochondrion genome was characterized by the existence of many extra genes compared with the animal mtDNA such as SDH gene, additional NAD and ATP genes and ribosomal protein genes (RPS and RPL genes); genes that encode for 23S, 16S and 5S rRNAs; a complete or almost complete set of tRNA genes; the genome was almost only coding sequence, without or maybe only a few introns in the sequence; typical eubacteria-like gene clusters; a standard genetic code [6].

The period of time between conversion of once free-living organism into interdependent cell organelle some crucial steps ensured integration of the organelle into the metabolic pathways and in a large amount of signalling networks of the cell [9].

Occurred a great reduction of the genome with the loss of protein-coding genes tRNA genes and rRNA genes. Protein-coding genes had an accelerated rate of sequence divergence [6].

But was not only the genome that turned shorter, also significant proteome modifications occurred, some of the proteins like the ones involved in cell envelope synthesis have virtually disappeared. The proto-mitochondria passed to import from the

cytosol a great quantity of proteins like proteins involved in replication, transcription, cell division, transport, regulation, and signal transduction. In fact only 15 to 20% of all proteins of actual mitochondria can be traced back to α-proteobacterial ancestor [10]. Consequently more than half of the remaining functions from the α-proteobacterial ancestor in modern mitochondria correspond to translation, including post-translational modifications, and to metabolic pathways that are directly, or indirectly, involved in energy conversion.

Remarkably, mitochondria developed a special mitochondrial codon usage. The codon UGA encodes for tryptophan instead of a stop codon; AUA encodes for methionine instead of isoleucine and AGA and AGG are stop codons instead coding for arginine [11].

1.2 Biogenesis, structure and mitochondrial functions

Mitochondria are organelles limited by two membranes, which have different proprieties. With these two different membranes is possible to create five different environments, the outer membrane, the intermembrane space and inner membrane, the inner membrane cristae, and the matrix (Figure 1). Each one of these environment has different functions [12].

The outer membrane is constituted by phospholipids and proteins in one proportion of 1:1. It is involved in the lipid metabolism, and calcium signalling to endoplasmic reticulum [12].

The intermembrane space is formed between the outer membrane and the inner membrane. The intermembrane space has a similar composition of small molecules and ions as the cytosol, due to the permeability of the outer membrane. However the large proteins need to have a signalling sequence to pass through the outer membrane, as the example of cytochrome c [12, 13].

The inner membrane is similar to the bacterial membrane in lipid constitution. This membrane has some of the most important functions in the mitochondria metabolism. It forms cristae that increase the area of the inner membrane, so the productivity of the mitochondria is higher. The membrane contains proteins to perform redox of oxidative phosphorylation, NADH, succinate dehydrogenases, ATP synthases, mitochondria's fusion

and fission proteins and proteins that regulate the transport of proteins and metabolites [12].

At last, the matrix, where are located the machinery for protein synthesis and the mitochondrial DNA. Contains also enzymes for fatty acids and pyruvate oxidation and for the citric acid cycle [12].

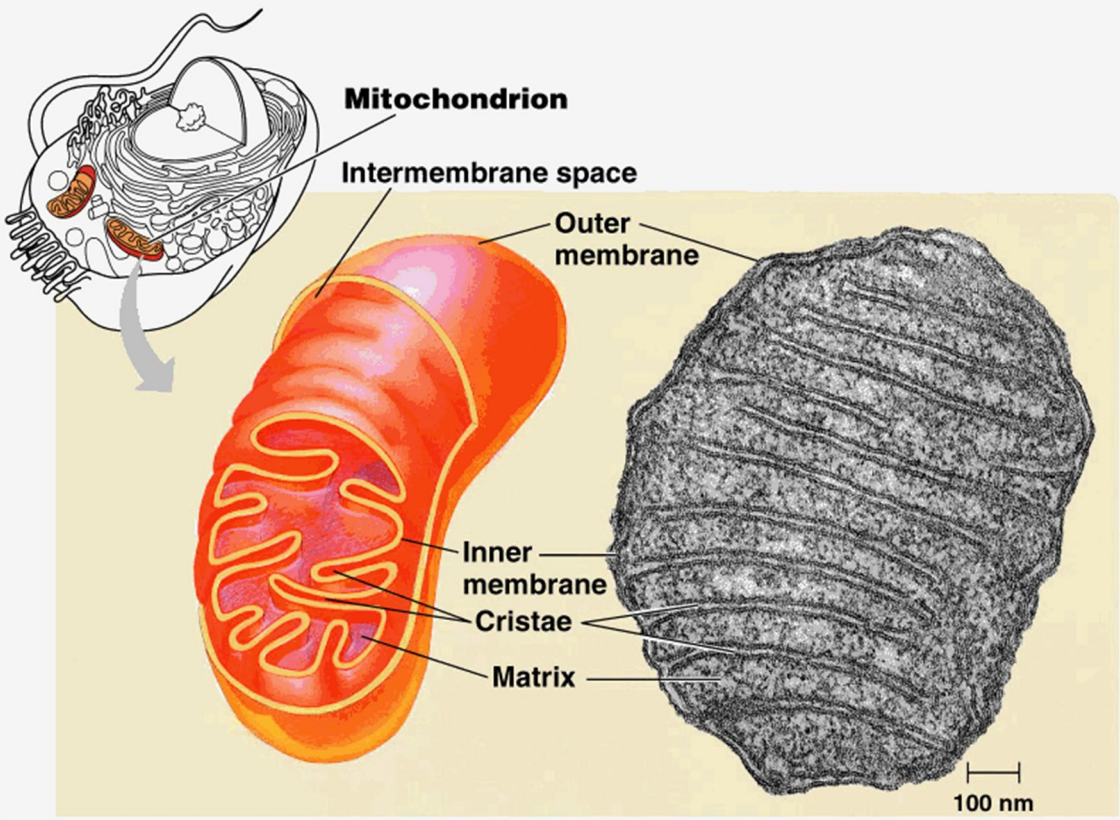

Figure 1: Mitochondria structure and organisation.

Mitochondria are not made *de novo*. They grow by the incorporation of proteins and by production of phospholipids and transport from others organelles. As these ancestors the mitochondrial growth is accompanied by division [14].

Notwithstanding, a great number of nuclear encoded proteins are directly or indirectly involved in the mitochondrial morphology. For example mutations on FZO1 gene (encode for Fis1p protein) produce small and fragmented mitochondria [15].

The shape, morphology, and number of the mitochondria are controlled by the balance between fusion and fission events and are regulated by specific proteins: Dnp1p,

Mdv1p and Fis1p for fission event and Mgm1p and Ugo1p for fusion. These events have also impact on mitochondrial function and distribution on the cell [16-18].

1.3 Human mitochondrial DNA

The mitochondrial DNA (mtDNA) is a small (16569 pairs of nucleotides), circular double strand molecule of DNA (Figure 2), which is present in all mitochondria and has the capacity to replicate independently of the nuclear genome replication [19].

Each mitochondrion has between two and ten mtDNA molecules that encodes for a global of thirty seven genes [19].

The mtDNA encodes for thirteen protein subunits corresponding to four of the five large oxidative phosphorylation (OXPHOS) complexes in the inner mitochondrial membrane [20]. The others subunits of these complexes are recruited from the cytosol. The mtDNA encodes two rRNAs genes, (12S and 16S rRNA). Twenty two tRNAs (a complete set with two isoccepting species for serine and leucine [21]. The mt protein machinery is not completely independent from the nucleus information because the mt ribosomal proteins and the tRNA aminoacyl-tRNA synthetases (aaRS) are recruited from the cytosol.

Long polycistronic primary transcripts are produced from each transcribed strand that must be processed to generate the mature mRNAs, rRNAs and tRNAs. The tRNAs, which flank the mRNA and rRNA genes, are the structural signals for the processing enzymes (the so-called “tRNA punctuation model”) [22]. Human mtDNA contains very few noncoding sequences because of the high-density gene organization. The major exception is the D-loop regulatory region, that contains three promoters required for transcription initiation, one L-strand promoter (LSP) and two H-strand promoters (HSP1 and HSP2), along with evolutionarily conserved regulatory sequences involved in DNA replication and D-loop formation (Figure 2) [23-25].

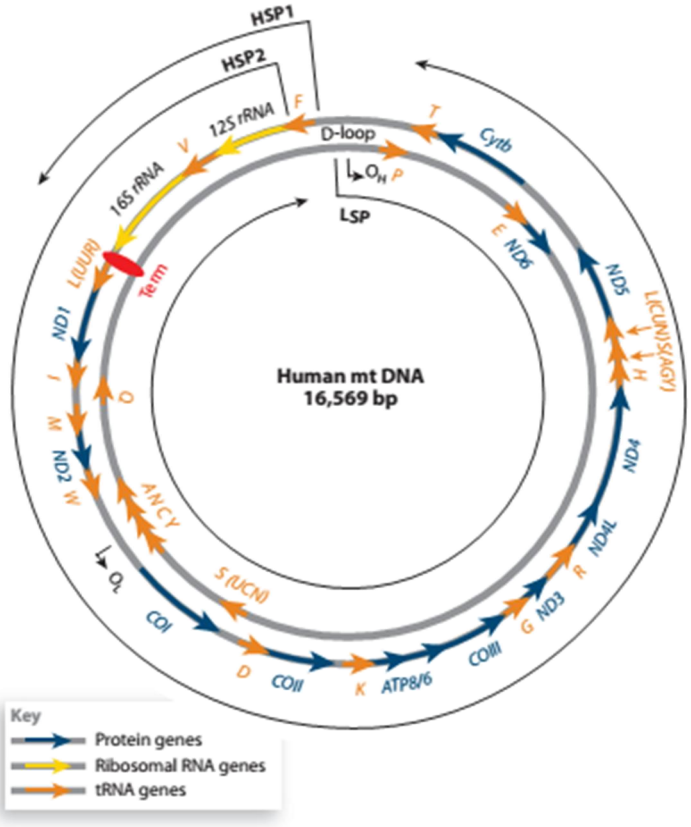

Figure 2: Structure of human mitochondrial DNA.

In yellow the two rRNA-encoding genes, in orange the twenty two tRNA-encoding genes, in blue are the genes that encode for the thirteen proteins and in blue the control region (D-loop). Arrows indicate the replication units [26].

1.4 Mitochondrial diseases

Since 1988 when was discovered the first mtDNA mutation that leads to a disease a great amount of mutations, that are correlated to pathologies in the mitochondria, were discovered. Nowadays there are estimated that one in five thousand people suffers of a mitochondrial disease and one in two hundred of living born has a mutation in the cord blood that has the potential to lead to a mitochondrial disease [27].

Mitochondrial diseases are devastating neurodegenerative pathologies and are related not only to mtDNA's mutations but also with mutations in nuclear genes involved in mitochondrial biogenesis, in which the great part is not discovered yet. The clinical presentations are normally heterogeneous and multisystemic [28].

As it is known, and previously explained, mitochondria are responsible for the production of energy in the cell, so the tissues that needed more energy generally are the most affected. Energetic defects have been implicated in forms of blindness, deafness, movement disorders, dementias, cardiomyopathy, myopathy, renal dysfunction, aging [29] and there are not any discovered cures for them (Figure 3) [27]. All the organs can have problems due to mitochondrial dysfunction.

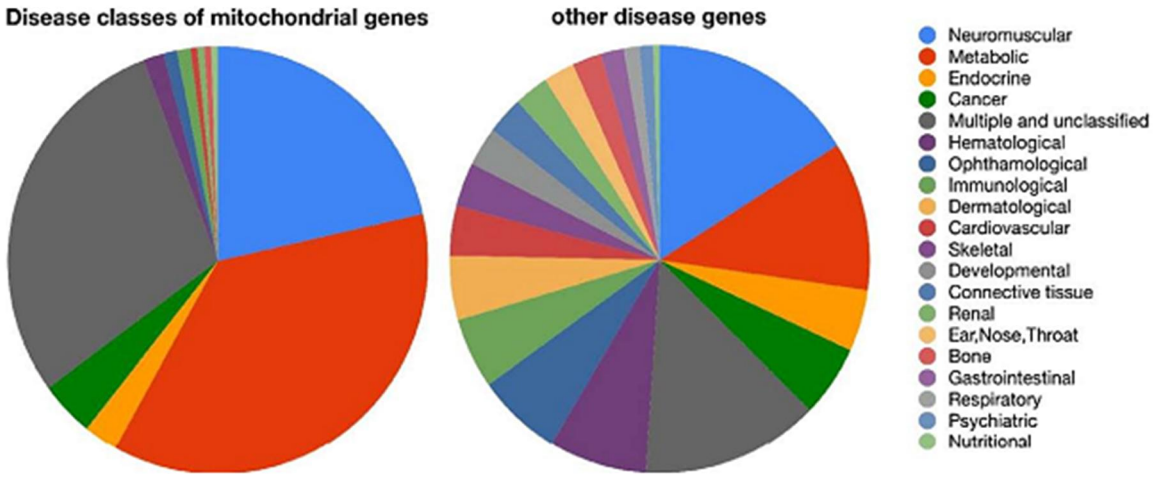

Figure 3: Comparison between diseases caused by mutations in mitochondrial genes (left) and disease caused by nuclear genes (right) [30].

Diseases due to mitochondrial mutations frequently affect numerous physiological systems at the same time, leading to a relatively large set of "Multiple and Unclassified" pathologies in the classification (e.g. Mitochondrial myopathy, Encephalopathy, Lactic acidosis, and Stroke-like episodes known by the acronym of MELAS). The mitochondrial diseases that can be classified have a distinct profile from the other genetic diseases, reflecting the metabolic, energy-converting function of the organelle [30].

1.5 Inheritance of mitochondrial DNA (homoplasmy and heteroplasmy)

With rarely exceptions the mtDNA is maternally inherited, that are the mitochondria that are in the oocyte [31]. The spermatozoid's mitochondria generally have the proteins from the outer membrane ubiquitinated, and when they enter in the oocyte, they are degraded. However sometimes this process does not work well and allows the entrance and "survive" of the paternal mitochondria. This event will give birth to an organism with two species of mtDNA and in some generations could give origin of heteroplasmic mitochondria [32, 33].

Each cell in the human body could contain between 1000 and 10000 mtDNA molecules [31]. In addition, these molecules are more susceptible to the mutation agents then the nuclear DNA due to the oxidative reactions that occur in mitochondria [34]. A mutation can be present in the mtDNA population of the oocyte or may arise in a specific tissue during life producing a heteroplasmic cell with two different DNA molecules (one wild type and another mutated) [35].

When a cell enters in mitosis, the mitochondria and so the mtDNA are randomly distributed in the daughter cells. So is possible to have different degrees of heteroplasmy, due to the independent segregation of mitochondria, and in some generation, lead to the resurgence of homoplasmic cells, either mutant or wild-type homoplasmic cells. Furthermore, the mitochondrial DNA replicates independently of the cell cycle, so in one cell the proportion of *wild-type* (WT) and mutated DNA could change depending on the molecules that replicate more during the successive cell divisions (Figure 4) [36].

But how could one inherited mutation increase their number by the pass of only a generation?

The "genetic bottleneck" hypothesis explain this phenomenon: in the initial division states of the zygote there is not any mtDNA replication, so the molecules are divided among the daughter cells. When the primordial germ cells appear, they have about 185 molecules that will originate the nearly 500,000 molecules in the mature oocyte. In this process only a few molecules will produce all the mtDNA molecules in the oocyte differently distributed in the oocytes; consequently a healthy mother could give birth a child with a severe pathological mitochondrial mutation if the oocyte has a high quantity of mutant mtDNAs (Figure 5) [37].

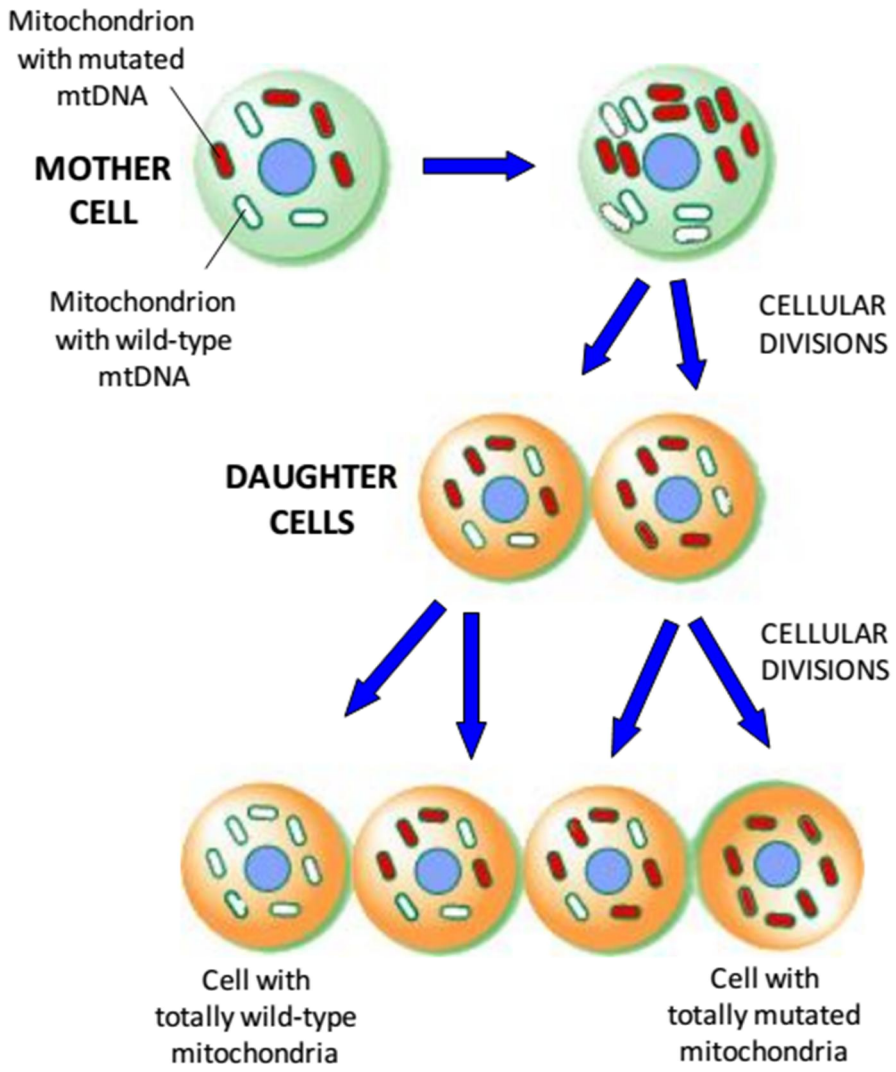

Figure 4: Inheritance of mitochondria during the cell division and random segregation of heteroplasmic mtDNA mutations.

Mitochondrial energetic function declines with the increase of percentage of mutant mtDNAs. The pathology appears with thresholds typically between 60 and 90% of mitochondrial dysfunction, caused by the extent of heteroplasmy combined with effectiveness of the mutation [38]. When energy output is insufficient for normal tissue function, symptoms appear and apoptosis or necrosis may be initiated, and when the percentage of mtDNA mutants is too high could lead to the death of the organism [39].

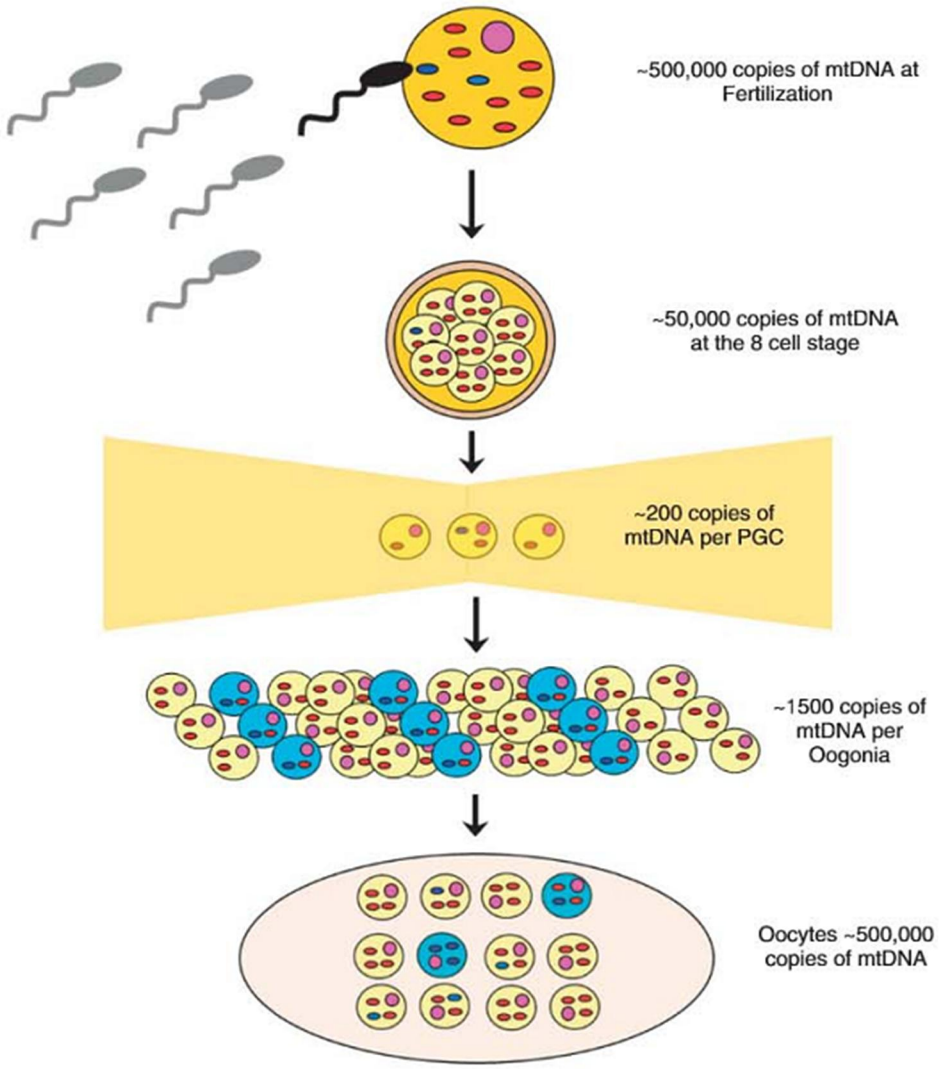

Figure 5: "Bottleneck" hypothesis in the germ line generation [4].

