UNIVERSITY OF PARMA

Department of Life Sciences

Ph.D. in Biotechnologies

XXV COURSE

Mitochondria and lifespan extension in *Saccharomyces cerevisiae:* the longevity mutations *sch9Δ* and *rei1Δ* contribute to mitochondrial DNA stability

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To me
To the four of us
To M.

"*The most beautiful experience we can have is the mysterious - the fundamental emotion which stands at the cradle of true art and true science.*"

-Albert Einstein-

"In scientific research neither the level of intelligence nor the capacity to conduct and the undertaken task are essential elements for the success. Both of them are more accounted for total dedication and for closing the eyes in front of the difficulties: in this way we can face those problems that others, more critical and acute, won't face."

-Rita Levi Montalcini-

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Age related mitochondrial decline has been reported in different organisms, and is accompanied by a reduction in mitochondrial respiratory activity, morphological alterations and progressive accumulation of mutations. Besides their role in ATP production, mitochondria take also part in the transduction of intracellular signals, in cell death and they are the main sites of production of oxygen reactive species (ROS), detrimental molecules that promote mitochondrial dysfunction and ageing.

Recent evidence showed that ROS may act also as signaling molecules to regulate cell growth, immune response and lifespan extension and that mutations that reduce the activity of the nutritional signaling pathways TOR/SCH9 and RAS/PKA induce an increase in chronological lifespan (CLS) extension in different model organisms, redirecting cells towards a respiratory metabolism and promoting stress resistance.

In order to assess the relationship between mitochondrial function and ageing we investigated whether the increased CLS of some well-known longevity mutants correlated with an increase in mitochondrial DNA stability. Among the different longevity mutations analyzed only two, *sch9Δ* and *rei1Δ*, accumulate large-scale rearrangements on mtDNA at lower rate compared to the parental strain, while there is no difference in the accumulation of point-mutations.

This result suggests that large-scale rearrangements on mtDNA have a primary role in the senescence process as mtDNA integrity is strictly important to maintain proper mitochondrial function and respiratory activity, a key determinant of lifespan extension. Moreover, the evidence that only two of the eight longevity mutations analyzed were able to increase mitochondrial DNA stability indicates that maintenance of mtDNA is only one of the actors involved in the regulation of the ageing process and that different mechanisms cooperate to regulate lifespan extension in yeast.

Sch9Δ and *rei1Δ* longevity mutants show high rate of respiratory activity accompanied by no significant difference in mtDNA amount, suggesting that the increased respiratory rate is imputable to an increased OXPHOS complexes content. We have been able to identify two different mechanisms through which the two longevity mutants promote lifespan extension and mtDNA stability. Deletion of *SCH9* leads to an increase of ROS production early during growth to promote an adaptive signal that stimulates lifespan extension and reduces oxidative damage in stationary cells, activating a stress response program mediated by Sod2p overexpression. *REI1* belongs to the RiBi genes regulon and the increase in mitochondrial DNA stability upon *REI1* deletion was observed also in other genes involved in ribosome biogenesis. In addition, we showed that reduced mtDNA rearrangements and increased respiratory activity in *rei1Δ* longevity mutant depend on a mechanism completely different from the adaptive response observed in *sch9Δ* mutant, and appear to rely on a direct stabilization of mitochondrial DNA through overexpression of nucleoid components.

CHAPTER I

Mitochondrial function and ageing pathways

1.1. MITOCHONDRIA

1.1.1. Mitochondrial structure and function

Mitochondria are eukaryotic organelles originated, according to the endosymbiotic theory, through an ancestral symbiotic event (Margulis 1975; Margulis & Bermudes 1985). They are commonly referred as the "powerhouse" of the cell because of their main function which is to generate chemical energy in the form of adenosine triphosphate (ATP), through the processes of aerobic respiration and oxidative phosphorylation.

In addition to their essential role in cellular energy supply, other several cellular processes occur in mitochondria such as heme and Fe/S clusters biogenesis, calcium homeostasis, pyruvate oxidation, the reactions of the tri-carboxylic acid cycle (TCA, Krebs cycle) and of metabolism of fatty acids, amino acids, cholesterol, steroids and nucleotides (Scheffler, 2008). Mitochondria take also part in the transduction of intracellular signals, in cell death, aging and they are the main sites of production of oxygen reactive species (ROS).

The multitude of different mitochondrial functions reflects into the complexity of their structure. Mitochondria are elliptic organelles of variable size, generally between 3-4 μm of length and 0.2-1 μm of diameter. Mitochondria are surrounded by a double membrane, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which create different compartments that carry out specialized functions (McBride et al. 2006).

The OMM is a lipid rich layer and contains large number of integrated proteins (porins), which form channels that allow molecules smaller than 10 kDa to freely diffuse across the membrane in both directions. The transfer of larger nuclear encoded proteins is mediated by the recognition of import sequences at their N-terminus by a multisubunit protein called translocase of the outer membrane (TOM), which actively moves them across the membrane (J M Herrmann & W Neupert 2000).

The IMM shows the highest ratio protein-phospholipid among the biological membranes and is enriched with the unusual phospholipid, cardiolipin (J M Herrmann, 2011). This particular composition makes the IMM highly impermeable but the presence of specific carriers (among them is worth to mention the translocase of the inner mitochondrial membrane, TIM) allows the exchange of metabolites between

mitochondria and cytoplasm and the formation of a ionic gradient (Guérin, 1991). The space between the OMM and the IMM is called inter-membrane space, while the space enclosed by the IMM is the mitochondrial matrix.

A) B)

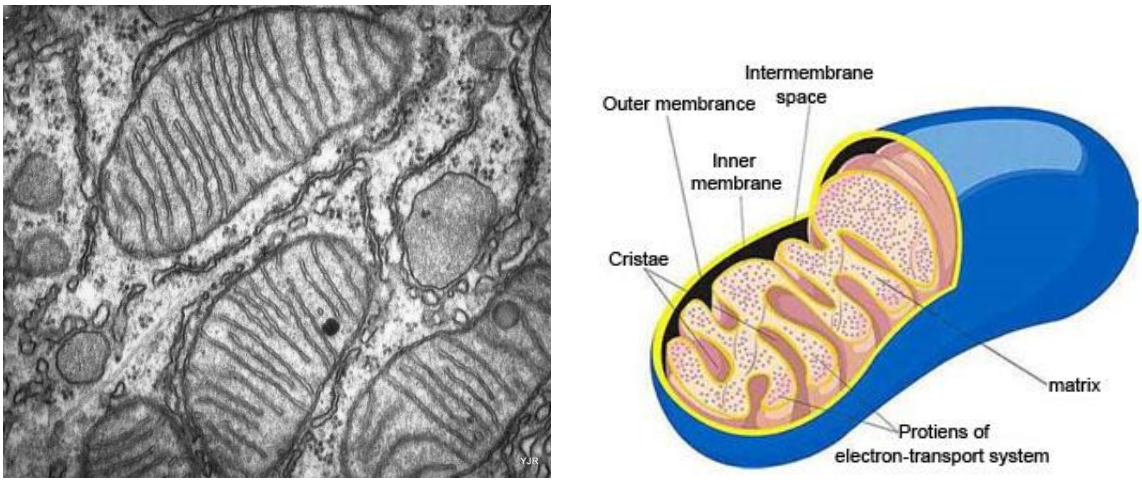

Figure 1.1. A) Electron microscopy image of a mitochondrion; B) Graphic representation of mitochondria.

The IMM is folded and compartmentalized into numerous invaginations called *cristae* where all the respiratory complexes are localized; the organization in these structures expand the surface area of the membrane enhancing its ability to produce ATP.

The number and morphology of *cristae* reflect the response of mitochondria to the energy demand of the cell. Highly folded lamellar *cristae,* with a large surface area, are typically found in tissues with high energy demand such as muscle, brain and hearth (Scheffler, 2008).

The number of mitochondria varies depending on the cell type we consider and the metabolic state of the cell; their distribution inside the cell depends on the site where mitochondria output is required (Bereiter-Hahn & Vöth 1994; Warren & Wickner 1996).

Inside the cell the mitochondria are not dispersed as isolated organelles but they are organized in a highly branched, reticulated structure (Hoffmann & Avers 1973; Stevens 1981).

Mitochondria have to be considered dynamic organelles whose morphology constantly change through the coordination of fusion and fission events (Nunnari et al. 1997; Dimmer et al. 2002; McBride et al. 2006). These processes are believed to provide a

mitochondrial quality control system and enable an effective adaptation of the mitochondrial compartment to the metabolic needs of the cell (Westermann, 2010).
A peculiar aspect that characterizes mitochondria is the fact that they retain their own genome (mtDNA) and machinery for protein synthesis.
Damage and subsequent dysfunction in mitochondria can lead to various human diseases, due to their influence in cell metabolism. Mitochondrial disorders include a wide range of clinical phenotypes and often present themselves as neurological disorders, but can also manifest as myopathy, diabetes, or multiple endocrinopathy (D C Wallace 2001; Massimo Zeviani & Di Donato 2004).

1.1.2. Mitochondrial DNA

Mitochondrial DNA is present into mitochondria in multiple copies and their number vary depending on growth conditions, environmental factors and in metazoans on the tissues we consider.
The majority of studied organisms present a circular molecule of mitochondrial DNA (mtDNA), whose extension varies considerably between metazoan, plants and fungi (Table 1.1). The different size of mtDNA does not necessarily correlates with the number of genes retained by these genomes.

Table 1.1. Mitochondrial DNA size in different organisms and number of genes encoded by the organelle genome (Lang *et al.*, 1999).

Class	Genome size (Kb)	Proteins coding genes	RNA coding genes
Fungi	19-100	8-14	10-28
Protists	6-100	3-62	2-29
Plants	186-366	27-34	21-30
Animals	16-17	13	4-24

In many organisms (e.g. the ciliate *Paramecium Aurelia*, the algae *Chlamydomonas reinhadtii* and the fungi *Candida, Pichia*) mtDNA is found as a linearly organized DNA (J Nosek et al. 1998). In some plants and fungi, including *Saccharomyces cerevisiae, in vivo* the linear form of mtDNA is the prevalent one and is organized in linear multiconcatamers (Bendich 1996; J Nosek et al. 1998; Jozef Nosek & Lubomír Tomáska 2003).

Human mtDNA is a double-stranded circular molecule of 16.5 Kb (fig.1.2) whose complete sequence has been characterized, and usually are present 100-10,000 copies of mtDNA per cell (S. Anderson et al. 1981). The nucleotide content of the two strands of mtDNA is different: the guanine rich strand is referred as the heavy strand and the cytosine rich strand is referred as the light strand.