1.6 Pathologies related with mitochondrial tRNA mutations

Mitochondrial tRNA (mt tRNA) pathogenic mutations can affect in different degrees of severity the energy metabolism of the cell. To understand the molecular mechanisms underlying their genotype and phenotype relationships, various aspects were explored, such as the aminoacylation properties and steady-state levels of aminoacyl-tRNA in mitochondria, mitochondrial tRNA turnover, base modifications (enzymes which modify specific tRNA bases), structure, stability and interaction with partners of the

translation machinery, such as aminoacyl-tRNA synthetases, mitochondrial protein synthesis Elongation Factor (EF-Tu), and the subunits of mitochondrial ribosomes [4].

Mitochondrial tRNA pathologies are frequently associated to heteroplasmy, when a critical threshold value is exceeded (usually between 70-90% mutated mtDNA molecules), the phenotype becomes evident; higher the degree of mutated DNA is, more severe could be the disease [40].

As shown in the Table 1 there are pathogenic mutations in all mitochondrial tRNA genes and the tRNA leucine (UUR) is the one that have the greatest amount of substitutions, with 33 pathogenic point mutations.

Table 1: Pathogenic [41] and polymorphic substitutions[42] in mitochondrial tRNAs.

tRNA	Pathogenic mutations	Polymorphic positions
Leucine (UUR)	33	6
Lysine	21	10
Isoleucine	20	7
Phenylalanine	15	9
Glutamine	14	15
Tryptophan	13	11
Serine (UCN)	12	10
Glutamate	12	10
Leucine (CUN)	10	9
Threonine	10	29
Serine (AGY)	10	14
Valine	9	10
Methionine	8	5
Alanine	6	11
Cysteine	6	10
Glycine	5	12
Proline	5	10
Asparagine	5	4
Histidine	5	10
Arginine	5	8
Aspartate	3	9
Tyrosine	2	5
Total	231	224

The great part of pathogenic mutations affects conserved nucleotides during the evolution, whereas most polymorphisms affect non-conserved nucleotides. This does not mean that only pathogenic mutations are in the conserved regions and polymorphisms are in the non-conserved regions [43].

The diseases related with mutations in the mitochondrial tRNAs are extremely difficult to diagnose, because the same mutation can lead to different phenotypes, and the same phenotype can be cause by different mutations [44]. So there is not a clear relationship between the site of specific mt RNA mutation and the resulting clinical phenotype [45].

As example I can report the mutation on A3243G in the tRNA leucine (UUR). This mutation has been related to a series of diseases, syndrome of mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), chronic progressive external ophthalmoplegia (CPEO), or maternally inherited diabetes and deafness (MIDD). Two or more of these diseases can occur within the same family; these phenotypes are not related specifically to A3243G mutation, but with a lot of others mutations [41, 46].

There are a lot of variables that is needed to have in mind when we study mitochondrial diseases. First of all it is needed to consider the rate of heteroplasmy in the cells that, as anteriorly explained, is an important factor to the expression of mitochondrial mutation; in addiction different tissues have different behaviours dependently on the needs of energy. Other important fact is that the mtDNA is in a separated compartment, mitochondria. At last, different nuclear products and their amounts can differently influence the metabolic pathway which in synergy with the mitochondria or alone contributes to mitochondrial biogenesis modulating the energetic failure. For this reason one of the major's challenges on the mitochondrial researches is the creation of a good model for studying mitochondrial diseases [38].

1.7 Models to study mitochondrial diseases

One of the biggest difficult to study and search a possible therapy for mitochondrial DNA mutations pathologies is to have a good cell or animal model for this kind of

diseases. Actually there are some works to create a mice model for mtDNA pathological specific mutations, but until now there are not any one effective. The use of mice models for mtDNA mutations will be very important in the future, but nowadays we need to use other kind of models [4].

However, some technics have been developed in the lasts years. One of them is using cytoplasts carrying a different mitochondrial genotype and fuse with oocytes carrying another mitochondrial genotypes, and the offspring will be heteroplasmic [47]. Other technic is isolating mitochondria from one mouse and micro-inject them into oocytes of other mouse. The problem is that these technics are unable to introduce mitochondrial mutations, these two technics only allows creating heteroplasmic cell, or heteroplasmic mice [47, 48].

Although there are two others technics in which is possible to introduce mitochondrial DNA mutated. The first one was achieved by introducing chloramphenicol resistant (CAP^r) mtDNA in the embryonic stem cells, and then injecting into blastocysts and creating chimeric mice. However this technic has a problem, the CAP^r mtDNA decrease too rapidly by the serial transmission [47, 49].

The second technic was performed by capture mutant mtDNA in cybrids, which do not have mtDNA (rho^o). Afterwards the cybrids with respiratory defects due to the mtDNA mutation are isolated and at last the mtDNA is introduced into zygotes, fusing the zygote with the cybrid enucleated. These mutations could be transmitted maternally to the offspring [49].

Transmitochondrial cybrid cells; in this case this cells are generated by fusing rho^o (mt DNA less) cells with cytoplasts containing the specific mtDNA variant of interest, that allows investigation of mtDNA variants in a control nuclear background. These cells are useful in modelling mitochondrial diseases [50].

The fact of the cybrids are created by using cancer cells like mioblastome or ostheosarcome could be the biggest problem in using these cells because their nuclear context is immortalized in order to maintain their viability in culture. Consequently could be possible that these cells require a different amount of energy to survive and so the mitochondria functionality may be different, when compared with normal cells due to the nuclear mutations [51].

As alternative we can use fibroblasts cultures from patients, but generally these cells do not show the defective phenotype observed in other tissues like the cardiac or skeletal muscle [27].

At last, but not less important the *Saccharomyces cerevisiae* model, this is a relatable model to study mitochondrial diseases because the genes involved in mt biogenesis are highly conserved between yeast and human [52, 53].

1.8 Yeast as a model to study mitochondrial mutations

The fact of the absence of any complex model available to study mitochondrial diseases, like mice models, increase the importance of simpler models like yeasts. Among them we have the *S. cerevisiae* that is non-pathogenic yeast considered a very good model to study a great quantity of biological processes, such as transcription, protein synthesis, cell cycle, membrane transport among others. This only is possible because this organism is one of the well-studied and characterized organism, both at the physiologic and genetic level, by having a quickly cell cycle and by being easy to isolate there mutants.

In addition, yeast is very easy to manipulate and it is possible to integrate a large spectrum of DNA molecules, like plasmid DNA by transformation, endogenous/exogenous DNA and replicative and integrative molecules by homologous recombination in a specific position of the genome.

The genome of this organism is totally sequenced since 1997, and has about 12,06 Mbp and contains about six thousand genes organized in sixteen linear chromosomes [54]. The functions of more than 75% of all opening reading frames (ORFs) are known (Figure 6) [55].

Yeast is an important model to study biological conserved processes among the eukaryotes and it is used to study human hereditary diseases since around 50% of all diseases related genes have an homologous gene in yeast [56], that turn this simple organism in a great model to study the biological processes of the diseases.

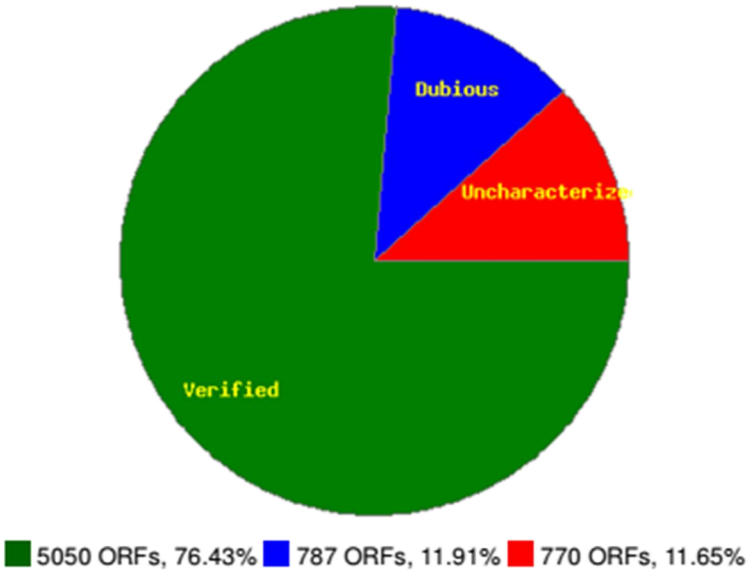

Figure 6: Pie chart with the percentages and the number of verified ORFs in *Saccharomyces cerevisiae*.
There are 5050 (76,43%) of ORFs verified (green), 787 (11,91%) with a dubious function (blue) and 770 (11,65%) uncharacterized ORFs (red) [57].

S. cerevisiae is capable of grow both haploid and diploid state. The haploid cells could be one of two genders, a or α. Two cells of opposite gender can mate by fusing (conjugation). As diploid state they could go into two different paths, one by grow and replicate in by mitosis and the other they could go to meiosis and originate four haploid cells, two a cells and two α cells (Figure 7) [58].

This remarkable organism is an optimum model for the study of human mitochondrial pathologies. First of all it is an aerobic facultative organism, so the cell can survive by producing energy by fermentation [59], even with a lethal mitochondrial mutation and it is possible to analyse the morphologic changes in the cell.

Other great advantage is that when *S. cerevisiae* is in a heteroplasmic situation (occurred in a 2n cell after conjugation) in only a few generations pass to a homoplastic situation and so we can do all the studies caused by a homoplasmic point mutation. Moreover the use a strictly respirable carbon source, like a glycerol containing medium permits to evaluate the possible defective phenotype of the mutant. In this cell model it has

been also isolated suppressor genes that when overexpressed rescue the respiration defect due to the mt tRNA mutation [60].

Human equivalent mt tRNA mutants have been obtained in yeast by introducing mitochondrial mutations by biolistic procedure, revealed that mutations having severe pathogenic effects in humans gave origin to severely defective respiratory phenotypes in the yeast. The mutations were amplified by PCR and introduced in the mitochondria by shooting the fragments attached in tungsten particles. The biolistic procedure that introduces a site specific mutation in the mt genes is possible only in the *S.cerevisiae* cell [61].

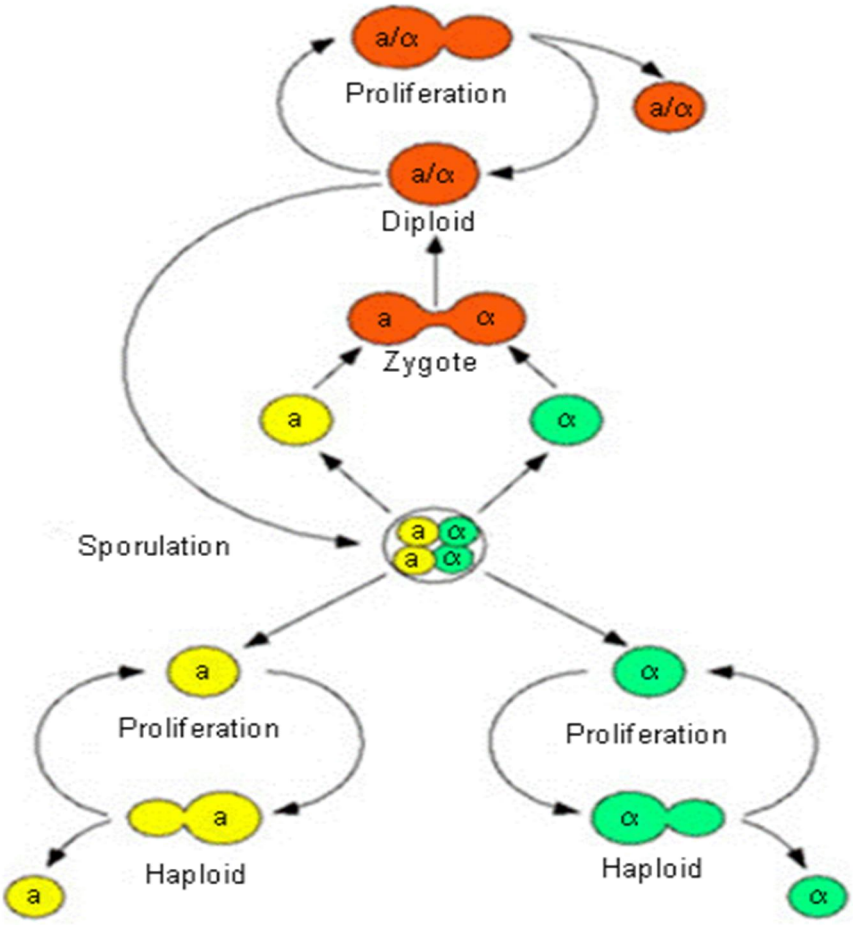

Figure 7: *Saccharomyces cerevisiae* life cycle

1.9 *Saccharomyces cerevisiae* mitochondrial DNA

The *S. cerevisiae* mitochondrial genome was completed sequenced in 1998 by Foury et al. The molecule size may varies from 68 to 85,8kbp, depending on the presence of intergenic sequences and introns. Intergenic regions are composed almost by A+T nucleotides (62% of the entire genome) and around 30% in C+G content distributed in 200 G+C clusters interspersed in the mt genome. Some of these clusters which form structures called "hairpin loops" are "hotspots" for rearrangements of the mtDNA [62]. It has been observed that GC clusters may have a role also in replication [63] and transcript maturation [64].

S. cerevisiae has a very similar mitochondrial genome compared with the human one. As the human molecule contains genes for cytochrome c oxidase subunits I, II and III (cox1, cox2 and cox3 respectively); ATPs synthases subunits 6, 8 and 9 (atp6, atp8 and atp9 respectively); Apocytochrome b (cytb); a ribosomal protein (var1) and several intron related ORFs [62].

Moreover it contains between 7 and 8 origin-like (ori) elements; genes that encode for 21S and 15S ribosomal RNAs (rRNA); 24 tRNAs genes and 9S RNA component of RNase P (RPM1). All the genes are transcribed from the same strand, except tRNAthr1 (Figure 8) [65].

Yeast and human mtDNA have nearly the same gene information. The only important differences with the human counterpart is the existence of intron genes, two code for maturase enzymes while third one code for an endonuclease subunit involved in mitochondrial recombination, and the long intergenic sequences [23, 62].

In around 1000 proteins that constitute the mitochondria [66] only eight are encoded by mtDNA and synthesized on mitochondrial ribosomes. The rest of the proteins are encoded in the nucleus, and imported from the cytosol as distinct classes of precursors that are specifically sorted into one of the four sub-compartments of the organelle [67].

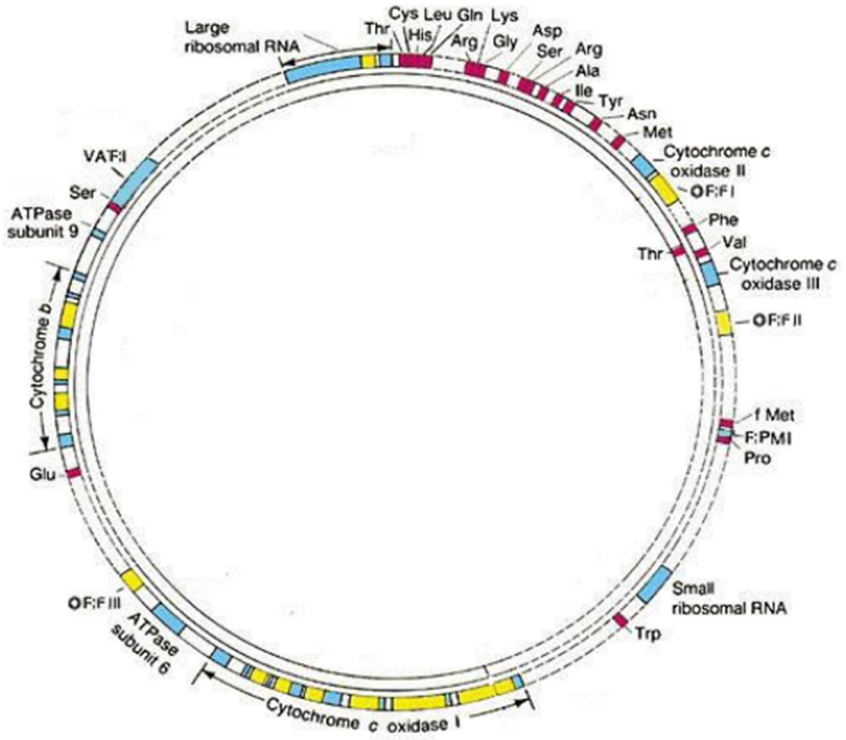

Figure 8: Structure of *S. cerevisiae* mitochondrial DNA. In red is represented the tRNA genes, and in blue protein and ribosomal genes. In yellow the intronic sequences.

1.10 Yeast mitochondrial mutants

The mitochondrial genome is more susceptive to mutation then the nuclear one due several aspects. Mitochondrial DNA replicates more often than nuclear DNA and consequently the presence of incomplete systems of repairing for DNA damages produce high percentage of mt mutations [68].

Mitochondrial mutant cells can be classified as:

Rho^{+} are the WT cells with a WT DNA content. In the opposite site there are the *rho*° mutants characterize by the total loss of mitochondrial genome. *Rho*$^{-}$ mutants are characterized by large deletions of mtDNA, up to 90%. The remaining DNA is amplified until reach the size of the wild-type mtDNA [69]. Rho^{o} and rho^{-} mutants form small colonies in plates containing glucose as carbon source because cells, due their incapacity to respire, grow slowly compared to the WT cells; these colonies are commonly called "*petite*". The *syn* mutants that are characterised by having point mutations or small

deletions in tRNAs or rRNAs genes, which prejudice the mitochondrial protein synthesis. As the *syn* mutants, *mit* mutants have small deletions or point mutations but in this case in genes coding for respiratory chain enzyme complexes. All this mutants are unable to grow on a medium with a strictly respirable carbon source as glycerol.

At last the *antR* mutants have small deletions or point mutations that allow growth on media containing antibiotics or other inhibitors, which normally interfere with respiratory processes or mitochondrial protein synthesis.

On other hand there are mutants with a wild-type mtDNA, but due to nuclear deletions or point mutations they are unable to perform the mitochondrial proteins and to grow on glycerol containing media, they are called *pet* mutants.

1.11 Mitochondrial tRNAs

TRNAs have a critical role for the correct translation of mRNA in proteins. As the name says (transfer RNA), they are responsible for the transport, transfer of the aminoacids until the ribosomes.

These smalls RNAs interact with several of other cellular components such as mRNA, aminoacyl-tRNA synthetases (aaRSs), initiation and elongation factors of protein synthesis and ribosomal machinery.

All tRNAs have a cloverleaf secondary structure (Figure 9A), composed by four stems and three loops, and a L-shaped tertiary structure (Figure 9B). The cloverleaf structure is characterized by their arms and which can be decomposed in two parts, first the double-stranded regions, called “stem” formed by intramolecular bonds, and the loop part in the end of the stem composed by single strand RNA.

More specifically a tRNA can be characterized by one acceptor stem, that has the function of transport the specific aminoacid, linked by one ester bond between the carboxyl group of the aminoacid and the hydroxyl group 2' or 3' of adenosine at 3' terminal of tRNA; by the anticodon loop, which links the respective codon in the mRNA during the translation; the D loop, where is always located a dihydrouridine base; by variable loop; and at last by the TψC loop that contains ribothymidine (T) and pseudouridine (ψ) [4].