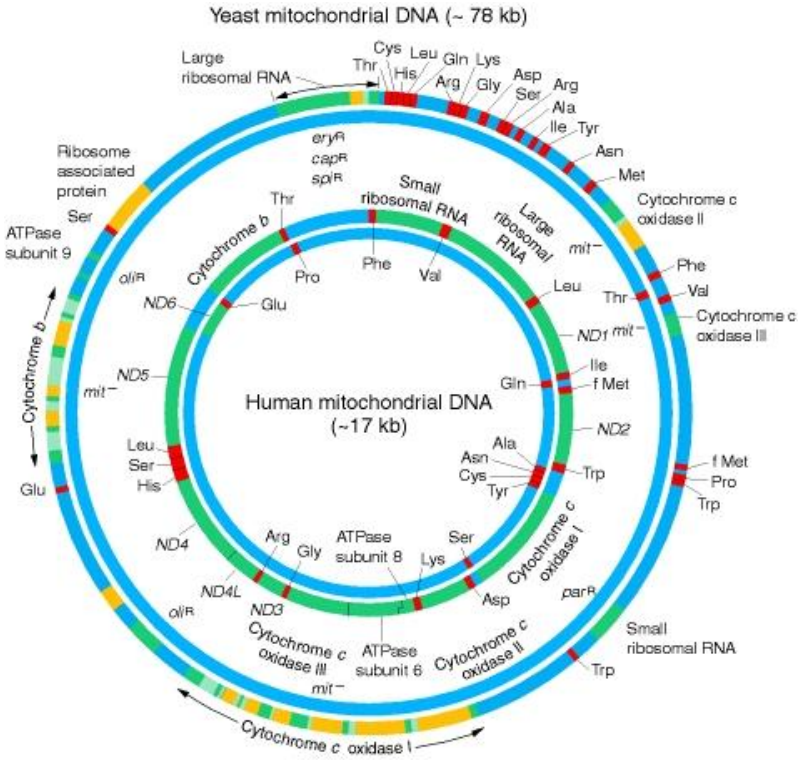

Figure 1.2. Maps of yeast and human mtDNAs. Each map is shown as two concentric circles corresponding to the two strands of the DNA helix. Green : exons and uninterrupted genes, red : tRNA genes, yellow: URFs (unassigned reading frames). tRNA genes are shown by their amino acid abbreviations; ND genes code for subunits of NADH dehydrogenase. Note that the human map is not drawn to the same scale as the yeast map. From: Inheritance of Organelle Genes, 1999, W. H. Freeman and Company.

Most of the original mitochondrial genes have been transferred to the nuclear genome, which now harbors the vast majority of the genes encoding for the *ca.* ~1500 proteins localized to mitochondria. Among the 37 genes contained in human mtDNA, 28 genes are encoded by the heavy strand and 9 by the light strand: 13 genes encodes for proteins of respiratory complexes, 22 encodes for mitochondrial tRNAs and 2 for two mitochondrial rRNAs (12S and 18S) essential for mitochondrial translation. The genetic information in human mtDNA is highly compacted with genes separated by few nucleotides or overlapping and no introns or UTR regions (Scheffler, 2008).

Yeast mtDNA is organized both in linear and circular molecules and has a size ranging from 68 Kb (short strains) to 86 Kb (long strains), whose difference is imputable to non-coding and intronic sequences. In particular, the genome of *Saccharomyces cerevisiae* is 86 Kb and the complete sequence was first published in 1998 (F Foury et al. 1998).

Unlike human mtDNA, in yeast there are few introns interspersed between coding sequences, non-coding regions and the degree of compaction is lower. The yeast mitochondrial genome contains genes for cytochrome c oxidase subunits (cox1, cox2, cox3), ATP synthase subunits (atp6, atp8, atp9), cytochrome b in complex III, a single ribosomal protein Var1p, and several intron related ORFs.

The introns contained in 21S rRNA, *COX1* and *COB* are translated independently or in frame with their upstream exons to produce maturases, reverse transcriptase and endonucleases (F Foury et al. 1998). However, it is worth of noting that unlike human mtDNA, in yeast there are no genes for complex I subunits that consists of only one protein encoded by nuclear genome.

In addition mtDNA encodes for 21S and 15S ribosomal RNAs, 24 tRNAs that recognize all the codons, the 9S RNA component of RNAase P and seven to eight replication origin-like regions.

1.1.3. Mitochondrial DNA organization

Unlike nuclear DNA, mtDNA is not associate with histones and organized in nucleosomes. Several proteins interact with mitochondrial DNA, packing it in a structure called nucleoid which was found to be associate with the inner mitochondrial membrane (Miyakawa et al. 1987; X. J. Chen & Ronald A Butow 2005; Malka et al. 2006; Kucej & Ronald a Butow 2007).

Haploid cells of the yeast *Saccharomyces cerevisiae* contain 40 nucleoids per cell, each one holding more than one copy of mtDNA and representing an inheritable unit (Williamson & Fennell 1979; H T Jacobs et al. 2000).

Nucleoid is a dynamic structure whose organization change according to metabolic cues and whose proper distribution requires frequent event of fission and fusion (fig.1.3) (MacAlpine et al. 2000; Kucej et al. 2008).

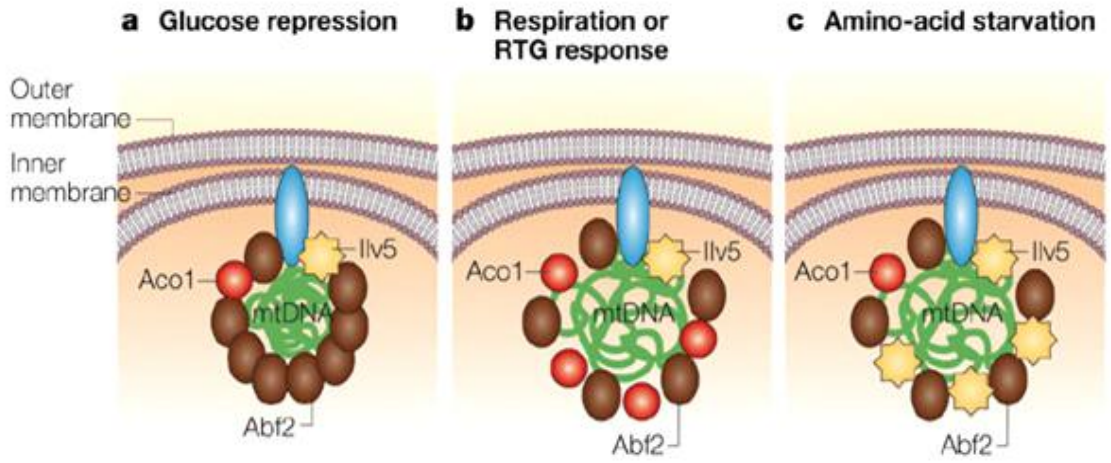

Figure 1.3. a) In the presence of glucose, mtDNA is tightly packaged by the protein Abf2, limiting mtDNA transactions. b) When cells are shifted to respiratory conditions, *ACO1* expression is increased. c) Similarly, in conditions of amino-acid starvation, the expression of *ILV5* is activated by the general amino-acid control pathway and Ilv5p accumulates in mitochondrial nucleoids. The increased levels of Aco1p and Ilv5p substitute for Abf2p packaging mtDNA into a metabolically favorable conformation and/or protect mtDNA in the remodeled conformation (from Chen & Butow, 2005).

The packing of mtDNA requires both proteins directly involved in mitochondrial DNA maintenance and proteins with apparently unrelated functions (X. J. Chen et al. 2005). In the yeast *Saccharomyces cerevisiae* have been identified 22 proteins involved in nucleoid formation, and they can be grouped in two classes: proteins associated to mtDNA and involved in DNA replication, transcription, repair, recombination, and proteins involved in cytoskeletal organization, import and mitochondrial biogenesis, metabolism or proteins responsible of protein quality control.

Abf2 is a protein that belongs to the first functional category and is able to bend mtDNA, inducing supercoiling in the presence of a topoisomerase. In addition to its role in mtDNA packing, Abf2p has been shown to be involved in replication and in the recruitment of other mitochondrial proteins to nucleoids (Newman et al. 1996; Zelenaya-Troitskaya et al. 1998; Friddle et al. 2004). Deletion of *ABF2* leads to a strong mtDNA instability. Other proteins have been shown to bend mtDNA such as Rim1p (protein that bind ssDNA), Mgm101p (enzyme involved in mtDNA repair) and Sls1p (involved in mtDNA transcription).

In the other functional class we find many bi-functional proteins: mtHsp60, mtHsp70, mtHsp10 are proteins involved both in mitochondrial protein import and nucleoid organization; deletion of HSP60 impairs nucleoids segregation with subsequent loss of mtDNA in daughter cells. Many metabolic enzymes involved in Krebs cycle, glycolysis

or amino acids metabolism are actually mtDNA stabilizing proteins (B Kaufman et al. 2000).

Among these there is aconitase, whose function in nucleoid is independent of his enzymatic activity (X. J. Chen et al. 2005; X. J. Chen et al. 2007), and Ilv5 which is a protein involved in the biosynthesis of isoleucine, leucine and valine and required for mtDNA stability. Expression of the ILV5 gene is regulated by the general amino acids control pathway and is a target for the transcription activator Gcn4 (MacAlpine et al. 2000).

Ilv5p rescues the high mtDNA instability due to *ABF2* deletion and seems to be required to maintain the stoichiometric ratio between nucleoids and mtDNA copy number. Proteins having a dual function both as metabolic enzymes and stabilizers of mtDNA are interesting because they provide a mechanism for coupling mtDNA maintenance to metabolism regulation.

One possibility is that bi-functional proteins have evolved as sensors for metabolic changes that can be transmitted by nucleoids to regulate mtDNA maintenance. For example proteins such as aconitase and Ilv5 are crucial for carbohydrate and nitrogen metabolism respectively and, at the same time, for mtDNA stability. Their presence in nucleoids suggests a mechanism for the metabolic control of mtDNA and nucleoid activity; in this way metabolic signals maybe translated altering nucleoids structure to control mtDNA maintenance (Kucej & Ronald a Butow 2007).

Mitochondrial DNA accumulates mutations at a significantly higher rate compared to nuclear genome for several reasons: the lack of protective histones, the high replication rate increasing the likelihood of errors and the proximity to inner mitochondrial membrane and electron transport chain, which is the major source of oxygen reactive species. For all that reasons the nucleoid has a pivotal role in packing mtDNA to prevent oxidative damage and genome instability.

1.2. FUNCTIONS

As pointed out before mitochondria are involved in a large number of processes, although their main role is to generate the majority of ATP for cellular functions, *via* the oxidative phosphorylation. The most important energy sources for the cell are represented by sugars, fatty acids and proteins whose oxidative degradation take place

in cytosol. In aerobic organisms these substrates are oxidized to CO_2 and H_2O, coupled to ATP production through a series of reactions known as cellular respiration.

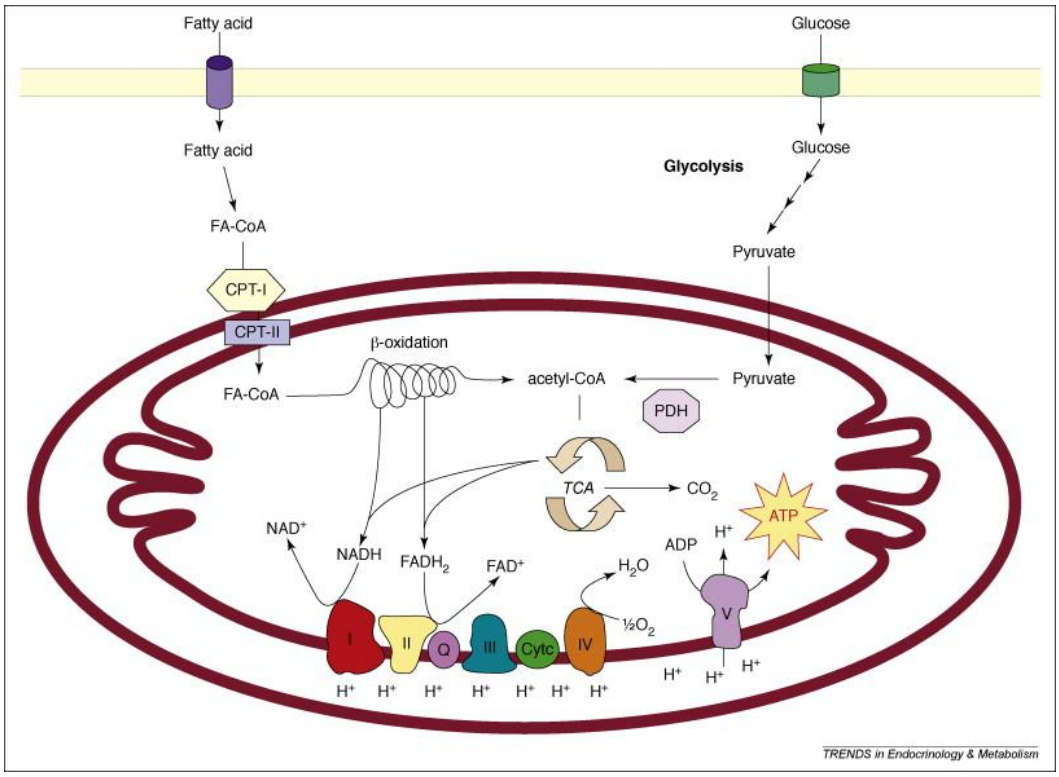

Figure 1.4. Some metabolic pathways that take place inside of mitochondria for energy supply (from Turner and Heilbronn, 2008).