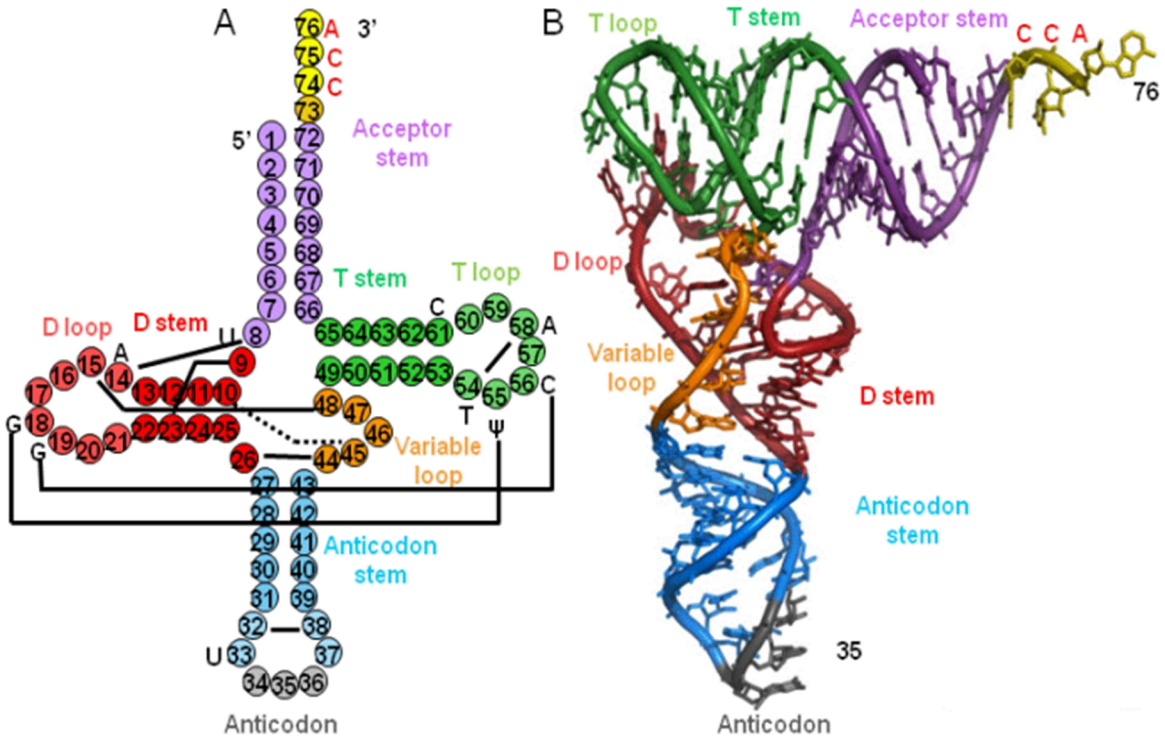

Figure 9: Secondary (A) and tertiary (B) canonical structures of a generic tRNA.

The L-shaped tertiary structure is formed by two domains: the acceptor domain consists of the T stem and the acceptor stem, and the anticodon domain made by the D stem and the anticodon stem. The T stem and the D stem form an important structure called “DT region” which interactions stabilize the tRNA; moreover the DT is the region where the mitochondrial protein synthesis Elongation factor Tu (EF-Tu) binds.

Since the tRNAs are excised from the polycistronic tracts, to mature the tRNA molecules several post-transcriptional modifications are needed. The RNaseP digests at the5’-phosphate end whereas the RNaseZ release the 3’end. After that a mitochondrial-tRNA nucleotidyltransferase adds the CCA sequence to the 3’end. At last, to acquire functionality and to stabilize the tertiary structure several factors introduce modifications in specific bases of the tRNA [70].

1.12 Suppressors of tRNA mutations

In the last years it has been discovered some nuclear products able to correct or supress the defective growth phenotype in yeast due to mitochondrial tRNA mutations. The molecules are nuclear encoded factors that bind the mutated tRNA and are essential for the mitochondrial protein synthesis process.

Among these interactors with suppression effect are the mitochondrial aminoacyl-tRNA synthetases and the mitochondrial protein synthesis Elongation Factor Tu. These two molecules are capable of rescue the growth in glycerol media of some mutated tRNA *S. cerevisiae* [52, 61, 71, 72].

1.12.1 Aminoacyl-tRNA synthetases

The aminoacyl-tRNA synthetases have an important role in the protein translation. They are responsible for the ligation of the aminoacids to the cognate tRNA. In other words they catalyze the esterification of one aminoacid or its precursor to the specific tRNA and form an aminoacyl-tRNA. This is a two steps reaction, in the first the aminoacid is condensed with an ATP molecule to form aminoacyl-AMP, then in the second step this molecule is transferred to adenosine on 3' of its cognate tRNA releasing an AMP [12].

1) ATP + aminoacid (aa) ↔ aa-AMP + PPi
2) aa-AMP + tRNAaa → aa-tRNAaa

All aaRS are encoded in the nucleus, but in general the mitochondrial proteins are coded from different genes of the ones that code the cytosol counterpart. In fact only three of them work in simultaneous, in the cytosol and in the mitochondria. Then proteins such as the aaRS, that pass from the cytosol to the mitochondria matrix carry an N-terminal extension (pre-sequence) that directs the protein across both outer and inner membranes into the matrix where the pre-sequence is cleaved off [73].

The aaRS usually is characterized by three domains, one binds the ATP molecule, the second binds the aminoacid and the third one that binds the tRNA.

The aaRS molecules are divided in two classes based on differences in the primary and tertiary structures and different reaction mechanisms. Class I has two highly conserved motifs and aminoacylates the 2' –OH and class II has a highly conserved sequence (HIGH), and have three highly conserved sequence motifs and aminoacylates the 3' –OH.

Class I charges the tRNA in two steps whereas class II transfer the aminoacid directly to the tRNA. Other difference in these two classes is in the recognition of the cognate tRNA, class I recognize the tRNA on the D-Loop and this interaction distorts the structure of the tRNA acceptor site, whereas class II enzymes bind the cognate tRNA recognizing the variable loop [74].

In the aaRS exists some nucleotides that are crucial for the interaction with its tRNA and so called "identity elements". In general, they are located in the acceptor arm and in the anticodon loop but the correct tRNA structure have a great importance in maintaining the functionality. Mutations within these elements could change drastically the tRNA's conformational structures and could lead to problems with the impairment of aaRS [75].

It has been suggested that some aaRSs may not charge the correct aminoacid due to the difficulty to discriminate among very similar aminoacids (leucine, valine and isoleucine). To minimise this problem class I synthetases have acquired the editing activity (editing domain) with a "double sieve" mechanism. In the first "sieve" pass the similar aminoacids and the second one pick the correct one as exemplified in Figure 10 [76].

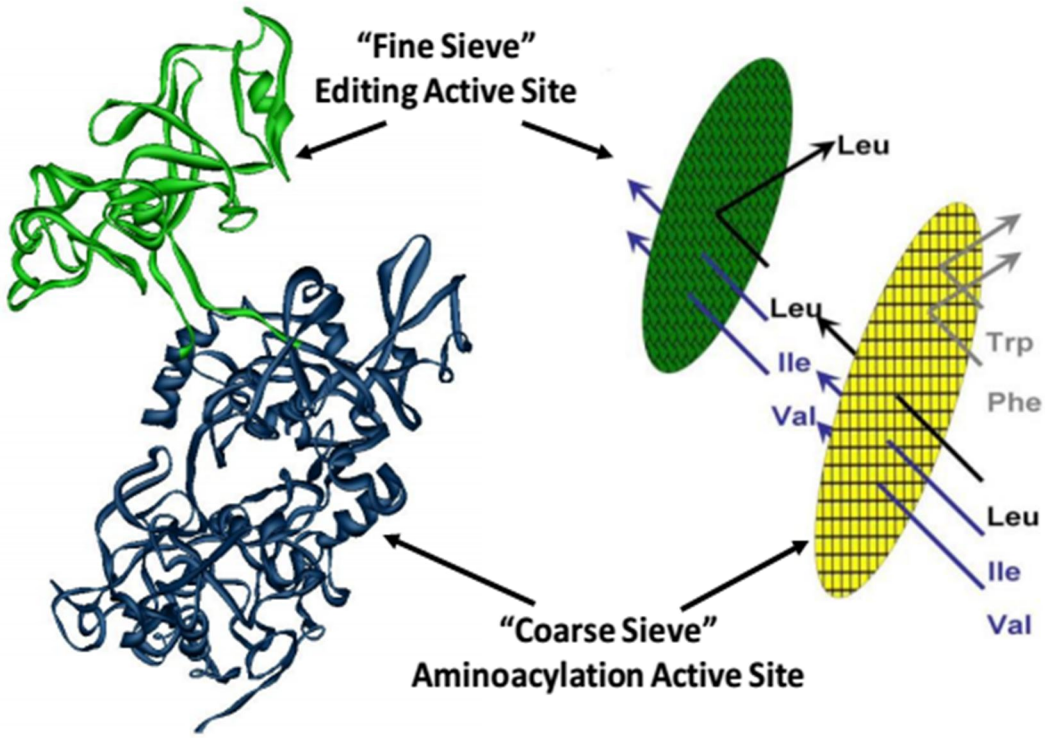

Figure 10: Yeast mitochondrial leuRS Double Sieve mechanism.

1.12.2 Elongation Factor Tu

Elongation Factor Tu is a GTPase encoded in the nucleus (TUF1 gene) that have a crucial role in the translation. This factor has the role of deliver the animoacyl-tRNA (aa-tRNA) to the translation site of the ribosome. The ternary complex of EF-Tu, aa-tRNA and GTP binds to the ribosomal A-site in the initial step of decoding. The interaction in the A-site between the codon of the mRNA and the anticodon of the tRNA leads to GTP hydrolysis by EF-Tu and subsequent dissociation of EF-Tu•GDP from the ribosome [77].

EF-Tu is composed by three domains highly conserved from *archaea* to humans. The domain I, called G domain, has the function to bind the GTP, the domains II and III are two β barrels, and they interact with the T stem and the acceptor stem of the tRNA (Figure 11).

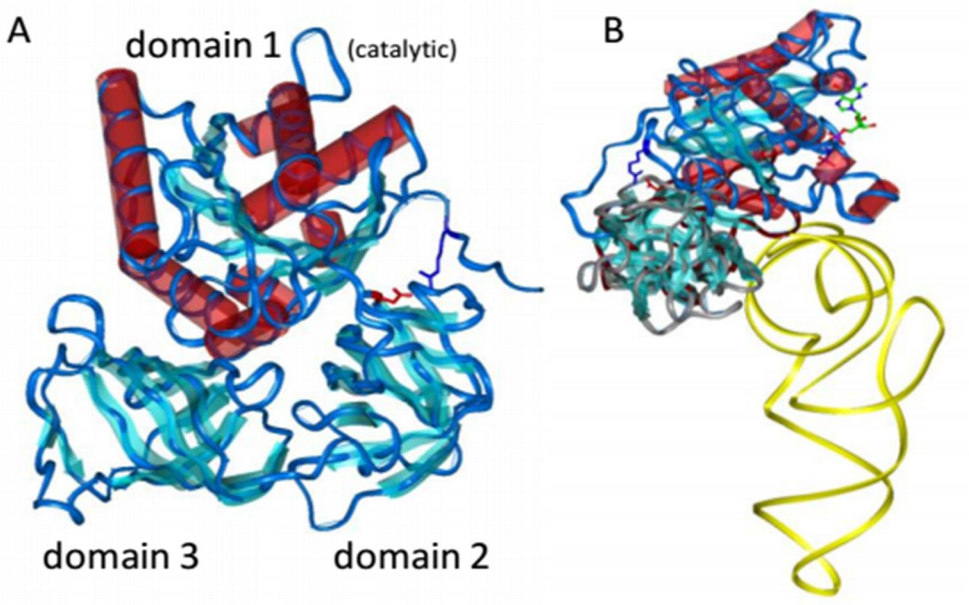

Figure 11: A- schematic structure of EF-Tu and their domains. B- Interaction between EF-Tu and tRNA.

An additional exchange elongation factor (EF-Ts) is required in human and in *Escherichia coli* (but not *S. cerevisiae*) [78]. This difference is difficult to explain in so highly conserved EF-Tu factors (Figure 12) and it has been suggested that EF-Ts could become necessary only when the expression level of the translation factors is very low, as is the case for higher eukaryotes. In spite of the high similarity between the human and the yeast factor (Figure 12) the human *TUFM* cannot substitute the yeast orthologous gene [78]. Several other, mainly regulatory, functions of the *TUF1* gene product have been described including aa-tRNA surveillance in mammalian mitochondria [79].

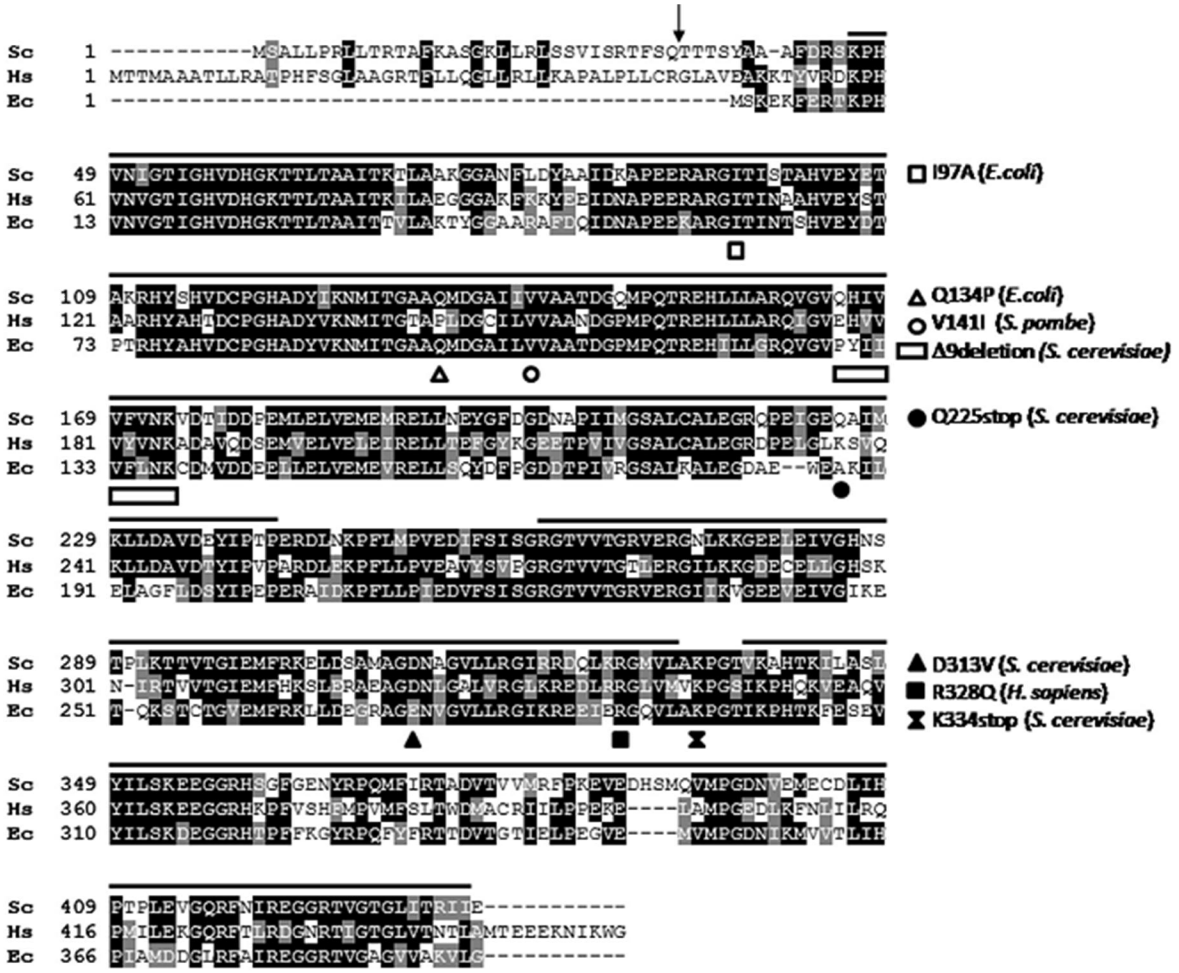

Figure 12: Alignment of yeast (Sc), human (Hs) and *E. coli* (Ec) EF-Tu.

In black are represented the conserved aminoacids, in grey different aminoacids with similar proprieties and in white different aminoacids with different proprieties. Alignment created using the web tool **ClustalW2** [80].

Material and Methods

2.1 Strains and growth conditions

***S. cerevisiae* wild-type strains:**

MCC123 MAT a, ade2, ura3-52, Δleu, kar1-1, Kan^R, rho° and rho^+ (kindly provided by Prof. M. Bolotin-Fukuhara)

D273-10B/A1 MAT α, met^-, ura3, Δleu, KanR, rho° and rho^+ [81]

W303-1B MAT α, his3-11, ade2-1, leu2-3,112, ura3-1, trp1-D2, can1-100, rho° and rho^+ [82]

FF1210-6C MAT α, ura1-2, rho° and rho+ (kindly provided by Prof. M BolotinFukuhara)

E. coli strain:

DH5α: end A, hsd R17, sup E44, thi-1, recA1, gyrA, relA1 D (lacZYA-argF), U169 (F80lacZ ΔM15).

Yeast growth media:

- Yeast Extract Peptone (YP): 1% yeast extract and 1% peptone, supplemented with:

2% glucose , 0.25% glucose, 3% glycerol, 0.1% galactose-3% glycerol or 2% raffinose

- Minimum media, 10X Yeast Nitrogen Base (WO) (6.7 g in 100 ml) 2% glucose, supplemented by the required amino acids (50X, 1 mg/ml).

E. coli growth media:

- Lysogeny broth media (LB): 1% tryptone, 1% NaCl, 0.5% yeast extract, supplemented with ampicillin (1 mg/ml) when required.

Solid media contain 1.5% agar.

2.2 Oligonucleotides

Appendix 1

2.3 Vectors

Appendix 2

2.4 DNA restriction analysis

Restriction enzymes recognize DNA specific sequences and generating fragments of variable length, having blunt or protruding ends. Each enzyme requires specific condition of buffer and temperature. Digested fragments were checked on 0.7-1% agarose gel with 1X TBE buffer (10X stock solution: 1M Tris base pH 7.4, 0.9 M boric acid, 0.01 M EDTA pH 7). The electrophoresis was conducted at 30 mA for the necessary time and the fragments were detected with Ethidium Bromide (1µl/ml) staining. Lambda phage digested EcoRI/HindIII was used as marker.

2.5 Polymerase Chain Reaction (PCR)

Two oligonucleotide primers were used to amplify specific sequences of DNA using the Polymerase Chain Reaction technique (PCR). The reaction mixture of 100 µl was prepared as follow: 1 µg of template DNA, 10 µl of 2 mMMgCl 2, 0.2 µM of each primer, 10µl of 10X PCR buffer (10 mMTris-HCl pH 9, 50 mMKCl, 0.2 ng/ml BSA). This mixture was denaturated for 5 minutes at 95°C and placed immediately on dry-ice plus ethanol; subsequently, the reaction mixture was placed at the annealing temperature of 56°C. 2µl of dNTP (0.2 mM) and 0.5 µl of Taq DNA polymerase 2.5 U were added to the mixture. The amplification reaction was: an annealing step of 2 minutes at 56°C, continuing for 3 minutes at 72°C for polymerisation, and a denaturation step at 94°C for 1 minute for denaturation. These conditions were repeated for 44 cycles. 6 µl of PCR products were checked on agarose gel.

2.6 Cytoductantcross

The mitochondrial tRNA mutants were obtained by cytoductant cross between the MCC123 rho° and different biolistic mutants. In this cross, the mutation kar1-1 prevented the nuclear fusion producing a small percentage of diploids (10%-20%) [83]. From the

cross four different cellular types could be distinguished: the original strains (MCC123 rho° and the biolistic mutants) the diploids and the new MCC123 mutant with mutated mtDNA associated with MCC123 nuclear context. The screening was facilitated by the presence of the ade2 mutation that enabled us to distinguish colonies with functional mitochondria, which accumulate a red pigment, from colonies with dysfunctional mitochondria, which appeared white [84]. The same procedure was also performed with other rho° strains crossing the MCC123 mutants with the FF1210-6C, W303-1B and 273-10B/A1 rho° and then selecting the cells with mutated mitochondria associated with different nuclear contexts.

2.7 Cultivation in bioreactor and respiration studies.

It was used a BiostatQ (B-Braun) bioreactor endowed with four 1-litre vessels, each containing 600 ml of YP 0.25% glucose and inoculated with late log pre-cultures, grown in YP containing 2% glucose, at starting cell concentrations of $0.5–1\times10^7$cell/ml. Incubations were performed at 28°C with magnetic stirring (300 rpm) and air supply (0.4 l/min).

2.8 Oxygen consumption

The oxygraph is an instrument that allows you to measure the breathing capability of a sample over time considering the progressive reduction of dissolved oxygen in a reaction chamber; the concentration change is expressed in $[O_2]$ nmol/ml. The data recorded by the instrument can be displayed in a table and a chart, whose slope is therefore index of breathe capability $d[O_2]/dt$ of the sample.

The purpose of the oxygraph experiments were to assess the breathe capability of the WT and mutant strains.

- Instrument preparing:

The instrument consists of two parts as shown in Figure 13:

- A reaction chamber, consisting on a separate thermostatable cylinder (by adding water) and a borosilicate glass cable tube, in which the sample was

inserted, that is located inside the cylinder. A special "screw" quarry closed the reaction chamber.

- A Type-Clark oxygen electrode (Hansatech Instruments). In 1956 Clark was the first to develop this technique for measuring the blood oxygen concentration.

The cathode and the anode were respectively made of platinum and of a silver ring. The electrode should be well moistened with KCl (17.5 g in 100 ml of deionised water), covered with a map to handle drum which was superimposed to a square, of the same size, of PTFT (Polytetrafluoroethylene) membrane. All the parts of the electrode were secured with a rubber O-ring. After membrane fixing KCl was added to form a salt bridge that connected the cathode with the anode. The reaction chamber and the electrode were assembled and placed on a platform connected with a USB cable to the computer.

- Sample preparation:

The samples were prepared from single colonies cells grown on plates. This method was preferred to that of growth in liquid media, because only in plates it was possible to discriminate between petite (rho^-/rho°) and rho^+ colonies.

Since the oxygen consumption is influenced by the physiological and metabolic state of the culture, the plates of *wild-type* and mutant strains were grown in identical conditions.

- Procedure:

0.03 g of cells collected from the large colonies (rho^+) were refreshed in liquid medium (0.25% glucose) for 2-3 hours. After cells centrifugation, the pellet was washed with 1 ml of sterile water in *Eppendorf*. The supernatant was removed and the pellet obtained was resuspended in 200 µl of phosphate buffer (10 mM $NaHPO_4Na_2PO_4$ and at pH 7.5 addicted with 20 mM glucose) to make it adaptable to the reaction mixture conditions. After centrifugation we removed the supernatant and the cells were resuspended in 20 µl of the same reaction buffer. 1 ml of phosphate buffer was added to the reaction chamber which was then closed with the "screw" quarry.

With a fine tip the cells were transferred in the chamber. During the experiment a small stirrer maintained the culture in constant agitation. The instrument measured the oxygen concentration every tenth of a second and the oxygen consumption trend due to cellular breath could be followed on the chart.

Figure 13: Oxygraph electrode and reaction chamber.

2.9 Stress resistance assays

Sensitivity to EGTA was performed by spotting serial dilution of exponentially growing cells on YP-2% glucose medium containing 20 or 30 mM EGTA.

Sensitivity to hydrogen peroxide was assayed by halo assay performed by plating exponentially growing cells (approximately 2×10^7 cells/ml in YP containing 2% glucose liquid medium) at high density on YP containing 2% glucose; a filter disk saturated 50 mM H_2O_2 was then placed on the medium surface. After an overnight incubation at 28°C the halo sizes of growth inhibition were measured.

2.10 Band shift

The transcription of human mt tRNALeu was performed with HiScirbe T7 transcription kit by Biolabs, substituting in the reaction mixture (final volume 40 ul) the ribonucleotide mix supplied in the kit, with 2µl of ATP, CTP, GTP 10mM each, and 2µl of

labelled UTP (γP34UTP) 10mM (from NEN NEG007H250UC). After 1 hour at 37°C 2µl of UTP 500µM were added and the incubated for 30 min more.

Then, 4.8µl of $MgCl_2$ 100 mM and 3µl of DNase (10 U/µl) were added to the reaction and let incubate for one hour at 37ºC. After that the sample was precipitated by add 5µl of sodium acetate in a concentration of 3M, pH of 4.5, 150 µl of ethanol 100% and 4µl of glycogen 20 ng/ml. After precipitation and centrifugation the pellet was eluted in TE and Geiger tested.

The bind assay was performed in an *Eppendorf* tube with 3 mRad of tRNA, 1µl of EMSA, 2µl of the tested peptide (5 µl final volume). The reaction was conducted for 1 hour at room temperature. Samples were loaded in a 15% polyacrylamide TBE gel and let it run overnight in cold room (120mV constant voltage).

2.11 Preparation and transformation of competent *E. coli* cells

A single colony of *E. coli* DH5α was inoculated in 2 ml of LB medium and incubated overnight at 37°C. 400 µl of this culture were inoculated in 40 ml of LB medium until the concentration of 0.2-0.3 OD measured at 550 nm. This culture was incubated on ice for 20 minutes and then centrifuged at 3,500 rpm for 5 minutes. The pellet was resuspended in 20 ml of a sterile ice-cold sterile solution containing 0.05 M $CaCl_2$. After 30 minutes on ice the cells were centrifuged and resuspended in 4 ml of the solution above described and incubated for 3-4 hours. This treatment causes cell wall modifications which increase the permeability of the cells to exogenous DNA. The cell pellet was resuspended in ice-cold sterile glycerol/CaCl2 solution (glycerol, 15% (v/v); 50 mM $CaCl_2$) and stored in 300 µl aliquot s at -70°C.

To transform of *E. coli* 40-50 ng of DNA were added to 300 µl of competent E. coli DH5α cells. The mixture was incubated for 40 minutes on ice. After a heat shock of 3 minutes at 42°C, which allows the exogenous DNA to enter the cells, the mixture was incubated for 10 minutes at room temperature. 1 ml of LB was added, and the cells were incubated at 37°C for 30-60 minutes. 200 µl of the mixture containing the transformed cells were plated on LB plus ampicillin, to select the transformants.

2.12 Miniprep of *E. coli* pDNA

1.5 ml of an *E. coli* DH5α overnight culture was centrifuged and resuspended in 100 µl of a solution containing 50 mM glucose, 10 mM EDTA, 25mM Tris-HCl pH 8. After 5 minutes at room temperature, 200 µl of a fresh solution, containing 0.2 M NaOH, 1% SDS, were added. After 5 minutes in ice, 150 µl of ice-cold high salt buffer (3M K-Acetate, 5 M Acetic Acid) were added and the mixture was incubated for 5 minutes on ice. After 5 minutes of centrifugation at 13,000 rpm, two volumes of 95% ethanol were added and the mixture was centrifuged for 10 minutes. The pellet was washed with 70% ethanol, dried and resuspended in 30 µl of 1X TE containing RNase (20 µg/ml). 2 µl were checked on agarose gel. This preparation of DNA can be stored at -20ºC.

2.13 Maxiprep of *E. coli* pDNA

To obtain large amount of *E. coli* DH5α purified plasmid DNA, a colony from transformed cells was inoculated in 50 ml of LB ampicillin and incubated overnight at 37°C. The pDNA was extracted following the instructions of the QiagenHiSpeedPlasmid Maxiprep Kit manual. The protocol is based on a modified alkaline lysis procedure, followed by binding of DNA to Qiagen anion-exchange resin on appropriate low-salt and pH conditions.

The pDNA was then simply eluted from the column with TE pH 7.4.

2.14 Transformation of *S. cerevisiae* using LiAc procedure

Yeast cells were grown in 100 ml of YP-2% glucose medium to $5x10^6$-$2x10^7$cells/ml (log phase). The pellet obtained after centrifugation for 5 minutes at 3,000 rpm was washed with 10 ml of TE and resuspended in 1ml of TE containing 0.1 M LiAc.

After 60 minutes at 30°C, 25µl of cells were transferred into *Eppendorf* tubes. 5 µl of 10 mg/ml salmon sperm DNA, as a carrier, and the appropriate amount of pDNA (2-10 µg) were added to the cells.

After an incubation of 30 minutes at 30°C, 1 ml of 40% PEG 4000 was added and the mixture was incubated for 60 minutes at 30°C. 5 minutes at 42°C provided the heat shock necessary to allow the pDNA to enter the cells. The cells were then centrifuged for 1 minute at 3,000 rpm and washed with water twice.

300 µl of YP containing 2% of glucose was added to the pellet and the cells were plated on selective media.

A spot of non-transformed cells on selective media was made for control.

2.15 DASPMI staining

Cells from exponential cultures were stained with 1 µM DASPMI (2-(4 dimethylaminostyryl)-N-methylpyridinium iodide), as described by Rafael and Nicholls [85], and were observed immediately by fluorescence microscopy at the wavelength of 350 nm.

2.16 Growth curves

Growth curves are graph in which biomass drift was reported, measured by Spectrophotometer as Optical Density (OD 600 nm) versus time (h). Optical Density measurements were performed for each culture beginning at time t=0 hours and repeating the measure every two or three hours.

2.17 Life span curves

For chronological lifespan determination, a single colony was inoculated in YP containing 2% glucose or 2% of raffinose liquid medium. As the culture reached the stationary phase, the same volumes of 10^{-5} dilutions were plated every two-three days. Colonies were counted after 4-days incubation at 28°C. In the same plates, we discriminated the *petite* colonies formation from the rho^{+} and we calculated the $rho°/rho^{-}$ percentages. Data were mean values of three independent experiments and standard deviations were calculated. Statistical analysis was performed with the Student's t-test.

2.18 Suppression activity

For study of cross suppression it was used the MCC123 strain. After transformation the cell were plated on selective WO medium and let them to grow for three or four days. After that the colonies were transferred on YP plates containing 3% of glycerol, and grow for five days at 28ºC and 37ºC.

Results and Discussion

3.1 *Wild-type* nuclear backgrounds of *Saccharomyces cerevisiae* affect strain phenotypes and mitochondrial "pathogenic" tRNA mutations

3.1.1 Comparison of growth, cell morphology and respiration among *wild-type* strains

Different nuclear backgrounds have different responses to mitochondrial point mutations [53]. To understand better what is the cause we decided to do some preliminary assays among the WT strains. I evaluate the capability of three WT strains (MCC123, W303-1B and D273-10B/A1) to grow on a strictly respirable medium (YP containing 3% glycerol as carbon source), and on fermentative medium (YP with 2% of glucose) and at last the respiration capability of each strain.

First of all is important to emphasize that on glucose all the three strains have a similar growth capacity either at 28ºC and 37ºC. However in glycerol medium two of the three strains (MCC123 and D273-10B/A1) grew slowly compared to the W303-1B and this effect is much evident when the plates are cultivated at 37ºC. So we can conclude that these three strains have a similar fermentative profile but a very different respirable profile (Figure 14).

Was also analysed the morphology of each WT strain by microscopy, using DASPMI staining that permit to visualize active mitochondrial membranes.

Figure 15 shows the different size of the cell strains, where D273-10B/A1 cells are smaller and more elongated. Whereas the MCC123 and W303-1B cells were round and occupied largely by a vacuole. This was especially evident for the MCC123 strain where the mt network was squeezed between the cell wall and the vacuole.

In the others strains we can see the mitochondria more scattered throw the cell. Importantly we observed the D273-10B/A1 cells have a mitochondrial network more complex than the others strains.

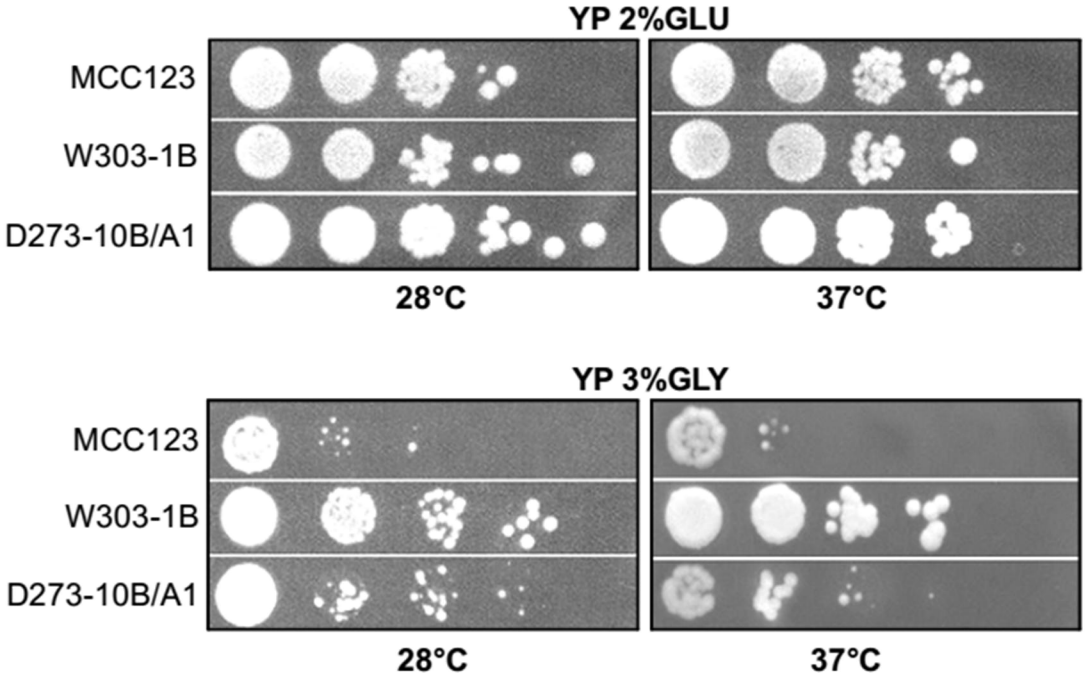

Figure 14: Growth capability of WT strains.

Serial dilutions of cultures from three WT strains were spotted on YP plates containing 2% glucose or 3% glycerol as carbon sources and incubated at 28°C and 37°C as indicated. Pictures were acquired after for 2-3 days of growth for plates containing glucose, and after 5 days of growth for plates containing glycerol as carbon source.

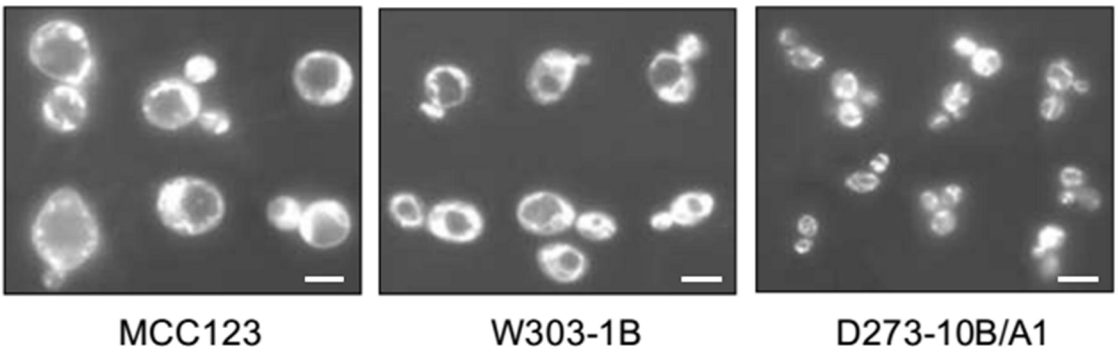

Figure 15: Wild-type cell's morphology.

Image obtained by fluorescence microscopy using DASPMI staining. Magnification is the same in all pictures. Bar = 10 μm.

To better understand the differences observed in growth capability the oxygen consumption was evaluated. 0,03g of resting cells, previously grown in YP media containing 0.25% or 2% glucose as carbon sources (Figure 16), were loaded in an Oxygen Reaction Chamber.

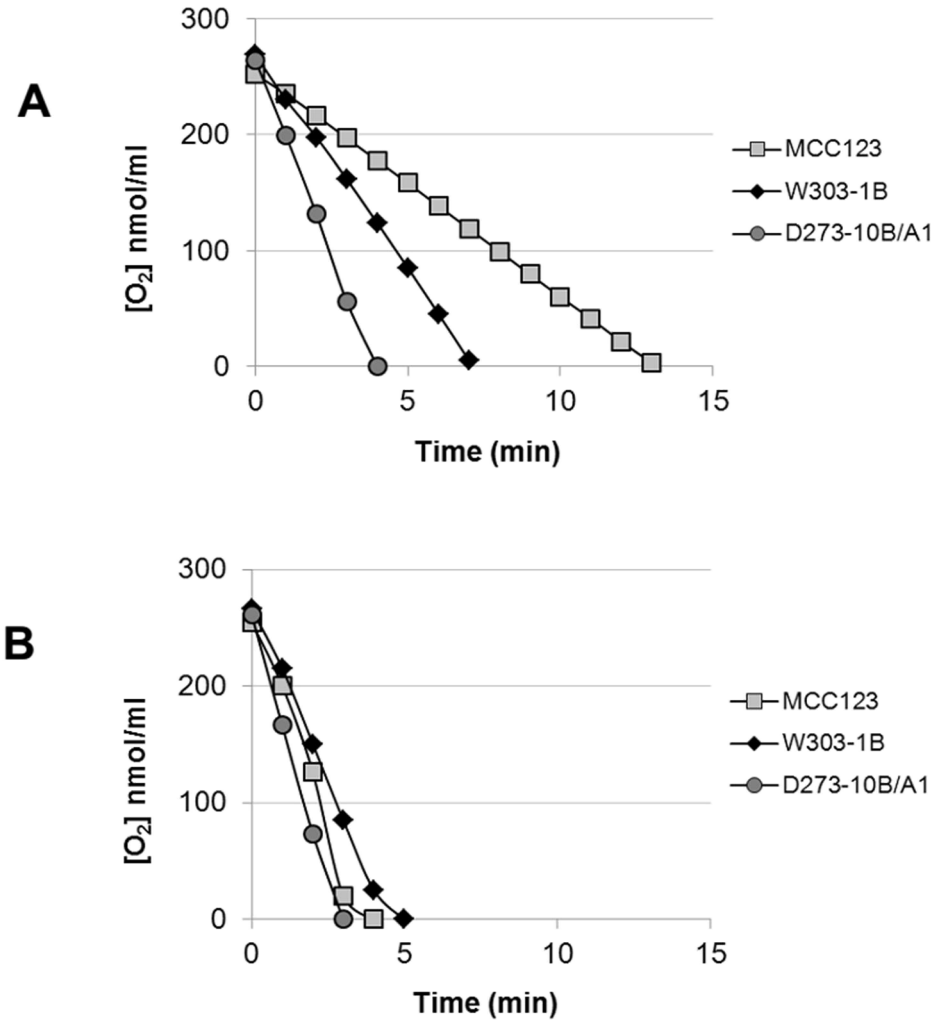

Figure 16: Oxygen consumption curves of resting WT strains.
Comparison of Oxygen consumption rates in reaction chamber of WT cells in resting condition. Cells were previously grown in YP medium containing 2% (A) or 0.25% glucose (B).

Surprisingly among the WT strains the oxygen consumption rates were different (D273-10B/A1 > W303-1B > MCC123; see Figure 16A) for cells grown on

fermentative/glucose-repression conditions (2% glucose) whereas minor differences were observed when cells were allowed/forced to respire by the low glucose concentration (0.25%; see Figure 16B).

To verify if the difference in growth capability observed on YP plates containing 3% glycerol as carbon source (Figure 14) could be ascribed to different cell duplication times due to possible variable respiratory efficiency, we performed the growth curve in bioreactor for the three WT strains, measuring the optical density of the cultures. In parallel we monitored the dissolved oxygen concentration with in situ electrodes. The experiment was performed in YP medium containing 0.25% glucose. Low glucose concentration was chosen to minimize the glucose repression and allowing respiratory metabolism (Figure16). We did not use glycerol that is metabolized only by respiring cells in order to permit the comparison of growth capability with mt mutant strains unable to grow on this carbon source (see below).

Figure 16 shows that indeed using a low concentration glucose medium (0.25% instead 2%) the cells use the respiratory pathway in advantage of fermentative pathway.

In the figure 17A is represented the cellular density by time of all three WT strains. The WT strains have a similar growth curve. The D273-10B/A1 cells reach first the stationary phase at 16.5 hours followed by MCC123 at 19.1 hours and at last W303-1B at 22.75 hours. That could be explained by small differences in the exponential phase.

Comparison of the growth curves (Figure 17A) showed a substantial difference in the duplication rate (calculated at the exponential growth phase) for the MCC123 of 1.8 h and the W303-1B of 2.8 h with the D273-10B/A1 showing an intermediate duplication rate of 2.1 h. The OD yields at the stationary phase were 9-9.5 for the D273-10B, 6.2 for the MCC123 and 5.5-5.7 for the W303-1B.

Oxygen consumption was faster and continuous in the D273-10B/A1 and MCC123 cultures compared to the W303-1B, where oxygen consumption slowed down at the end of the exponential growth before entering in the stationary phase (Figure 17B). All together the profiles of figure 17 shows that D273-10B/A1 culture rapidly reached the stationary phase and abruptly stopped to respire after 16.5 h, whereas the W303-1B strain shifted slowly to stationary phase after 22.75 h. The MCC123 culture showed a fast oxygen consumption similar to the D273-10B/A1 but with a difference in the extent of respiration;

in fact the MCC123 cells continued to respire also in the beginning of the stationary phase until 19.1h.

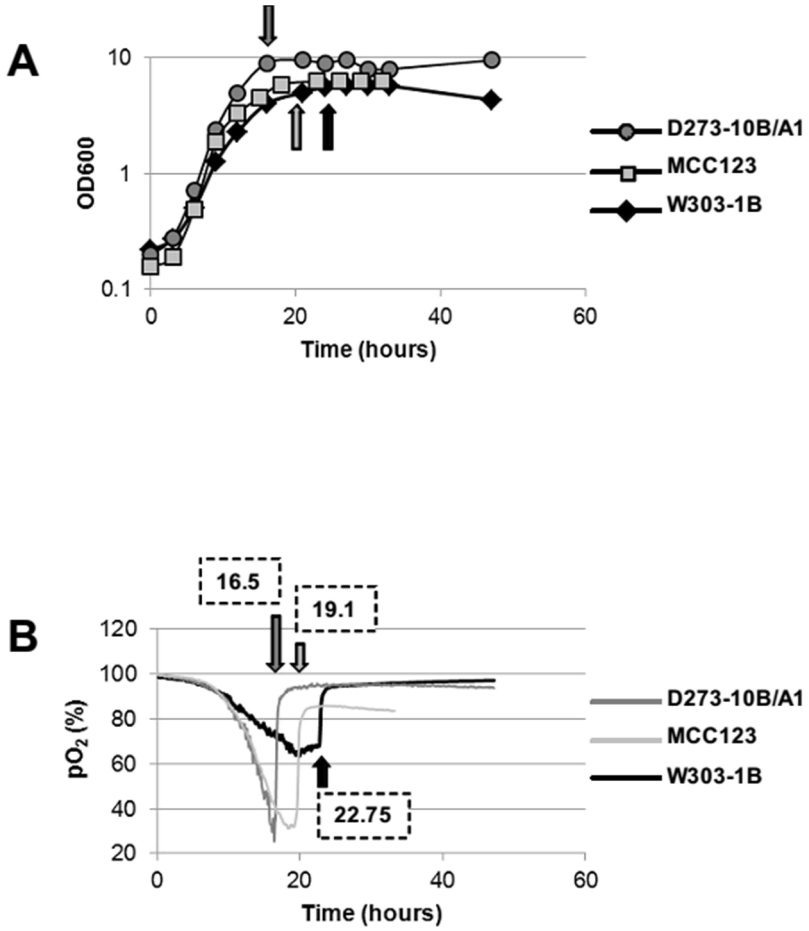

Figure 17: Cultivation in bioreactor of WT cells.