1.2.1. Sugars and fatty acids oxidation

Different catabolic pathways are involved in the oxidation of sugars and fatty acids: cytosolic glycolysis is a central pathway that allows the conversion of glucose in two molecules of pyruvate, 2 ATP and 2 NADH for each molecule of glucose processed. In aerobiosis, the glycolysis represents only the first step of glucose degradation: each pyruvate molecule produced is actively transported inside mitochondria, where it undergoes further oxidation by pyruvate dehydrogenase to acetyl-CoA that enters in Krebs cycle, with a net gain in ATP production (Perham 2000).

In anaerobiosis, pyruvate is converted to lactate or, in yeast, to ethanol by lactic or alcoholic fermentation. Fatty acids are highly negatively charged molecules and they need to be activated and converted in acyl-CoA in cytosol. Once activated, fatty acids

are imported in mitochondria, through an acetyl-carnitine carrier and oxidized to acetyl-CoA by a four steps reaction commonly known as ß-oxidation.
In animals cells the degradation of very long fatty acids is carried out by peroxisomes, where fatty acids are shortened to be easily accepted by the mitochondrial system (Scheffler 2008). In yeast strains ß-oxidation is restricted to peroxisomes, without coupling the process to ATP synthesis; the high potential electrons are transferred to O_2 yielding H_2O_2. The enzyme catalase converts hydrogen peroxide into water and oxygen. The acetyl-CoA units generated in peroxisomes are then transferred into mitochondria by the carnitine shuttle.
Acetyl-CoA is a key component of metabolism and represents the conversion point of different degradation pathways. The acetyl-CoA units obtained both from glycolysis, fatty acids oxidation and from catabolism of some amino acids (glutamate, lysine) enter in the Krebs cycle and are oxidized to CO_2.

1.2.2. Krebs cycle

Krebs cycle, also known as tricarboxylic acid cycle (TCA), consists of eight reactions used by all aerobic organisms and occupies a central position in metabolism. The Krebs cycle is an amphibolic pathway: many catabolic processes converge on it and many intermediates produced by the cycle are precursors used in anabolic reactions (e.g. α-ketoglutarate is precursor for amino acids and nucleotides, succinil-CoA is used for heme biosynthesis) (Scheffler, 2008).
In eukaryotic cells the TCA cycle occurs in mitochondrial matrix where acetyl-CoA is oxidized to CO_2 with the reduction of NAD^+, FAD^+ and ubiquinone. Stoichiometrically 1 molecule of acetyl-CoA is converted in 2 CO_2 with production of 3 NADH, 1 $FADH_2$ and one high energy GTP molecule. All the enzymes of the citric acid cycle are soluble enzymes in mitochondrial matrix, with the only exception of succinate dehydrogenase. This enzyme is the only one in Krebs cycle directly linked to the electron transfer chain (complex II), and represents a connection between TCA cycle and oxidative phosphorylation.
The succinate dehydrogenase consists of four subunits: in yeast Sdh1p and Sdh2p form the catalytic core, which faces mitochondrial matrix, while Sdh3p and Sdh4p are two integral membrane proteins that anchors the complex to the inner mitochondrial membrane (Lemire & Oyedotun 2002). The cycle is useful to increase the cells ATP

production potential by generating reduced carriers whose electrons are passed through the electron transport chain.

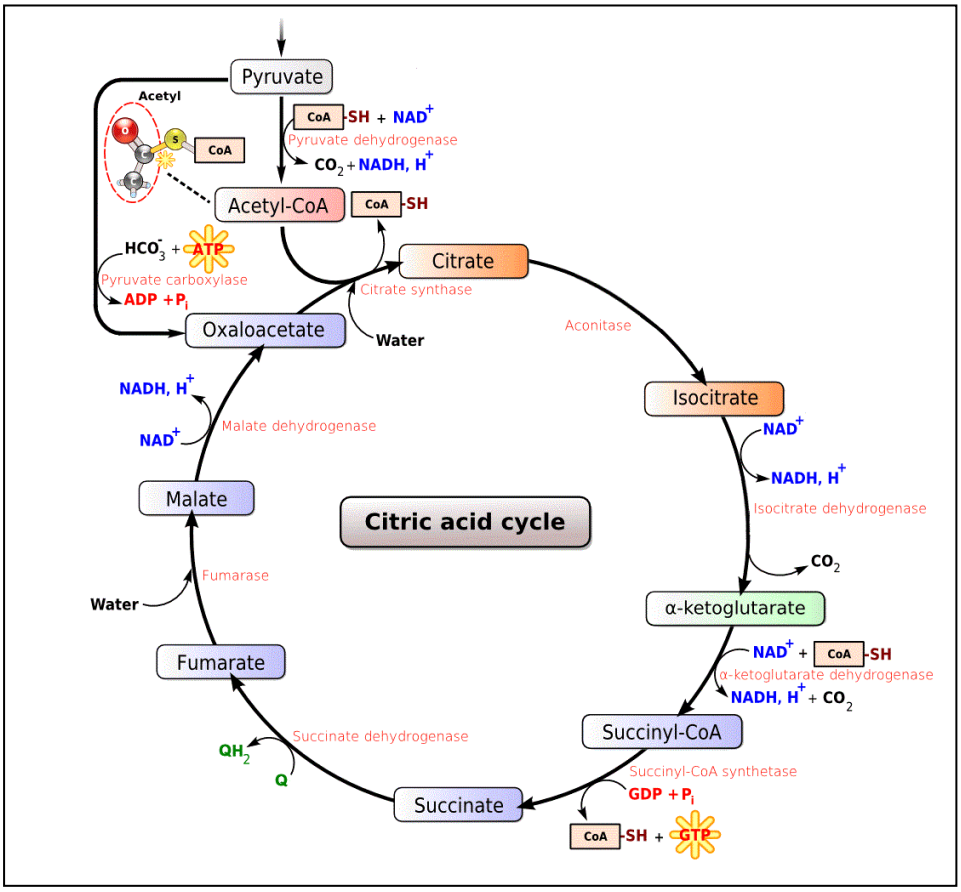

Figure 1.5. Schematic representation of all the reactions and intermediates involved in Krebs cycle.

1.2.3. Oxidative phosphorylation

Catabolic pathways involved in the oxidation of carbohydrates, proteins and fatty acids flow together into the final step of cellular respiration, wherein the energy released by oxidation processes is used for ATP synthesis. During oxidative phosphorylation the electrons derived from NADH or $FADH_2$ are passed through an electron transport chain (ETC) consisting of carriers of increasing reduction potential, ultimately being deposited on molecular oxygen with formation of water. The free energy derived from the electron flow is used to actively pump out protons from the mitochondrial matrix to the intermembrane space (D C Wallace et al. 1988; Salvatore DiMauro & Eric A Schon 2003).

This generates an electrochemical potential across the membrane, which is used to drive ATP synthesis, by allowing protons to flow back across the IMM. NADH electrons are sufficient to produce three ATP molecules, while two ATP molecules are obtained from $FADH_2$ electrons.

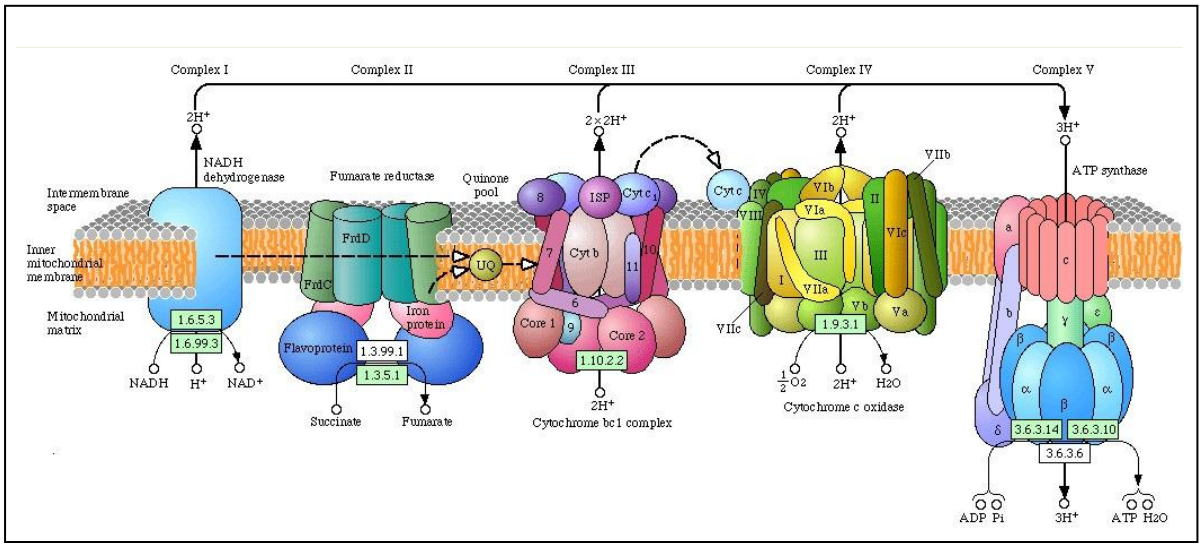

Figure 1.6. Mitochondrial electron transport chain complexes and soluble carriers. The image is taken from KEGG pathway.

The flow of electrons involves both soluble and protein-bound transfer molecules, that are cyclically reduced and oxidized. In ETC tree groups of transporters are involved:

- Coenzyme Q or ubiquinone: small and highly hydrophobic molecule that freely diffuse in the inner mitochondrial membrane and may act as a shuttle between other membrane-bound transporters. Coenzyme Q carries both electrons and protons and exists in three redox states: fully oxidized (ubiquinone), semiquinone (ubisemiquinone), and fully reduced (ubiquinol).
- Cytochromes: mitochondrial proteins that contain heme groups, bound either covalently or not. They are involved only in the electrons flow, transferring them by the reduction and oxidation of a metal ion (usually an iron atom), that the protein holds within the heme group. Three classes of cytochromes exist in mitochondria and they are distinguishable by their visible adsorption maxima; in a-type cytochromes the maximum adsorption peak is around 602 nm, in b-type cytochromes at around 560 nm and in c-type cytochromes at about 550 nm. In cytochromes a and b the heme group is strongly but not covalently bounded to

the protein, while in c-type cytochromes is covalently associated to cysteine residues of the protein.

- Iron-sulfur clusters: the iron atom is bound to inorganic sulfur atoms and/or to sulfur atoms of cysteine residues in the protein. The clusters can be really simple, with one iron atom coordinated by four sulfur atoms, or more complex with two or four iron atoms. The Fe-S cluster proteins are involved in redox reactions in which only one electron at time is transferred, with change of the iron redox state.