Comparison of growth curves and of O_2 consumption rates of WT strains (panels A and B) performed in YP- 0.25% glucose containing medium. In panels B the times of respiration arrest are indicated by arrows flagged with time values. The same symbols are reported in panel A at the corresponding time points.

3.1.2 Comparison of growth and respiration among mitochondrial tRNA mutants

After evaluate the growth of WT strains the next step was to evaluate to evaluate how the different nuclear context can influence the respiratory phenotype of the cells bearing an identical mt tRNA substitution. The bioreactor was inoculated with mutant cells bearing the tRNA valine substitution C25T. This mutation is equivalent to the human mutation m.1624C>T that cause Leigh Syndrome [86]. We introduced the mutation from the MCC123 nuclear context (M/ValC25T) to W303-1B and D273-10B/A1 rho^{o} cells strains by cytoduction. The mutants were named by their mutation type and position, according to the standard tRNA numbering [87] and the first letter corresponded to their isogenic WT strain.

Previously in this research unit was studied the phenotype caused of the mutant LeuA29G, corresponding to the human mutation m.3260A>G. This mutation is responsible for causing the myopathy and cardiomyopathy (MMC) disease [88]. This mutation has different levels of severity of respiratory defects according to different nuclear contexts. The severity followed this trend: D273-10B/A1 > MCC123 > W303-1B. This behaviour should be ascribed only to the influence of the nuclear background since the mutated mitochondria were transferred by cytoduction crosses in the different rho° strains. By following this procedure, both nuclear and mitochondrial inter-strains recombination was prevented [53].

The most affected mutant was D/ValC25T with a short exponential phase with only 8.25 hours of growth and with a duplication time of 2.8 hours. On the contrary the optic densities of M/ValC25T and W/ValC25T strains have similar duplication time as shown by the growth curves (Figure 18).

D/ValC25T strain only had 8.25 hours using the respiratory pathway instead in W/ValC25T only stopped respiring after 26.5 h, similarly to the isogenic WT and the M/ValC25T exhibited an intermediate behaviour with an arrest in respiration after 19.7 h (Figure 18B). The W303-1B and MCC123 strains maintained more or less the same time respiring compering to the WT strains and so not suffer any problem in there growth rate (Figure 18A).

Despite that, the rate of oxygen consumption decreases with a maximum of consumption in M/ValC25T of 40%, in W/ValC25T of approximately 20% and D/ValC25T only about 2% (Figure 18B).

In fact, growth of D/ValC25T in YP with 0.25% of glucose containing medium was supported only for the early exponential growth phase when presumably the cells consumed the endogenous pool of metabolites until exhaustion.

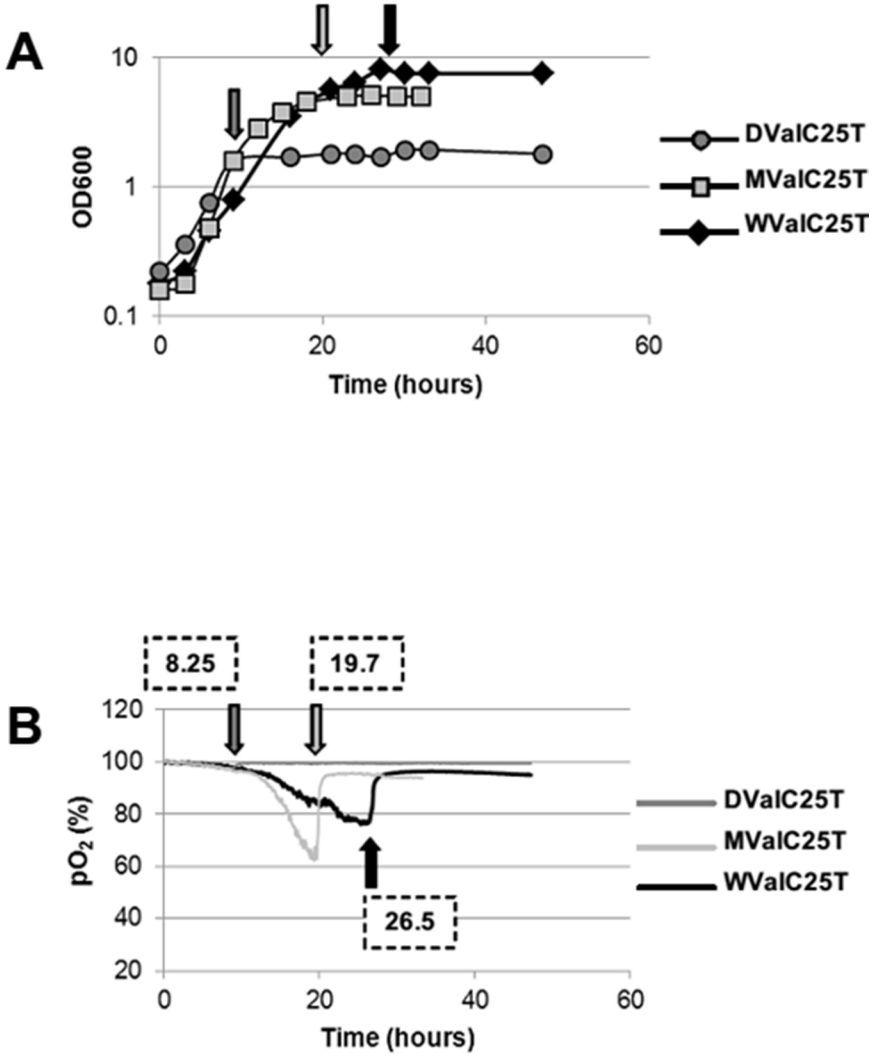

Figure 18: Cultivation in bioreactor of mutant cells.

Comparison of growth curves (panel A) and of O_2 consumption rates (panel B) of W/ValC25T, M/ValC25T and D/ValC25T mutants performed in YP containing 0.25% glucose containing medium. In panels B the times of respiration arrest are indicated by arrows flagged with time values. The same symbols are reported in panel A at the corresponding time points.

To compare the growth capability of ValC25T, LeuT20C and LeuA29G tRNA mutants, serial culture dilutions was spotted on plates containing 3% glycerol as carbon sources and incubated at 28 and 37°C (Figure 19). The LeuT20C is a human equivalent mutation(m.3250T>C) responsible for MM/CPEO disease, obtained by biolistic transformation [89].

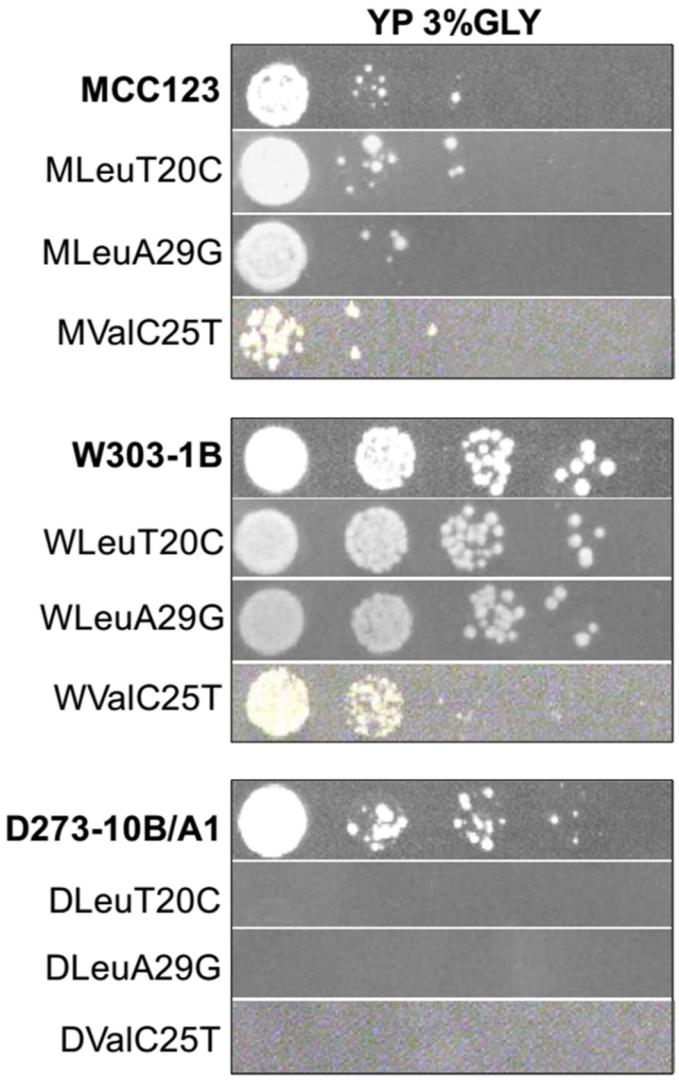

Figure 19: Influence of three different nuclear backgrounds on the glycerol growth phenotype of mt tRNA mutants.

Serial dilutions of LeuT20C, LeuA29G and ValC25T yeast mutant strains, having MCC123, W303-1B and D273-10B/A1 as nuclear background, grown overnight in YP liquid medium containing 2% glucose, were spotted on a YP plate containing 3% glycerol and incubated at 28 °C for 5 days.

In the MCC123 and W303-1B nuclear backgrounds the mutations did not avoid the growth in glycerol containing medium with the exception of W/ValC25T that showed mild defective growth. But in the case of D273-10B/A1 nuclear background the mutant is unable to grow in glycerol medium (Figure 19).

It means that the D273-10B/A1 nuclear background is more susceptive to mutations than other two contexts.

In conclusion, the ValC25T mutation in the D273-10B/A1 nuclear background did not permit respiration; in the MCC123 background, the mutation induced a significant decrease of respiration (from 20 to 60% of residual O_2) while in the W303-1B nuclear background had only a minor effect.

3.1.3 Rho$^-$/rho° production in WT and tRNA mutants

Naturally the *S. cerevisiae* cells can lose partially or totality their mitochondrial DNA, generating rho$^-$ and rho^o cells respectively. In table 2 is represented the percentage of production of *petite* colonies by the studied strains (WT and mutant strains). It is important to mention that inhibition of mt protein synthesis increases the formation of *petite* mutants [90]. Because of that the percentage of formation of *petite* colonies within a specific nuclear context could be one indicator of the importance of the mt protein synthesis defect [53].

As it is possible to observe in Table 2, all the WT strains produce in average 2% of *petite* colonies after a growth overnight in YP containing 2% of glucose. This percentage is maintained in the D273-10B/A1 derived mutant. However a higher percentage of *petite* colonies is observed in mutants bearing the MCC123 nuclear context that reach a percentage of 45% of *petite* colonies for the M/LeuA29G mutation. Mutants with W303-1B nuclear context the productions of rho$^-$/rho^o colonies are not so higher than MCC123 nuclear context but reach 22% of production in the mutant W/ValC25T. That demonstrated that in those two nuclear backgrounds the mt genomes are very unstable depending on the mt tRNA substitution.

This behaviour has been observed for other mt tRNA mutations and the rho° production could reach 80% for the human equivalent MELAS tRNA leucine substitutions [52, 72].

Table 2: *Petite* colonies formation, in percentage, of WT and mutant strains.
The cells were plated in YP with 2% of glucose after an over-night growth in liquid YP with 2% of glucose [52, 53].

Strain	rho$^{-/o}$ percentage	Reference
MCC123	2%	Montanari et al. 2008
M/LeuT20C	35%	Montanari et al. 2008
M/LeuA29G	45%	De Luca et al.2009
M/ValC25T	27%	De Luca et al.2009
W303-1B	2%	Montanari et al. 2008
W/LeuT20C	13%	Montanari et al. 2008
W/LeuA29G	11%	This Thesis
W/ValC25T	22%	This Thesis
D273-10B/A1	2%	Montanari et al. 2008
D/LeuT20C	2%	Montanari et al. 2008
D/LeuA29G	2%	This Thesis
D/ValC25T	2%	This Thesis

3.1.4 Stress resistance assays comparison

The next step was to compare the capability of the strains to survive in media with toxic agents. First one we measured the halo size obtained when we put in the centre of the plate a filter disk containing 50 mM of oxygen peroxide (H_2O_2) and in the second one it was assayed the capability of growth in plates with 20 mM or 30 mM of EGTA.

Due to the respiratory process the mitochondria produce extremely oxidant compounds, such as superoxide (O_2^-) and oxygen peroxide, for that the cell needs to have a

pathway to degrade this oxidant compounds. Superoxide dismutase degrades the O_2^- whereas cytochrome c peroxidase and the catalase detoxify the H_2O_2 [91]. We studied the growth of the different strains in the presence of H_2O_2.

The results showed that strains having defective respiration (rho° cells and mt tRNA mutants) were in general all more sensitive to H_2O_2 compared to the isogenic WT (Figure 20). However, in the W303-1B background, where the presence of rho° mitochondria produced the higher increase of sensitivity, the mt tRNA mutations did not cause a significant variation of sensitivity compared to the isogenic WT. Only in the MCC123 background it was possible to observe a variable sensitivity among the different mt tRNA mutants, with the LeuA29G substitution exhibiting the higher sensitivity.

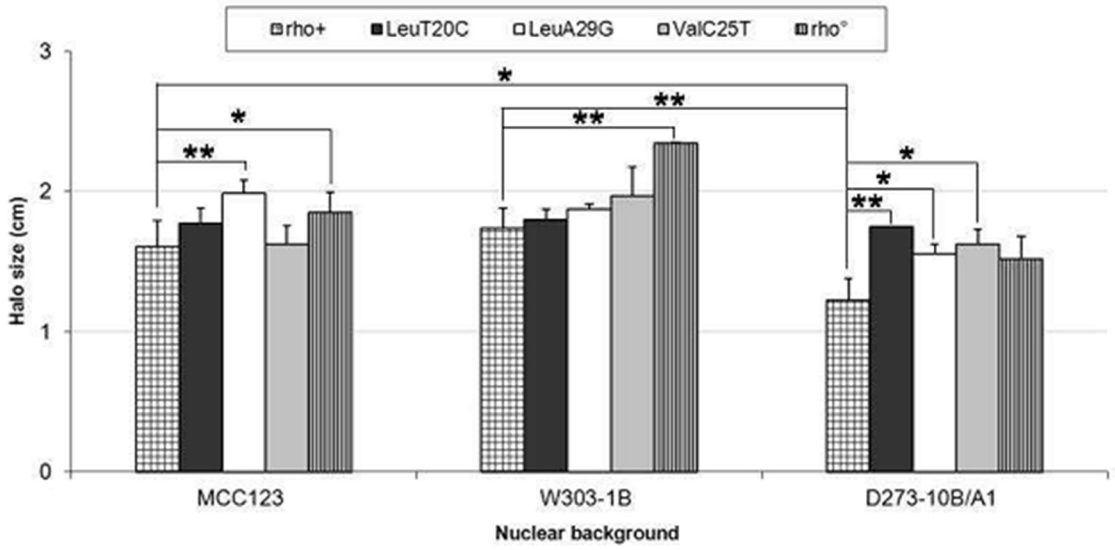

Figure 20: Hydrogen peroxide sensitivity of WT, rho° and mt tRNA mutants in MCC123, W303-1B and D273-10B/A1 nuclear backgrounds.

Comparison of the halo sizes of H_2O_2 growth inhibition obtained by plating $2x10^9$cells from exponential cultures. The values of halo sizes, expressed in cm, are means (with standard deviations) obtained from three independent experiments. * ($p< 0.05$) and ** ($p<0.01$) indicate the statistical significance of differences calculated by comparing the strains linked by the bars.

Mitochondria have one important role in the calcium ions homeostasis [4, 5]. To evaluate how the different nuclear contexts could influence this Ca^{2+} homeostasis we used

one cation chelator agent (EGTA) in the medium, to compare the growth capacity of the yeast strains (with the mutations and WT) in YP medium with 2% of glucose, with two different EGTA concentrations (20mM and 30mM) at 28ºC and 37ºC.

All the WT strains were resistant to 20mM of EGTA, and they could also grow in 30mM. In the opposite situation are the rhoº mutants; only the D273-10B/A1 rhoº grew in a concentration of 20mM.

The MCC123 mutants are completely sensitive to EGTA, neither one of the mutants grew in the presence of this cation chelator. In 20mM of EGTA the growth both, W303-1B and D273-10B/A1 mutants, was allowed; however in 30mM of EGTA only the D273-10B/A1 mutants were capable of grow.

The results shown figure 21 are related only to the ones incubated at 28ºC, because the temperature did not influence the EGTA restriction and so the results are not shown.

The data suggest that, at least at 28ºC, D273-10B/A1 nuclear background probably has an efficient mechanism of intracellular Ca^{2+} release also in absence of mt protein synthesis, because these was the only nuclear context where rhoº cells grew in EGTA.

The role of mitochondria in intracellular signalling has implications in development, aging, disease and environmental adaptation. Retrograde regulation is the general term for mt signalling and is broadly defined as cellular responses to changes in the functional state of mitochondria. Mt signalling is opposite in direction to that of the more familiar anterograde regulation characterized by the transfer of information and factors from the nucleus and cytoplasm to mitochondria.

Retrograde responses depend on metabolic signals or more direct pathways, such as mitochondria related changes in intracellular Ca^{2+} dynamics or in the efficiency of mt macromolecular synthesis, all of which produce changes in nuclear gene expression. These retrograde responses are mostly adaptive and represent cellular adjustments to altered mt states [92].

Due to the multiplicity of organelle functions, a variety of interlinked anterograde and retrograde pathways can be expected [93]. The extent to which different signals can be integrated into common pathways is not clear.

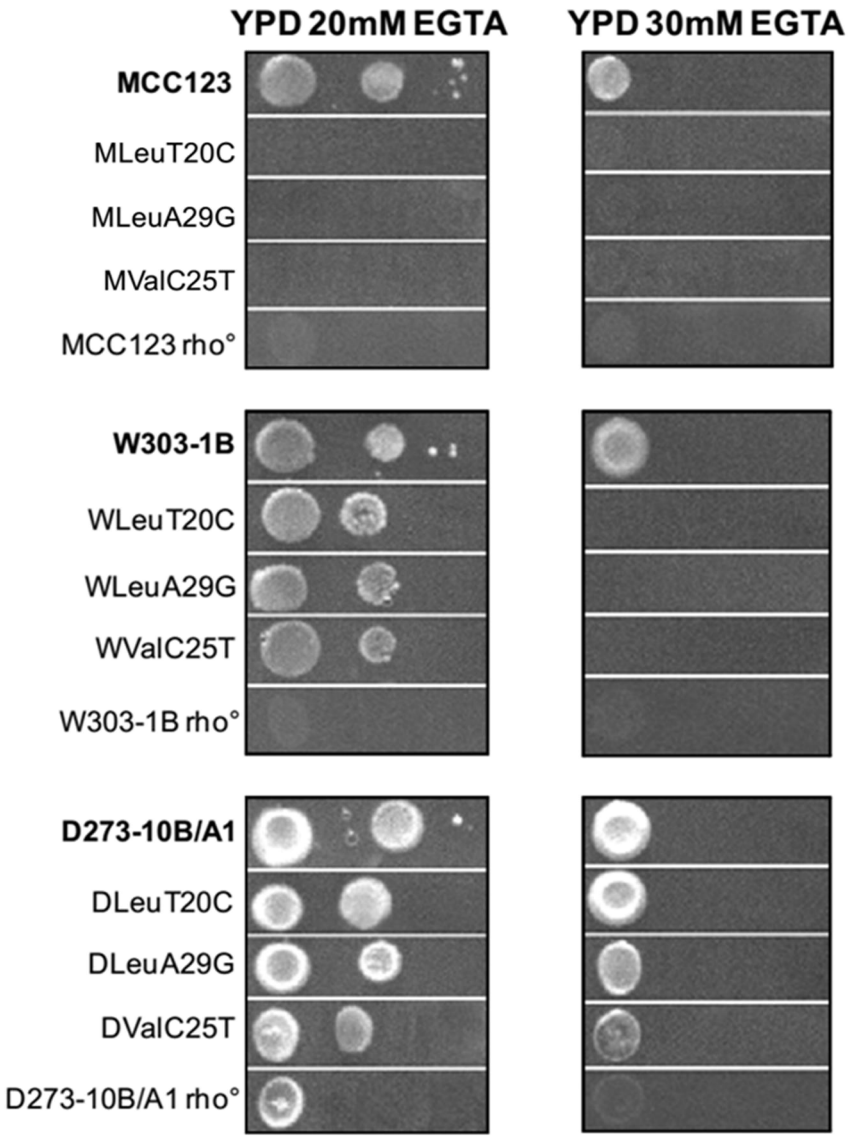

Figure 21: EGTA sensitivity of WT, rho° and mt tRNA mutants in MCC123, W303-1B and D273-10B/A1 nuclear backgrounds.

Exponential cultures grown overnight in YP liquid medium containing 2% glucose, were spotted on plates containing EGTA as indicated. Plates are incubated at 28°C for four days.

3.1.5 Chronological lifespan and petite production in WT strains

Nowadays mitochondria functionality is considered a very important contributor to longevity both in yeast and other eukaryotic organism and is connected with the general problem of diet restriction and aging.

As a contribution to this open discussion we wanted to analyse the chronological life span of the three WT and rho° isogenic strains in YP containing 2% glucose. Over the time there were evaluated the total number of colonies (viability) and the percentage of *petite* colonies formation from rho^+ cells.

Figure 22A shows that the viability of D273-10B/A1 and MCC123 decreased in 15 days while the viability of W303-1B was almost constant up to 30 days and reached 0 after 50 days. Figure 22B shows that in this strain a high increase of petite colonies were observed, starting after the fifth day and continuing up to the 35 days of growth. The profile of the first five days confirmed the similar percentage of petite formation (2%) reported in Table 2.

It might be proposed that *petites* can generate a lower oxidative stress than WT cells [94, 95] but we note that the petite formation reached about 7% in the first 30 days of growth. Later, when the W303-1B cells started to die, we observed an increase of rho°/rho^- cells (up to 15%) which did not account for the high longevity of W303-1B cells.

These results demonstrate that W303-1B cells have a higher longevity, but with a higher mitochondrial DNA loss (Figure 22B).

Interestingly, the longevity of the three rho^o cultures have a similar longevity so the MCC123 and D273-10B/A1 WT strains (Figure 23). It is possible to conclude that the increase of *petite* colonies formation in not a signal for the senescence of the cells.

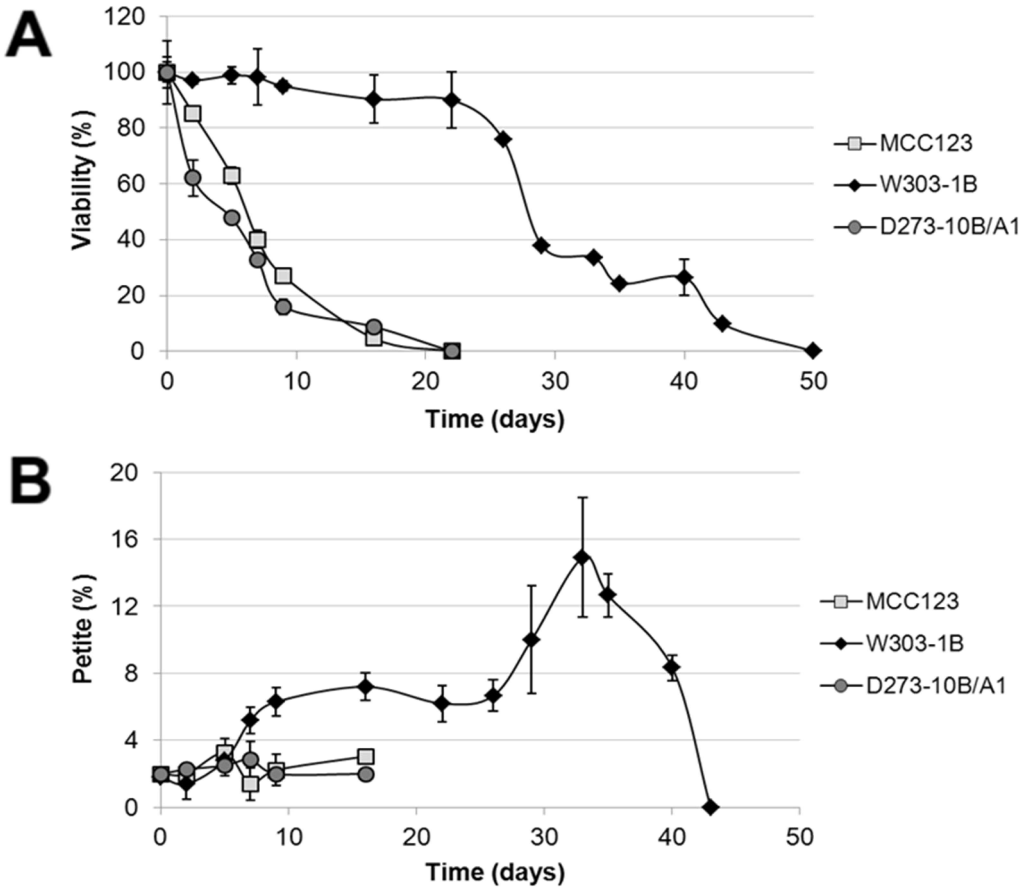

Figure 22: Chronological lifespan and petite formation of WT strains.

Panel A: comparison of chronological viability of WT strains grown in YP liquid medium containing 2% glucose, reported as percentages of colony forming units. Stationary phases were taken as 100%. Panel B: comparison of petite colonies formation during chronological survival as in panel A. For each analysis (A, B) the same aliquots of cultures were diluted and plated on solid medium. Large and *petite* colonies forming units were counted after 3 days. Data are means of at least three independent experiments, with reported standard deviations bars.

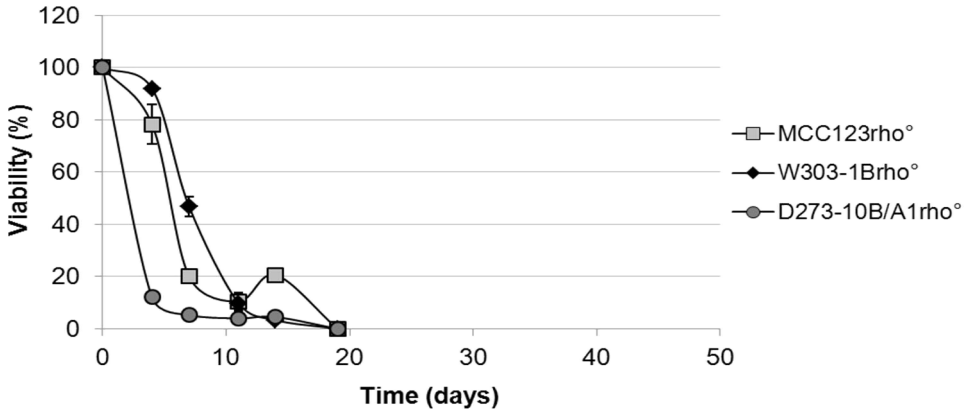

Figure 23: Chronological lifespan of rho^0 cells.
Comparison of chronological viability of rho^0 mutants grown in YP liquid medium containing 2% glucose, reported as percentages of colony forming units. Stationary phases were taken as 100%. For each analysis the same aliquots of cultures were diluted and plated on solid medium. Colonies forming units were counted after 3 days Data are means of at least three independent experiments, with reported standard deviations bars.

3.1.6 Nuclear context and the response to a point mutation

After we demonstrated that the absence of mt protein synthesis is not a signal for the senescence of the cells, it was decided to analyse the effect in the viability and *petite* colonies formation of mutant with defective mt protein synthesis. For this experiment were used W303-1B WT strain and two isogenic mutants, a mt tRNA mutant (W/ValC25T) and a nuclear mutant affecting a subunit of the cytochrome C complex (Oxi1) of the mt respiratory chain (W/TF145). Differently from the data shown in Figures 22 and 23, YP medium with 2% of raffinose was used in order to avoid the glucose repression.

As expected the WT W303-1B has high viability that is maintained this until more or less 20 days decreasing almost to 0% in 37 days. Interestingly the mutant's cultures lost the viability faster, with a similar trend like we have observed for the WT MCC123 and D273-10B/A1 grown in glucose containing media (Figure 24A). This demonstrates once more that W303-1B cells lose their higher longevity in the presence of defective mitochondria and that it is not important where the mutation is located (Mitochondria

versus nucleus). In raffinose the longevity and *petite* colonies formation trend is similar to that observed in 0.25% glucose containing media.

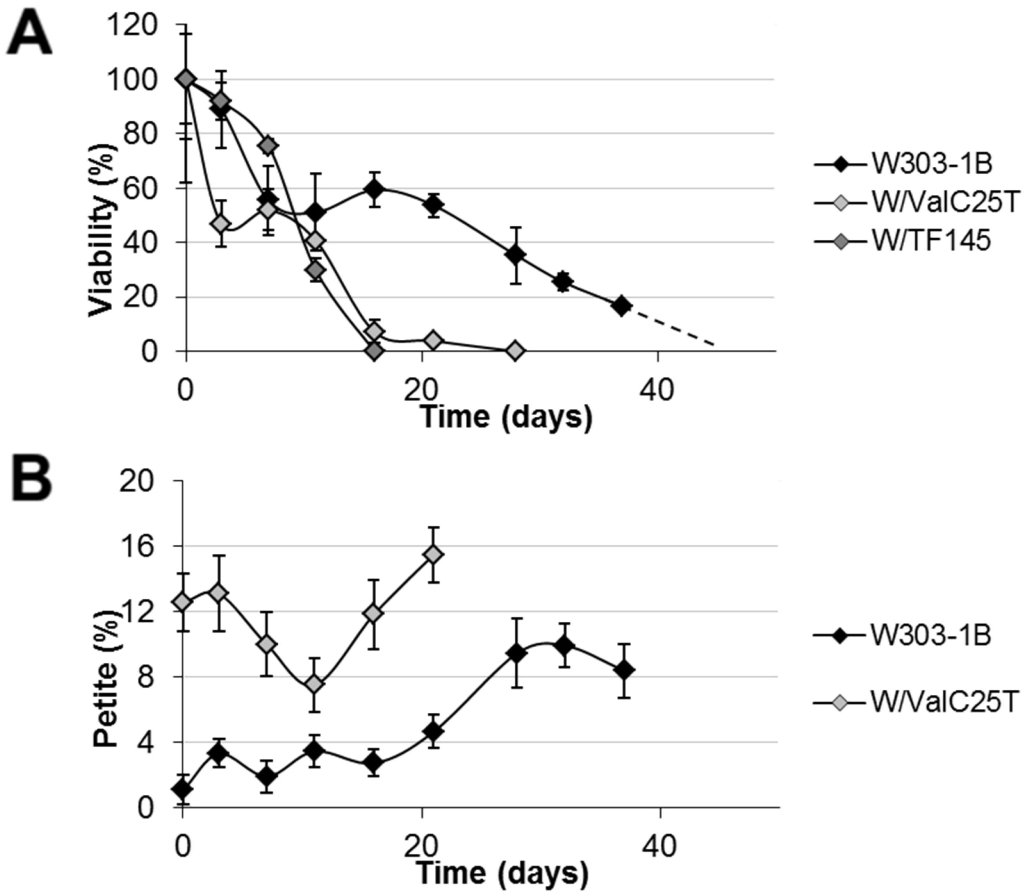

Figure 24: Chronological lifespan and petite formation of mutant.
Panel A: comparison of chronological viability of mutant strains grown in YP liquid medium containing 2% raffinose, reported as percentages of colony forming units. Stationary phases were taken as 100%. Panel B: comparison of petite colonies formation during chronological survival as in panel A. For each analysis (A, B) the same aliquots of cultures were diluted and plated on solid medium. Large and *petite* colonies forming units were counted after 3 days. Data are means of at least three independent experiments, with reported standard deviations bars.

Compare to the isogenic WT strain W/ValC25T produces a higher percentage of *petite* colonies reaching 14%. Differently from the 16% observed in glucose containing

media the WT cells grown in raffinose containing medium reach only about 10% in *petite* colonies formation and only in the last days of viability (Figure 24B) suggesting that indeed the glucose repression pathway is positively involved in *petite* formation.

In conclusion it is possible that a mitochondrial disorder (nuclear or mitochondrial mutation) leads to a decrease of life span in yeast. This could be related to mechanisms of usage of the carbon source (fermentation and respiration), but also to the fact that the mitochondria have an important role in the homeostasis, and in the production of important metabolites [4].

3.2 Cross suppression

3.2.1 Cross suppression of tRNA mutants aminoacylated by classII aaRS

It is proved in yeast that the some class I of aaRSs (IleRS, LeuRS and ValRS) have a suppressive effect on mutated phenotype due to point mutations in cognate or not cognate mt tRNA isoleucine, leucine and valine genes. The aaRS could correct and stabilize the tertiary structure of the mutated tRNA acting as a chaperone-like [96]. However, there is no evidence how this effect is extended or if it is present also in mutated tRNAs aminoacylated by class II aaRS. For this reason I performed suppression experiments with class II aaRS and cognate (or not cognate) tRNAs with point mutations obtained by random mutagenesis. These mutations are not correlated with any human disease. They only serve to study the suppressive mechanisms.

For the tests we studied the capacity of the cells to grow in a strictly respirable medium (YP containing 3% of glycerol).

To study the suppressive effects of class I and class II aaRSs it was used three different tRNA mutants in different positions, AspC61T and HisG51A, mutations in TΨC arm and GlyG30A mutated in the anticodon arm, as shown in the figure 25 and the isogenic MCC123 strain as control.

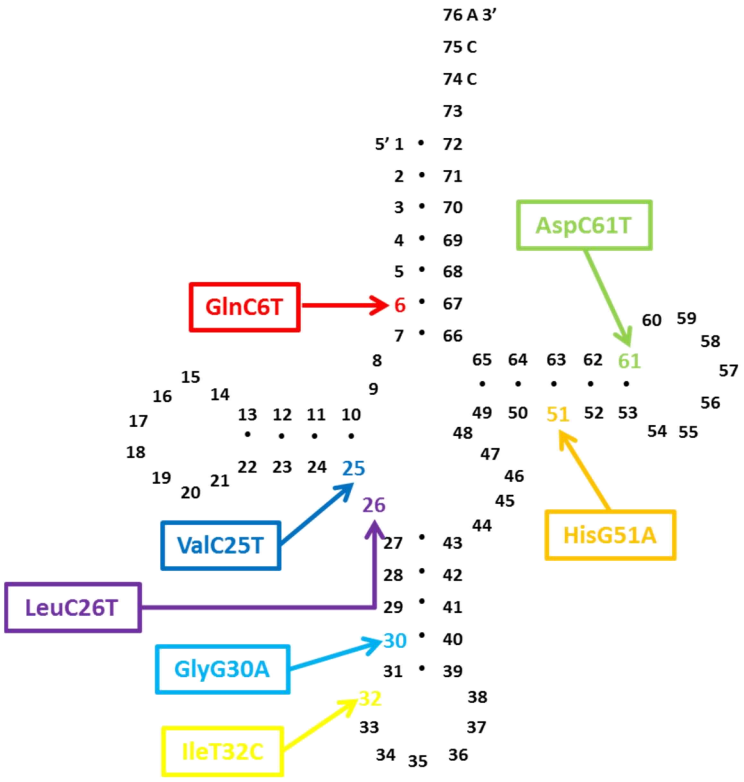

Figure 25: Positions in the mt tRNA of the point mutations GlnC6T, ValC25T, LeuC26T, GlyG30A, IleT32C, HisG51A and AspC61T.

The suppression experiments were conducted by transformation of yeast competent cells with different plasmids carrying the mt aaRS, after that they were plated in a selective solid medium at 28ºC. After three or four days we transferred the grown colonies to YP solid medium containing 3% of glycerol at 28ºC and 37ºC. The results were analysed after five days of growth.

Table 3 shows the growth capacity of three mt tRNA mutants overexpressing the genes HTS1, MSD1, MSK1 and MSF1 (yeast genes that encode for mt HisRS, AspRS, LysRS and PheRS respectively).

Only the defective phenotype of GlyG30A was not cross suppressed by any aaRS. This could be due the localization of the mutations, in anticodon arm, that might not binds a non-cognate synthetase.

Table 3: Suppression and cross suppression of the defective phenotype by Class II aaRS.
The phenotype of the transformants was checked after five days of growth on YP containing 3% glycerol. The second column represents the growth of the mutated strains without plasmid. "+" means that the mutant cells grow as the WT; "-" means that the cells did not grow; "+/-" means that the cells only have a slight growth.

tRNA Mutant	Growth on Glycerol 3% 28°C 37°C	Suppressor genes of class II aaRS							
		Mt HisRS (*HTS1*) IIa		Mt AspRS (*MSD1*) IIb		Mt LysRS (*MSK1*) IIb		Mt PheRS (*MSF1*) IIc	
		28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C
AspC61T	-	-	+	-	+	+	+	-	+	-
GlyG30A	-	-	-	-	-	-	-	-	-	-
HisG51A	+/-	-	+	+	+/-	-	+	+	+	+

Moreover Table 3 shows that not only the overexpression of the cognate synthetase is capable to rescue the defective phenotype of the mutants AspC61T and HisG51A, but they could be rescued by overexpressing others class II aaRS. AspC61T mutation was cross suppressed only at 28ºC by all the synthetases. Interestingly the HisG51A mutant phenotype was suppressed 28ºC and 37ºC by overexpressing all the mt aaRS except AspRS.

Table 4 shows the cross suppression obtained overexpressing class I mt aaRS in mutants bearing mutations in tRNA aminoacylated by class II RS It was overexpressed NAM2, VAS1, ISM1 MSE1 (yeast genes that encode for the mt LeuRS, ValRS, IleRS and GluRS respectively), LARS2 and VARS2 (human genes that encode for mt LeuRS and ValRS respectively).

The defective phenotype of AspC61T was partially suppressed by the overexpression of LARS2 and VARS2. Similar data were also obtained for HisG51A mutant which was also suppressed by ISM. Interestingly the overexpression of some class I aaRS genes (LARS2, VAS1 and VARS1) have partial suppressive activity in the GlyG30A mutant (only at 28ºC).

All together these data demonstrate that class I aaRS are not efficient suppressors tRNA mutation of the class II, but, could be possible to improve the suppressive effect utilising shorter or modified sequences?

Table 4: Cross suppression of the defective phenotype by Class II aaRS.

The phenotype of the transformants was checked after five days of growth in YP with 3% of glycerol. The second column represents the growth of the mutated strains without plasmid transformed aaRS. "+" means that the mutant cells grow as the WT; "-" means that the cells did not grow; "+/-" means that the cells only have a slight growth.

tRNA Mutant	Growth on Glycerol3% 28°C 37°C	Suppressor genes of class I aaRS					
		Mt LeuRS		Mt ValRS		Mt IleRS (*ISM1*) Ia 28°C37°C	Mt GluRS (*MSE1*) Ib 28°C 37°C
		Sc (*NAM2*) Ia 28°C 37°C	Hs (*LARS2*) Ia 28°C 37°C	Sc (*VAS1*) Ia 28°C 37°C	Hs (*VARS2*) Ia 28°C 37°C		
AspC61T	- -	- -	+ -	- -	+/- -	+ -	- -
GlyG30A	- -	- -	+ -	+ -	+ -	- -	- -
HisG51A	+/- -	+/- -	+ -	+/- -	+ -	+ -	+/- -

It is known that C-terminal domain of LeuRS is responsible for the suppression of tRNA mutants aminoacylated by class I aaRS (tRNA leucine, valine and Isoleucine) and that could be the key for the suppression activity. Importantly the separated C-terminal domain have a better function then the entire enzyme [97].

3.2.2 C-terminal of LARS2 and the its shorter sequences (β30-31 and β32-33) as suppressors of mutation in tRNA aminoacylated by class II aaRS

Previous data and also the one reported here, show that human LeuRS (encoded by LARS2) is the best cross suppressor. For this reason was imperative to search for the suppressive mechanism of this aaRS. Francisci et al. in 2011 subcloned the three domains of yeast LeuRS and was discovered that C-terminal domain (Figure 26) is the domain responsible for the correction of the defective phenotype [97].

At this point I studied the suppressive capability of LARS2 and of NAM2 C-terminal domains and of the two β motifs from the C-terminal of LARS2 (β30-31 and β32-33).