Four membrane-bound complexes have been identified in mitochondria, and they consist of multiple peptide intermembrane structures anchored to the IMM. Each complex is structurally in tight contact with the other ones, and functionally connected via mobile carriers (cytochrome c, ubiquinone). Complex I or NADH ubiquinone oxidoreductase catalyzes the following reaction:

$$NADH+Q+5H_i^+ \Rightarrow NAD^+ + QH_2 + 4H_0^+$$

with the transfer of four protons from the matrix to the intermembrane space. In mammalians mitochondria complex I is made of 45 subunits (>900 KDa), seven of which are encoded by mtDNA (ND1, ND2, ND3, ND4L, ND5, ND6). Complex I is tightly associated to the prosthetic group flavin mononucleotide (FMN), and contains different iron-sulfur clusters that transfer electrons from reduced FMN to coenzyme Q. High resolution electron microscopy showed that the complex has an L shape, with one arm dispersed into IMM and the other one facing mitochondrial matrix (Mckenzie & Ryan 2010).

Unlike mammals, common fission and budding yeasts lacks of complex I and use instead a completely different enzyme for electron transfer from NADH to ubiquinone, which does not pump protons out of the matrix.

Complex II, also known as succinate dehydrogenase, is the only enzyme that directly links Krebs cycle to ETC coupling the oxidation of succinate to fumarate with the reduction of coenzyme Q. The complex consists of four subunits: two integral membrane proteins anchors the complex to inner mitochondrial membrane while the two largest peptide organize the enzyme catalytic core. The latter transfers the

electrons from succinate to a flavin adenine dinucleotide cofactor (FAD), and then to three Fe-S clusters with the subsequent reduction of coenzyme Q.

Complex II does not pump electrons from matrix to IMS, because the amount of free energy originated from electron transfer from $FADH_2$ to CoQ is too low. Actually, this is the reason why the electrons flowed in ETC by $FADH_2$ produce only 2 ATP molecules instead of the three obtained from complex I NADH oxidation, which involves complex I.

The ubiquinol-cytochrome c oxireductase or simply cytochrome bc_1 is the third complex in ETC (complex III). This complex is a multi-subunits protein, whose components are quite similar in different species such as yeasts, animals and plants.

Only one peptide of the complex is encoded by mtDNA (cytochrome b), while the others (8 in yeast and 10 in mammals) are encoded by nuclear DNA and imported for assembly in the inner mitochondrial membrane. Complex III catalyzes the oxidation of coenzyme Q (QH_2), by transferring the electrons to cytochrome c and pumping two protons in the intermembrane space.

Functionally, the most important subunits of cytochrome bc_1 are the cytochromes b (b562 and b566) and c_1 and the Rieske iron-sulfur proteins. These are the only proteins participating in the reaction and actually in bacteria, the correspondent complex involved in electron transfer, includes only these three proteins. Anyway yeast mutants for each subunit of complex III have been shown to be respiratory deficient, indicating that even the subunits lacking the prosthetic groups have a relevant role, perhaps in the assembly and stabilization of the complex (Scheffler 2008).

Electrons flow from the reduced Q10 to cytochrome b and then they shuttle through the Fe-S centers to cytochrome c.

$$QH_2 + Cyt\ c^{3+} + 2H_i^{+} \Rightarrow Q + 2\ Cyt\ c^{2+} + 4H_o^{+}$$

Unlike other cytochromes, cytochrome c is not associated to additional proteins but is loosely bound to the IMM and is able to freely diffuse along the mitochondrial membrane. The reaction mechanism of complex III is known as the ubiquinone cycle: two ubiquinone reaction centers are present in complex III and involved in the cycle. The Q_n or quinone reduction center is located in the matrix side of the IMM, while the Q_p center is located close to the outer face of the IMM. Ubiquinol (QH_2) is therefore oxidized by transfer of one electron to the Rieske protein and one to cytochrome b;

once reduced by the electron coming from Q_p center, the Rieske protein shuttles the electron to cytochrome c_1 which in turns completes the reaction through the reduction of the soluble cytochrome c.

The other electron is transferred from one cytochrome b moiety to the second one, and then shuffled to the ubiquinone bound at the Q_n site. The next step is the reduction of ubiquinone to a semiquinone radical. A second Q cycle is necessary to complete the reduction of semiquinone to ubiquinol, which re-enters in the cycle, using the electron of one cytochrome b reduced by a second ubiquinol cofactor which is released as ubiquinone.

Once the cycle is completed four protons are released in the intermembrane space and two protons are taken up from the matrix, with a net transfer of two protons and the reduction of two molecules of cytochrome c.

The reduced cytochrome c is then reoxidized by complex IV (cytochrome c oxidase), which catalyzes the electron transfer from soluble cytochrome c to the final acceptor molecular oxygen, with production of two molecules of H_2O. This process involves two steps: two electrons are transferred on the IMS side, while four protons are taken up from the matrix side, resulting in the transfer of four positive charges across the membrane. In addition, an average of one proton is pumped through the enzyme for each electron transferred to oxygen (Brändén et al. 2006).

In mammals the complex consists of 13 subunits, while in yeast there are only 9 subunits. However in all organisms the three biggest subunits are encoded by mitochondrial DNA and synthesized in the matrix. The complex is a large integral membrane protein which includes two cytochromes (a and a_3) and copper centers involved in the electron transfer. The last non respiratory complex involved in oxidative phosphorylation is ATP synthase (F_1-F_0 ATPase) or complex V.

ATP synthase consists of two regions: the F_0 portion is embedded in the inner membrane and organizes the channel that translocate protons back in the matrix while the F_1 unit faces mitochondrial matrix, whose rotary motion is coupled to ATP production

During ETC, the efflux of protons from mitochondrial matrix creates an electrochemical gradient across IMM which is used by ATP synthase to generate chemical energy in the form of ATP, starting from ADP and inorganic phosphate. .

Once produced, ATP is exported in cytosol by an ATP/ADP carrier that at the same time imports ADP inside the matrix to be recycled. ATP synthase components are

encoded both by mitochondrial and nuclear genes; in yeast the nuclear genes appear to be constitutively expressed, regardless of the carbon source in contrast to nuclear genes for ETC (Ackerman & Alexander Tzagoloff 2005).