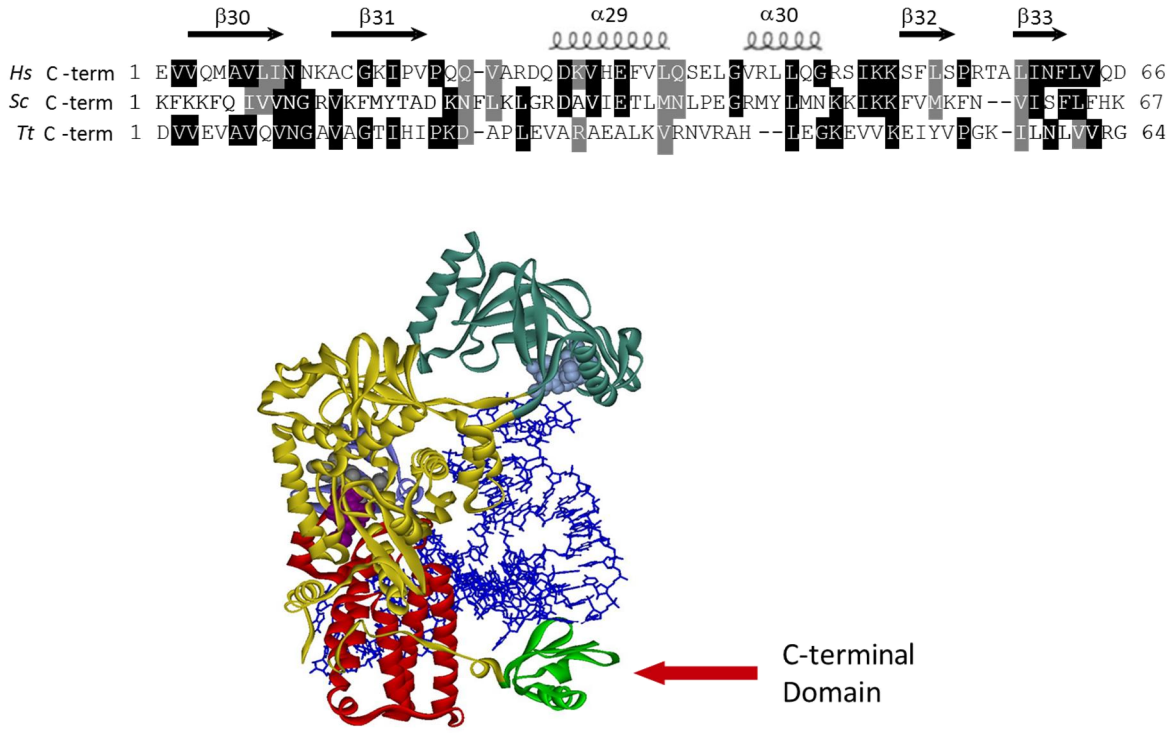

Figure 26: Top panel - Comparison of the aminoacidic sequence of the C-terminal Domain of LeuRS of Human (Hs), *Saccharomyces cerevisiae* (Sc) and *Thermus thermophilus* (Tt). Bottom panel–Structure of C-terminal domain in the *Thermus thermophilus* LeuRS [75].

Table 5 shows that the suppression of the defective phenotype of AspC61T is partially suppressed by overexpress the C-terminal of NAM2, the same happens with C-terminal of LARS2 and the suppressive activity increase when I used the fifteen aminoacids sequences of LARS2 (β30-31/β32-33).

Interestingly the HisG51A mutation is incredibly suppressed by shorter sequences of LeuRS with the following order: LeuRS<C-terminal domain<β30-31/β32-33. Shorter sequences led to a better suppression possibly because they can more easily bind and correct/stabilise the mutated tRNA and at the same time allowing the aminoacylation by the cognate synthetase (HisRS).

The same did not happen with the GlyG30A mutant, in this case the overexpression of these four sequences did not led to any suppressive effect. Further experiments are needed to evaluate the reason of the difficulty to supress this mutation.

Table 5: Suppression of the defective phenotype of tRNA mutants aminoacylated by class I aaRS using C-terminal of LeuRS and its derived peptides.
The phenotype was checked after five days of growth in YP with 3% of glycerol. The second column represents the growth of the mutated strains without any transformation. "+" means that the mutant cells grow as the WT; "-" means that the cells did not grow; "+/-" means that the cells only have a slight growth. *Growth on YP plates containing 3% of glycerol and 0.1% of galactose.

tRNA Mutant	Growth on Glycerol 3%		Glycerol growth of transformant strains									
			+pNAM2		+pLARS2		+pCtermNAM2		+pCtermLARS2		+pβ30-31 LARS2		+pβ32-33 LARS2	
	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C
AspC61T	-	-	- / + * -		+	-	++	-	++	-	+	-	+	-
GlyG30A	-	-	-	-	+	-	-	-	-	-	-	-	-	-
HisG51A	+/-	-	+/-	-	+	-	+ / ++ *	+	+ / ++ *	-	++ / +++ *	++	++ / ++ *	++

It is suggest that the C-terminal domain could bind the mutated tRNA (cognate or not cognate) correcting the modified structure. The explanation for the higher suppression activity with the small sequences of C-terminal (β30-31 and β32-33) could be that the contact is more easily and specific (Figure 27) [75].

It is possible these peptides act as chaperone-like modifying and stabilising the mutated tertiary structure of the tRNA (with a chaperone-like mechanism) and so the molecules that can easily bind the cognate synthetase. [97, 98].

For prove this hypothesis further experiments are needed (see perspectives to the future).

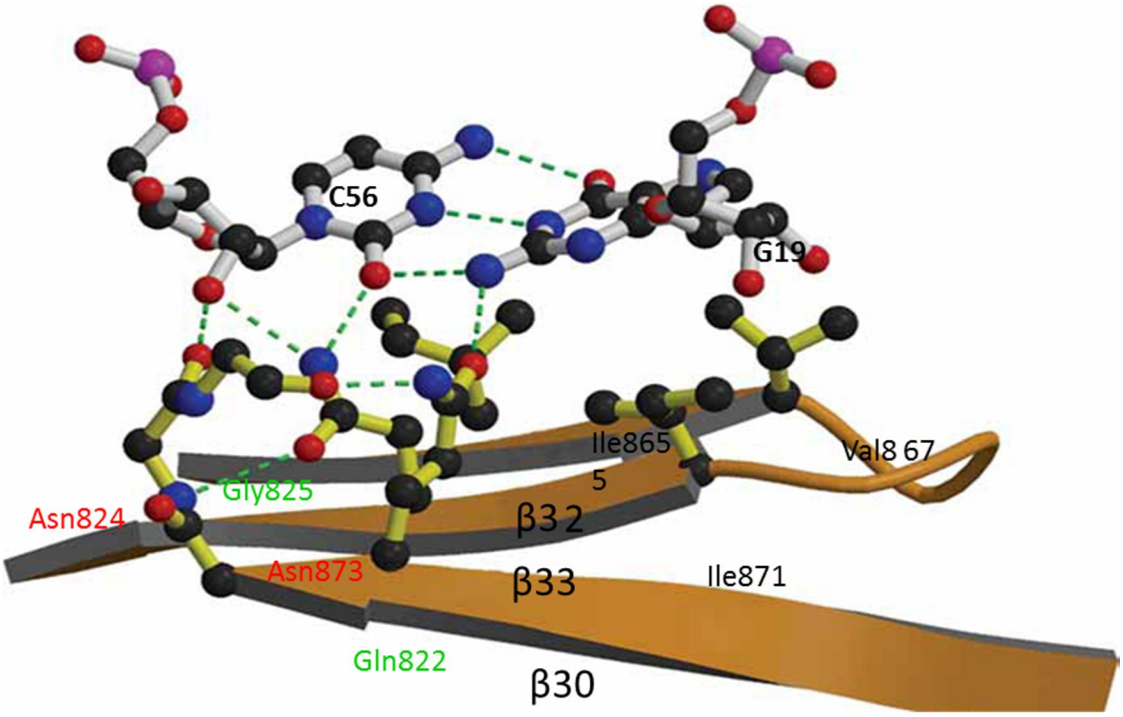

Figure 27: Interaction model between the C-terminal of LeuRS and mt tRNA leucine. Hydrophobic residues Ile865, Val867 and Ile871 form a platform for the base pair that is more precisely positioned by base-specific hydrogen bonds, notably to conserved Asn873 and Gln822. Colouring of residue labels reflects conservation among bacterial LeuRS: blue, conserved hydrophobic; red, absolutely conserved; green highly conserved [75].

3.2.3 Evaluation of β30-31 motif of C-terminal domain to bind the mt WT tRNA leucine and the suppressive effect of a short sequence on mutated tRNA aminoacylated by class I aaRSs.

The main goal to these studies is to find a possible therapy for pathologies due to mt tRNA point mutations. At the same time that we were working with mutations in tRNA aminoacylated by class II aaRS we were in forward experiments with three others mutations, the ValC25T, LeuC26T and IleT32C (Figure 25), aminoacylated by class I aaRS.

Previous results showed that the defective phenotypes of these mutants were suppressed not only by the entire LARS2 overexpression but also by overexpressing the C-terminal domain of LARS2, β30-31 and β32-33 sequences [97]. After that it was proposed to verify the interaction of the β30-31 C-terminal domain of human mitochondrial LeuRS motif with the WT human tRNA leucine (UUR), by band shift experiment.

The different mobility in 15% TBE polyacrylamide gel electrophoresis between the tRNA leucine alone and the tRNA leucine plus the β30-31 indicates that an interaction between the tRNA leucine and modified the peptide has occurred (Figure 28).

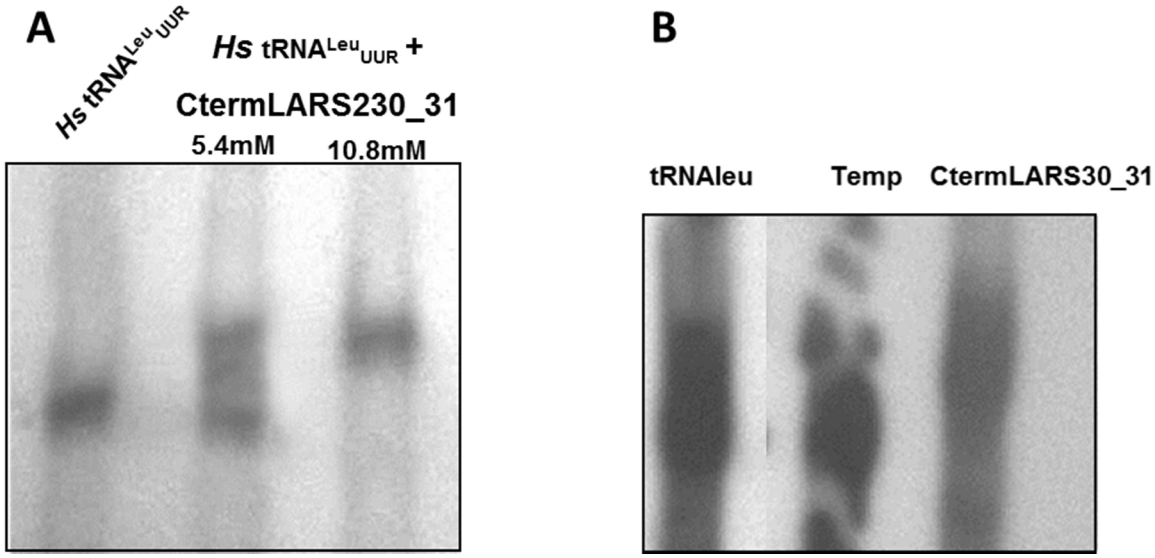

Figure 28: Band shift of β30-31 motif of C-terninal domain of human mitochondrial LeuRS incubated with the human mt tRNA leucine (UUR) *in vitro* labelled.
A: First slot loaded with only the human tRNA leucine (UUR). Second and third slots represent the shift of the tRNA leucine (UUR) with two different concentrations (5.4mM and 10.8mM) of CtermLARS2β30-31 peptide. B: First and second slots are loaded with only the tRNA leucine and with Temporine (a commercial peptide of 12 aminoacids) respectively. The last slot is loaded with the human tRNA leucine (UUR) incubated with the CtermLARS2β30-31.

After this evaluation was proposed to me to do suppression experiments overexpressing other sequences derived from the LARS2 C-terminal (Cterm6, *scramble*, Ctermα) and with the C-terminal of ISM1 (Yeast gene that encodes for the mt IleRS). The

plasmid bearing the Cterm6 encodes for a peptide of six aminoacids length (LINNKA) comprised in the β30-31; from this sequence is derived the *scramble* which codes the same aminoacids in random order (KIANLN). At last it was used the Ctermα (sequence of C-terminal of LARS2 that encode for α-helix 29); this peptide is not supposed to directly bind the tRNA (Figure 26) [75].

To perform this experiment we choose the LINNKA sequence because five in the six aminoacids contact directly with the tRNA, and it is more likely to have a suppressive effect. The *scramble* peptide and the Ctermα were used as negative controls [75].

After transformation with each one of the plasmids of the mutants with defective growth in glycerol contain in media I plated in selective medium and let them grow for three or four days. The transformed colonies were than replicated on YP containing 3% of glycerol to evaluate the suppression activity of the plasmid.

Table 6: Suppression of the defective phenotype of mutants which mutated tRNA is aminoacylated by class I tRNA by short peptides from C-terminal domain of LARS2 and by C-terminal of ISM1. The phenotype was checked after five days of growth in YP with 3% of glycerol. The second column represents the growth of the mutated strains without any transformation. The signal "+" means that the cells grow, the signal "-" means that the cells did not grow and "+/-" means that the cells only have a slight growth. * Data not present.

tRNA Mutant	Growth on Glycerol 3%		Glycerol growth of transformant strains									
	28°C	34°C	+pCterm6 28°C	+pCterm6 34°C	+pCtermα 28°C	+pCtermα 34°C	+pscramble 28°C	+pscramble 34°C	+pISM1 28°C	+pISM1 34°C	+pCtermISM1 28°C	+pCtermISM1 34°C
ValC25T	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+	+	+/-	+/-
LeuC26T	-	-	+	-	-	-	+/-	-	+	+	-	-
IleT32C	-	-	*		-	-	-	-	+	+	-	-

As expected the Ctermα and *scramble* peptide did not have any suppressive effect on the mutants, but at the same time ether the Cterm6 did not have any effective suppression with the exception of a partial rescue only on the LeuC26T mutant (at 28ºC) (Table 6). LINKKA is a short peptide, and this could be the reason for its inefficiency, and

unable to stabilise and change the tertiary structure of the mt mutated tRNAs. Very interesting point is that similarly to LeuRS and ValRS, the IleRS protein has the same cross-suppression property. On the contrary this activity is not conserved in its C-terminal domain. It will be necessary to study in detail and compare the amino acid sequences of these enzymes.

3.2.4 The particular case of tRNAs glutamine and glycine

Among the tRNA mutants that I have analysed two of them had the glycerol growth phenotype not rescued by the plasmids used for transformation generally active in the other mutants. Interestingly these two mutants have the substitution located in the acceptor stem (GlnC6T), and in the anticodon stem (GlyG30A).

Table 7 and 8 show the results obtained with GlnC6T and GlyG30A respectively.

The defective phenotype of the GlnC6T is suppressed only by the overexpression of NAM2 gene (mtLeuRS); we did not observe the rescue at 37°C by either the human orthologous gene and by its C-terminal or derived sequences. AspRS (belonging to class II) and LysRS (belonging to class I aaRS) overexpression were partially active in suppression. The mt gene coding for GlnRS have been not yet identified and it is possible that the cyt enzyme is also active in the mt protein synthesis. For this reason I cloned and I analyse the suppression capability the cyt GlnRS (encoded by GLN4) but we could not have positive results. It will be necessary to repeat the suppression experiments to confirm the results obtained overexpressing leuRS (human and yeast genes) but the data suggest that there is an extended inter and intra class cross suppression.

In the case of GlyG30A we obtained cross suppression only overexpressing the entire LARS2 gene (Human gene that encode for the mt LeuRS), VARS2 and VAS1 (Human and yeast genes encoding for Mitochondrial ValRS). These genes belong to class I aaRS and our results confirm the intercross class suppression. GRS1 is the gene that encodes the cytosolic and mitochondrial GlyRS and belongs to class II. The cognate suppression overexpressing GRS1 have not been yet performed.

Table 7: Suppression of the defective phenotype of GlnC6T mutant by Class I and class II aaRS. The phenotype was checked after five days of growth on YP plates containing 3% of glycerol as carbon source. The second column describes the gene sequence borne by the plasmid and the aaRS class; the third column reports the glycerol growth of the transformants. "+" means that the cells grow, the signal "-" means that the cells did not grow and "+/-" means that the cells only have a slight growth. In red is represented the suppression by their cytosolic synthetase of tRNA glutamine.

tRNA Mutant	Plasmid		Glycerol Growth 28°C	Glycerol Growth 37°C
GlnC6T	Empty		+/-	-
	Mt LeuRS	Sc (*NAM2*) Ia	+	+
		Hs (*LARS2*) Ia	+	-
	Mt ValRS	Sc *(VAS1)* Ia	+/-	-
		Hs (*VARS2*) Ia	+/-	-
	Mt IleRS (*ISM1*) Ia		+/-	-
	CytGlnRS (*GLN4*)Ib		+/-	-
	Mt GluRS (*MSE1*) Ib		+	-
	Mt TyrRS (*MSY1*) Ic		+/-	-
	Mt HisRS (*HTS1*) IIa		+/-	-
	Mt AspRS (*MSD1*) IIb		+	+/-
	Mt LysRS (*MSK1*) IIb		+	+/-
	Mt PheRS (*MSF1*)IIc		+	-
	CtermNAM2		+	-
	CtermLARS2		+	-
	β30_31 LARS2		+	-
	β32_33 LARS2		+	-

It is interesting to note that the C-terminal domain of LeuRS and shorter sequences were not able to rescue the defective phenotype of both mutants, GlnC6T and GlyG30A. We can hypothesize that the loss of suppressive effect could be due to the fact that since the interaction of the C-terminal domain do not involve the acceptor and the anti-codon arms where the GlnC6T and GlyG30A mutations are located respectively; consequently the chaperone function of the suppressor do not stabilize the mutated tRNA tertiary structure in that regions [99]. Further studies are needed to confirm this hypothesis (see Future perspectives).

Table 8: Suppression of the defective phenotype of GlyG30A mutant by Class I and class II aaRS. The phenotype was checked after five days of growth on YP plates containing 3% of glycerol as carbon source. The second column describes the gene sequence borne by the plasmid and the aaRS class; the third column reports the glycerol growth of the transformants. "+" means that the cells grow, the signal "-" means that the cells did not grow and "+/-" means that the cells only have a slight growth.

tRNA Mutant	Plasmid		Glycerol Growth 28ºC	37ºC
GlyG30A	Empty		-	-
	Mt LeuRS	Sc (*NAM2*) Ia	-	-
		Hs (*LARS2*) Ia	+	-
	Mt ValRS	Sc *(VAS1)* Ia	+	-
		Hs (*VARS2*) Ia	+	-
	Mt IleRS (*ISM1*) Ia		-	-
	Mt GluRS (*MSE1*) Ib		-	-
	Mt TyrRS (*MSY1*) Ic		-	-
	Mt HisRS (*HTS1*) IIa		-	-
	Mt AspRS (*MSD1*) IIb		-	-
	Mt LysRS (*MSK1*) IIb		-	-
	Mt PheRS (*MSF1*)IIc		-	-
	CtermNAM2		-	-
	CtermLARS2		-	-
	β30_31 LARS2		-	-
	β32_33 LARS2		-	-

3.3 Suppressive capabilities and mechanism of EF-Tu factor in mt tRNA mutations

As described in the topic 1.12.2 from the bibliographic review the elongation factor EF-Tu is a GTPase which role is the delivery of the aa-tRNA to the translation site of the ribosome.

Since 1997 is known that overexpression of TUF1 gene supresses the defective phenotypes of mitochondrial tRNA aspartate C61T yeast mutants [100].

After that it has been demonstrated that not only TUF1 but also TUFM (the human orthologous gene) suppress the glycerol growth defective phenotypes caused of many mt tRNAs mutations. In 2008, Sasarman et al proved that overexpression of TUFM could

partially supress the respiratory defects in myoblasts of a patient with MELAS caused by a mutation on tRNA lecine (UUR) (m.3243 A>G) [101].

In our laboratory was demonstrated that the overexpression of the TUF1 gene in yeast strains bearing equivalent human mt tRNA mutations LeuA14G, LeuC25T, LeuT60C and ValC25T corresponding to the pathogenic mutations m.3243 A>G [101], m.3256 C>T [102], m.3291T>C [103] and m.1624 C>T [86] respectively, was able to restore the glycerol growth capacity.

However the homoplastic human equivalent mutations IleT32C and IleT33C (corresponding to the pathogenic human mutations m. 4290T>C [104] and m.4291T>C [105] respectively) the overexpression of TUF1 did not have any suppressive effect. The possible explanation for this is the fact the anticodon loop, where the mutations are located, does not contact the EF-Tu molecule [106].

In this topic I report a further characterization of the molecular activity of mt yeast and human EF-Tu factors (TUF1 and TUFM genes respectively) in their capacity to sustain the mt protein synthesis (catalytic activity) and suppressing activities in *S. cerevisiae* cells. Both activities have been measured by glycerol growth capability. For this study I used the WT (MCC123), the strain with TUF1 gene deleted (MCC123ΔTUF1) and the isogenic mutant with a point mutation in the mt tRNA aspartate (AspC61T). This mutant was chosen because the TΨC stem, where the substitution is located, [107], contacts directly the EF-Tu factor. Both mutants are unable to perform the mt protein synthesis and consequently unable to grow on glycerol containing media.

Table 9 shows that neither the TUFM overexpression neither TUFM plus TSFM (human gene that encodes for Mitochondrial protein Elongation Factor Ts, binds and add the recycling of EF-Tu.) overexpression could rescue the defective phenotype of the MCC123ΔTUF1 strain. This means that the TUFM cannot substitute the endogenous TUF1 gene in yeast cells.

To evaluate the suppressive capability of the human gene on yeast I did the same assay with the AspC61T mutant (table 10). Interestingly the overexpression of the human gene shows suppression capability in yeast.

These data demonstrate that the catalytic activity of the suppressor gene is not necessary to rescue the defective phenotype of the mutant. So probably the EF-Tu factor

acts binding the tRNA and remodelling the defective structure of the mutated tRNA with a chaperone-like mechanism.

The effect of TUF1 variants have been studied, in the two strains mentioned above.

Starting in the domain 1 (see 1.12.2) I studied the effect of mutation I97A (isoleucine ATT > alanine GCT in the position 97) corresponding to the *E. coli* equivalent mutation I60A. In *E. coli* this mutation decreases by 35% the GTPase activity [86]. The decrease of catalytic activity is also confirmed in yeast and also the suppressive effect is reduced (Figure 29).

Table 9: Activity of overexpressed TUF1 and TUFM genes evaluated by growth on respiratory substrate of MCC123, WT or deleted of the endogenous TUF1 gene (ΔTUF1) transformed with plasmids bearing several variants of this gene TUF1.
"+" indicates wild-type growth and "-" indicates absence of growth.

Nuclear context	Mitochondria	Plasmid	Glycerol growth
MCC123 (MAT a, ade2, ura3-52, kar1-1)	Rho^+	No plasmid	+
MCC123 (MAT a, ade2, ura3-52, **ΔTuf1,** kar1-1, Kan^R)	Rho^+	Empty plasmid	-
		pETUFM	-
		pETSFM	-
		pETUFM+pETSFM	-
		pETUF1	+
		pETUF1(I97A)	+/-
		pETUF1(Q134P)	+/-
		pETUF1Δ9	-
		pETUF1(Q225stop)	-
		pETUF1(D313V)	+
		pCTUF1(R328Q)	+
		pETUF1(K334stop)	-

The mutation Q134P equivalent to the *E. coli* Q97P mutation in yeast this mutation maintain almost all their catalytic activity, but could not suppress the mutant phenotype of AspC61T (Figure 31). Limongelli el al showed that this mutation in *E. coli* is completely inactive [104]. The proline is present at this position in the human gene and the region has been shown to be important for factor interactions; e.g. in *E. coli* the equivalent mutation

produces a decrease in the GTP binding [104] and in the *Schizosaccharomyces pombe* orthologous gene the V141I adjacent mutation has been found by Chiron et al. to eliminate the requirement for EF-Ts [78].

I also studied the effect of TUF1 overexpression when the cloned gene has a deletion of nine aminoacids from the aminoacid 165 to 173. This deletion completely abolishes both the catalytic and the suppressing functions (Figure 29).

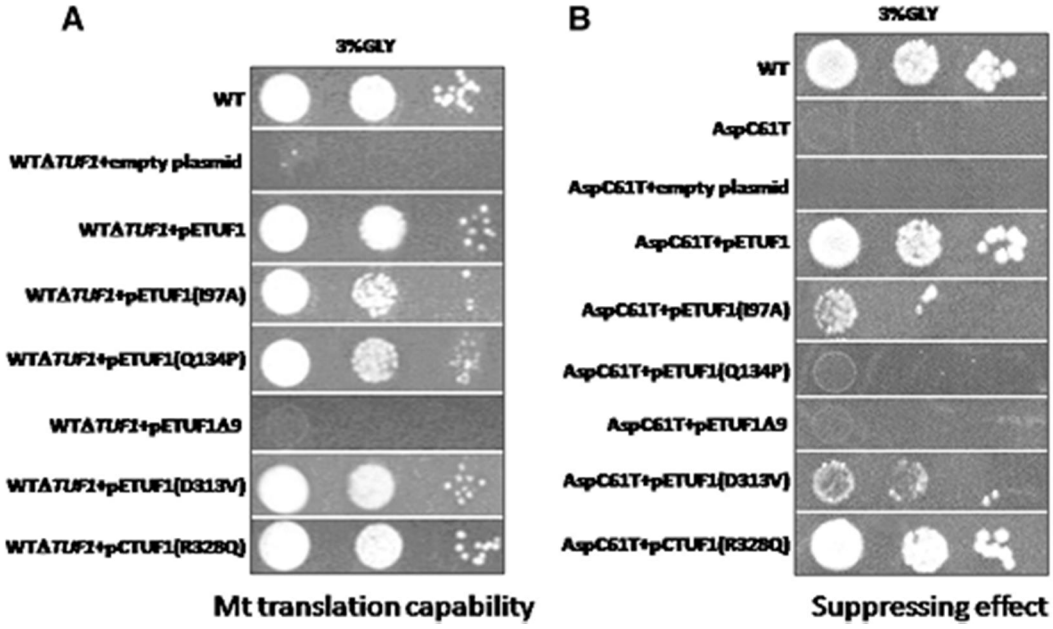

Figure 29: Mt protein synthesis and suppression capabilities of different overexpressed versions of TUFM and of TUF1evaluated by growth on respiratory substrate.
A) Glycerol growth of MCC123 (WT), and WT deleted of the endogenous TUF1gene (MCC123ΔTUF1) transformed with episomal (pE) or centromeric (pC) plasmid bearing differentTUF1gene versions. B) Glycerol growth phenotype of WT, mutant (AspC61T) and mutant cells transformed with the same plasmids as panel A. The cloned sequences were: the WT *S. cerevisiae* TUF1 (pETUF1), three mutated versions in domain 1 (I97A,Q134P andΔ9), one in the domain 2 (R328Q) and the D313V,TUF1natural variant isolated from D273-10B/A1 yeast strain. Serial dilutions were observed after three days on YP 3% glycerol containing media at 37 °C

The Q225stop mutation, by replacing CAG with TAG (stop codon), is obviously inactive both for catalytic and suppressive functions (Table 9 and 10).

In the domain two I overexpressed the variant D313V (replacing the codon GAC for GTG) present naturally in D273-10B/A1 strain. This variant has a normally catalytic activity however is interesting to note that in the D273-10B/A1 nuclear context mutations in mt tRNAs genes have a maximal severity. This may suggest that the slightly defective EF-Tu, acts as an endogenous "modifier gene" [105]. Despite this variant has a completely normal catalytic activity, and have a partial suppression of the defective phenotype (Figure 29).

I Also study the effect R328Q variant equivalent to the human R339Q mutation located in the second domain and originating a fatal encephalopathy [87]. However in yeast this variant maintains the catalytic and suppressive activities (Figure 31). The different behaviour of this mutation in human and yeast is also discussed by Valente et al. [108].

Table 10: Suppression activity of overexpressed TUF1 gene, analysed by glycerol growth capability of AspC61T mutant transformed with plasmids bearing TUFM, TSFM and different variants of TUF1.
"+" indicates wild-type growth and "-" indicates absence of growth.

Strain	Plasmid	Glycerol growth
AspC61T[107]	No plasmid	-
	Empty plasmid	-
	pETUFM	+
	pETSFM	-
	pETUFM+pETSFM	+
	pETUF1	+
	pETUF1(I97A)	+/-
	pETUF1(Q134P)	-
	pETUF1Δ9	-
	pETUF1(Q225stop)	-
	pETUF1(D313V)	+/-
	pCTUF1(R328Q)	+
	pETUF1(K334stop)	-

At last I evaluate the activity of the variant K334stop that corresponds to the deletion of the entire domain three. This variant also did not have neither suppressive neither catalytic activity (Table 6 and 7).

In conclusion by the use of the orthologous human gene we have demonstrated that the catalytic activity of FE-Tu is not necessary for suppression activity in mt tRNA mutants. Moreover we have shown that single base substitutions in the TUF1 gene that had no effects on the catalytic activity could impaired the suppression activity (Q134P, R328Q, D313V and I97A) suggesting that these positions are essential for the recognition and binding tRNA-EF-Tu. The interaction between EF-Tu and the mutated tRNA could be especially important if this interaction and the stabilization are essential to avoid the tRNA degradation by nucleases [109].

Conclusion

In the first part of my project research I characterized three *Saccharomyces cerevisiae* strains (W303-1B, MCC123 and D273-10B/A1) in order to understand the role of the nuclear background in the mitochondrial phenotype, and consequently the influence in the mt tRNA point mutations phenotype. The three strains showed morphological and metabolic differences.

W303-1B strain has a very high capability of growth on glycerol medium and extended respiration suggesting high efficiency of mitochondrially related metabolic functions. This strain can be utilized to study the molecular effect of severe mt tRNA mutations which would have catastrophic effect in other nuclear backgrounds.

Furthermore, the chronological and the replicative lifespans appear to be regulated by overlapping but distinct mechanisms [110] differentially regulated in the three strains. W303-1B cells are the best tool to study the mechanisms and to identify genes involved in longevity; in particular it will be useful to study which are the changes (at transcriptional and expression levels) that occur after 25 days of growth that are the signals to initiate death. Indeed it has been shown that in W303-1B strain the retrograde regulation is constantly active [111] and this probably explains why the mt mutations cause a milder phenotype compared with other nuclear backgrounds.

The strain D273-10B/A1 has the higher growth rate in glucose containing medium with a very rapid O_2 consumption, poorly influenced by glucose concentration. The presence of mt tRNA mutations do not increase *petite* production but have deleterious effect on the capacity of growth in medium containing either 3% glycerol or low glucose concentration. In this strain is possible that the retrograde effect is absent or strongly reduced. It can be used to study mutations with mild effect on mt functions.

At last, the MCC123 strain has the lowest growth rate in glycerol medium. The studied mt tRNA mutations increase the percentage of *petite* colonies and this value depends on the severity of the mutation. Therefore, the MCC123 might be preferentially used to test the phenotypic effects of mt tRNA substitutions and to study the molecular effect of mutations that cause severe impairs of mt protein synthesis. Due to the results it was hypothesize that the MCC123 have a inducible retrograde response that compensates the adverse conditions.

Our results demonstrate that yeast is a good model to study mitochondrial diseases but is very important the choice of the strain.

The second aim of this project was to study the effect of the overexpression of some class I and II aaRS genes, of the human LeuRS C-terminal domain and to identify possible shorter sequences there are able to suppress the defective glycerol growth phenotype due to mutations in mt tRNA aminoacylated by class II aaRS (AspC61T, GlyG30A and HisG51A). Overexpressing the shorter sequences i.e. fifteen aminoacids length (domain<β30-31 and β32-33) the suppression activity was even better than the entire C-terminal domain of mt LeuRS. We also concluded that class I aaRSs are cross suppressing enzymes active with mutated tRNA belonging to both classes; moreover C-terminal and the shorter sequences (β30-31 and β32-33) of mt LeuRS also maintained the suppressing capability in the tested tRNAs.

Further in order to understand the mechanism of suppression of EF-Tu, I studied the over expression of the human, of the yeast genes and of same yeast variants. All together the data show that the catalytic activity of the protein is not needed to suppress the defective phenotype of the mutant and suggest that a chaperone-like activity is involved.

Future perspectives

Some of the experiments that I did in this project are very preliminary, so is imperative to continue to achieve the main goal of all this experiments, that is to create a possible therapy for human mitochondria tRNA diseases.

We believe that our data provide good evidence for the potential therapeutic use of the C-terminal of LARS2 for most pathogenic human mt tRNA mutations. Future work aims to perform additional experiments in order to define the extent of the cross suppression. This can be performed by selecting shorter active peptides from the CtermLARS2 beta motifs, giving elucidation about the localization of the mutation involved in the suppression.

To verify the lack of binding with the selected C-terminal derivated peptides cloning and band shift experiments of tRNA glycine transcribed *in vitro* will be performed. Moreover the results obtained with WT and mutated tRNAs (obtained by site-directed mutagenesis) will be compared in order to understand the stability of the complex. To achieve this point other techniques as CD, Thermofluor and Biacore will be use.

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Appendix

Appendix 1 (Oligonucleotides)

- Aminoacyl-tRNA synthetase oligonucleotides:
  - Ism1+ : 5'-ATGAAGCGCTCCAGGCTTGTGCCTC-3'
  - Sma1ism1- : 5'-TCCCCCGGGGGATCAAGACATGAGATGATCCACAG-3'
  - petLARS+Bamh1 : 5'-CGGGATCCCGACAAGATGGCTTCTGTTTGG-3'
  - petLARS-Xba1 : 5'-GCTCTAGAGCGATATCGCCATGGTTGAGG-3'
  - MSE 5' : 5'-GAAAGGATCGTTCATAAAG-3'
  - MSE Pst : 5'-GCTGCAGCGTATATGACGTATTTACATG-3'
  - KpnICtermLARS2+: 5'-CGGGGTACCCCGATGGAGGTTGTCCAGATGGCAGTTC-3'
  - KpnICtermLARS2-: 5'-CGGGGTACCCCGGATATCTTGCACCAGGAAGTTGATG-3'
  - EcoR1CtermISM+ : 5'-CGGAATTCCGATGCGACATATTGCAATCATCTGC-3'
  - CtermLARS30.31E/B+ : 5'-CGGAATTCCGATGGCAGTTCTGATCAACAATAAAGCTTGTGGCAAAATTCCTGTGTGACGGGATCCCG-3'
  - CtermLARS30.31E/B-: 5'-CGGGATCCCGTCACACAGGAATTTTGCCACAAGCTTTATTGTTGATCAGAACTGCCATCGGAATTCCG-3'
  - CtermLARS32.33E/B+ : 5'-CGGAATTCCGATGAAGAAGTCCTTCCTTTCCCCGAGAACTGCCCTCATCAACTTCCTGGTGTGACGGGATCCCG-3'
  - CtermLARS32.33E/B-: 5'-CGGGATCCCGTCACACCAGGAAGTTGATGAGGGCAGTTCTCGGGGAAAGGAAGGACTTCTTCATCGGAATTCCG-3'
  - Cterm6E/B+: 5'-CGGAATTCCGATGCTGATCAACAATAAAGCTTGACGGGATCCCG-3'
  - Cterm6E/B-: 5'-CGGGATCCCGTCAAGCTTTATTGTTGATCAGCATCGGAATTCCG-3'
  - scrambleE/B + : 5'-CGGAATTCCGATGAAAATCGCTAACCTGAATTGACGGGATCCCG-3'
  - scrambleE/B-:5'-CGGGATCCCGTCAATTCAGGTTAGCGATTTTCATCGGAATTCCG-3'
  - alfaCtermE/B+ : 5'-CGGAATTCCGATGGACCAGGACAAAGTCCACGAATTTGTTCTTCAAAGCTGACGGGATCCCG-3'
  - alfaCtermE/B-: 5'-CGGGATCCCGTCAGCTTTGAAGAACAAATTCGTGGACTTTGTCCTGGTCCATCGGAATTCCG-3'

- TUF1 Oligonucleotides:
  - TUF7Not +: 5′-TTGCGGCCGCAATTGTAAACTATTTTGTGCT-3′
  - TUF4Not-: 5′-TTGCGGCCGCAAGAAATGAACAGAATATATAG-3′
  - TUFI97A+: 5′-AGCTCGTGGTGCTACAATTTCTAC-3′
  - TUFI97A−:-5′-GTAGAAATTGTAGCACCACGAGCT-3′
  - TUFQ134P+: 5′-CCGGTGCTGCTCCAATGGATGGTGC-3
  - TUFQ134P−: 5′-GCACCATCCATTGGAGCAGCACCTT-3′
  - TUFK334+: 5′-GGTCTTAGCTTAGCCAGGTACCG-3′
  - TUFK334−: 5′-CGGTACCTGGCTAAGCTAAGACC-3′
  - TUF1+ :5′-GTGGTATTACAATTTCTACTG-3′
  - TUF5−:5′-GATAGCACCATCCATTTG-3′
  - KANtuf+: 5′-CTTCTCTATCTCCGGTAGAGGTACTGTGGTCACTGGTCGGATATCAAGCTTGCCTCG-3′
  - KANtuf−: 5’-CATTCCATTTCAACATTGTCACCTGGCATAACTTGCATAGGTCGACACTGGATGGCGGC-3′

Appendix 2 (Vectors)

- Aminoacyl-tRNA synthetase gene containing vectors:
  - pNAM2 (kindly provided by Prof. Christopher Herbert) in which the NAM2 gene with its own promoter, is cloned into the multi-copy vector pFL44S [1].
  - pCtermNAM2 in which sequence of C-terminal domain of NAM2 has been cloned in multi-copy vector pYES2.1TOPO under the inducible Gal1 promoter.
  - pVAS1 (kindly provided by Prof. M. Bolotin-Fukuhara) was cloned into multi-copy vector pCM262 by the “gap repair” technique under the tetracycline promoter [2].

- pHTS1 (kindly provided by Prof. G. Fink), in which HTS1 gene with its own promoter, has been cloned in multi-copy vector YEp24 [3].
- pISM1 in which the amplified ISM1 gene using ISM1+ and SmaIISM1- primers, from wild-type genomic DNA was cloned into the multi-copy vector pYES2.1/V5-HisTOPO (pYES2.1TOPO TA Expression Kit, Invitrogen) under the inducible Gal1 promoter.
- pCtermISM1 in which the amplified C-terminal domain de ISM1 gene using EcoR1CtermISM+and SmaIISM1- primers, from wild-type genomic DNA was cloned into the multi-copy vector pYES2.1/V5-HisTOPO (pYES2.1TOPO TA Expression Kit, Invitrogen) under the inducible Gal1 promoter.
- pMSE1 in which the amplified MSE1gene using MSE 5'+ and MSE pst -into the yeast pYES2 transcription vector (Invitrogen) under the inducible Gal1 promoter.
- pGLN4 (kindly provided by Prof B. Krett) in which GLN4 has been cloned in multi-copy vector pRS416.
- pMSD1 in which MSD1 has been cloned in multi-copy vector pELAC181 [4].
- PMSK1 (kindly provided by prof. I. Tarassov) in which MSK1 has been cloned in multi-copy vector pELAC181.
- pMSF1in which MSF1 has been cloned in multi-copy vector pYEP13[5].
- pVARS2 in which the VARS2 gene was digested KpnI/NotI from pcDNA5/ FRT/TO (Invitrogen), a kind gift of Prof. R. Lightowlers and ligated into pYES2 transcription vector (Invitrogen) under the inducible Gal1 promoter.
- pLARS2 in which the amplified LARS2 gene using petLARSBamHI+ and petLARSXbaI- primers, from the pET32 plasmid (a kind gift of Prof S. A. Martinis) was digested BamHI/XbaI and ligated into the yeast pYES2 transcription vector(Invitrogen) under the inducible Gal1 promoter.
- pCtermLARS2 in which amplified sequence of C-terminal domain of LARS2 gene, using KpnICtermLARS2+ and KpnICtermLARS2- primers, has been cloned in multi-copy vector pYES2.1TOPO under the inducible Gal1 promoter.
- pCtermβ30-31 in which amplified sequence of the motif β30-31 from C-terminal domain of LARS2 gene, using CtermLARS30.31E/B+ and

CtermLARS30.31E/B- primers, has been cloned in multi-copy vector pYES2.1TOPO under the inducible Gal1 promoter.

- pCtermβ32-33 in which amplified sequence of the motif β32-33 from C-terminal domain of LARS2 gene, using CtermLARS32.33E/B+ and CtermLARS32.33E/B- primers, has been cloned in multi-copy vector pYES2.1TOPO under the inducible Gal1 promoter.
- pCterm6 in which amplified sequence of a six aminoacids peptide from C-terminal domain of LARS2 gene, using Cterm6E/B+ and Cterm6E/B- primers, has been cloned in multi-copy vector pYES2.1TOPO under the inducible Gal1 promoter.
- pscramble in which amplified sequence of scramble peptide of six aminoacids from C-terminal domain of LARS2 gene, using scrambleE/B + and scrambleE/B- primers, has been cloned in multi-copy vector pYES2.1TOPO under the inducible Gal1 promoter.
- pCtermα in which amplified sequence of α-helix 29 from C-terminal domain of LARS2 gene, using alfaCtermE/B+ and alfaCtermE/B- primers, has been cloned in multi-copy vector pYES2.1TOPO under the inducible Gal1 promoter.

- TUF1 containing vectors:
  - The TUF1 gene with its own promoter(pETUF1), recleaved from the YEpLAC181TUF1 [4], was cloned into the EcoRI restriction site of pFL61 vector. Plasmids pETUF1(I97A), pETUF1(Q134P) and pETUF1(K334stop) were obtained by site directed mutagenesis (Quick-change kit from Stratagene) amplifying the pETUF1 plasmid with opportune primers.
  - pETUF1(Q225stop) has been obtained propagating the pETUF1 into the XL1-Red competent cells (from Stratagene).
  - In pETUF1(D313V) theTUF1gene was PCR amplified with TUF7Not + and TUF4Not−primers from total DNA extracted from D273-10B/A1 strain and cloned in pFL61 vector digested with NotI under PGK1 promoter
  - pCTUF1(R328Q) contains the mutated gene cloned in pFL38 plasmid [6] and was a kind gift of C. Donnini.

- pETUF1Δ9 was selected from a plasmid pool obtained from the pETUF1 HpaI digestion treated with Bal31 DNase for 10 min followed by ligation. Sequence analysis confirmed the deletion of 9 aa (165–173) maintaining the correct frame.
- pTUFM the human TUFM gene was cloned in pYX212.
- pTSFM, the human TSFM gene (cloned in pFL46) was a kind gift of N. Bonnefoy [7].

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