Along ETC single electrons may escape prematurely, mainly from complex I and III, and react with oxygen leading to the formation of superoxide. This highly reactive ions can subsequently be converted into hydrogen peroxide and hydroxyl radicals, which are deleterious molecules commonly referred as reactive oxygen species (ROS). High levels of ROS are injurious and induce oxidative stress damaging proteins, lipids and mitochondrial DNA, while at lower concentrations they may act as a beneficial regulatory signal (Ristow & Schmeisser 2011).

In healthy cells the superoxide ions are transformed by the protein superoxide dismutase, which will be discussed later.

1.2.4. OXPHOS impairment

A wide range of pathologies are associated with an impairment of mitochondrial function, whose primary consequence is the accumulation of metabolic intermediates, increased production of ROS and the decrease in ATP production. In general all the clinical phenotypes associated with biochemical and genetic defects affecting oxidative phosphorylation are referred as mitochondrial disorders (Massimo Zeviani & Di Donato 2004).

Mitochondrial disorders may arise as the result of mutations in both mitochondrial DNA or nuclear genes that code for mitochondrial components. For instance in *S. cerevisiae* nuclear genome encodes for at least 500 proteins that localize in mitochondrion where they are involved in a wide range of functions (Lipinski et al. 2010).

It is worthy of note that mitochondrial biogenesis depends on the cooperation between nuclear and mitochondrial genomes. Only a few polypeptides of respiratory complexes are encoded by mtDNA, while the vast majority is encoded in the nucleus and imported into mitochondria.

Moreover transcription of mtDNA is carried out by a dedicated RNA polymerase and its associated transcription factors, all of them encoded by nuclear DNA. Mitochondrial translation apparatus is different from the one used to express nuclear genes and encoded by both nuclear and mitochondrial genome (all the protein components are

encoded by the nucleus, whereas all the RNA components have a mitochondrial origin) (Bonawitz et al. 2007).

Mitochondrial disorders may therefore originate not only from mutations in mitochondrial structural genes impairing oxidative phosphorylation, but also from mutations in both nuclear and mitochondrial DNA. The latter affects mitochondrial protein synthesis and hence the biogenesis of multiple complexes, mtDNA replication and transcription, repair, RNA processing, as well as defects in protein import (Scheffler 2008).

Mitochondrial diseases are heterogeneous and a considerable clinical variability exists in affected patients. One factor responsible for this variability lies in the homoplasmic or heteroplasmic status of mitochondria. A large number of copies of mtDNA is present within an eukaryotic cell, usually identical among them in a healthy individual at birth; this condition is referred to as homoplasmy.

By contrast, a mixture of wild-type and mutated mtDNA can be present, a condition known as heteroplasmy (Holt et al. 1988; Holt et al. 1990). The fraction of mutated mtDNA can range from barely detectable to high percentage, and therefore the expression of the defect can range from mild to very severe. Consequently the clinical phenotypes and their severity vary widely (Scheffler 2008).

It has been shown that the vast majority of mtDNA mutations are recessive and that the proportion of mutant mtDNA must exceed a critical threshold level, before the cell exhibit a biochemical defect (M. P. King & G Attardi 1988). Furthermore, the threshold level of mutant mtDNA may vary among individuals even within the same family, and also among different tissues and organs (Patrick F Chinnery 2000).

Mitochondrial disorders preferentially affect tissues whose function depend on aerobic metabolism such as brain, muscles, heart, peripheral nerves, renal and endocrine system, enhancing the clinical variability observed. They are usually progressive and frequently induce disability and premature death (Leonard & Schapira 2000; S DiMauro & E A Schon 2001).

The major clinical manifestations are myopathies, neuropathies, encephalomyopathies, ataxia, diabetes mellitus, mental retardation and ophthalmologic defects (McKenzie et al. 2004). As described above, mitochondrial diseases may arise both from nuclear and mitochondrial DNA mutations. Mitochondrial DNA mutations include single base substitutions and rearrangement mutations (deletions or partial duplications).

Point mutations are maternally inherited and may be both homoplasmic or eteroplasmic. They can be distinguished in missense mutations affecting structural genes or mutations in genes encoding for mitochondrial rRNA and tRNA, which globally compromise mitochondrial protein synthesis (D C Wallace 1999).

Among them it is worth to mention the A3243G mutation in the $tRNA^{LEU}$ gene which is responsible for mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) syndrome, and a set of mutations affecting complex I activity responsible for Leber's hereditary optic neuropathy (LHON).

Mitochondrial DNA rearrangements, deletions and duplications, are generally associated with severe progressive diseases, usually sporadic and lethal. Rearrangements frequently occur in correspondence of directly repeated sequences and there is reason to believe that are the result of a de novo mutational event, arising during oogenesis or early embryogenesis (Rötig 2010).

Large-scale rearrangements are mainly associated with two clinical conditions that have in common various symptoms: the first is the Kearns-Sayre syndrome (KSS) characterized by progressive ophthalmoparesis, pigmentary retinopathy, mental retardation and increased probability of complete hearth block. The second is the Pearson syndrome which induces pancytopenia and alteration in pancreatic exocrine function in childhood, while in adulthood it induces the typically clinical features of KSS (Scheffler 2008).

As pointed out before, mutations in nuclear genes may give rise to mitochondrial diseases by directly and indirectly compromising OXPHOS function and mitochondrial metabolism, or by destabilizing mtDNA (D C Wallace 1999).

The most common nuclear encoded mitochondrial disease is the Leigh syndrome, which is associated with an impairment of complex IV activity due to severe mutations in the *SURF1* as well as *SCO1* and *SCO2* genes, involved in the delivery of copper to the complex (Tiranti et al. 1998; Zhu et al. 1998; Tiranti et al. 1999; Papadopoulou et al. 1999). The characterization of patients with reduced cytochrome oxidase activity revealed that the defective nuclear genes were mainly involved in the complex assembly.

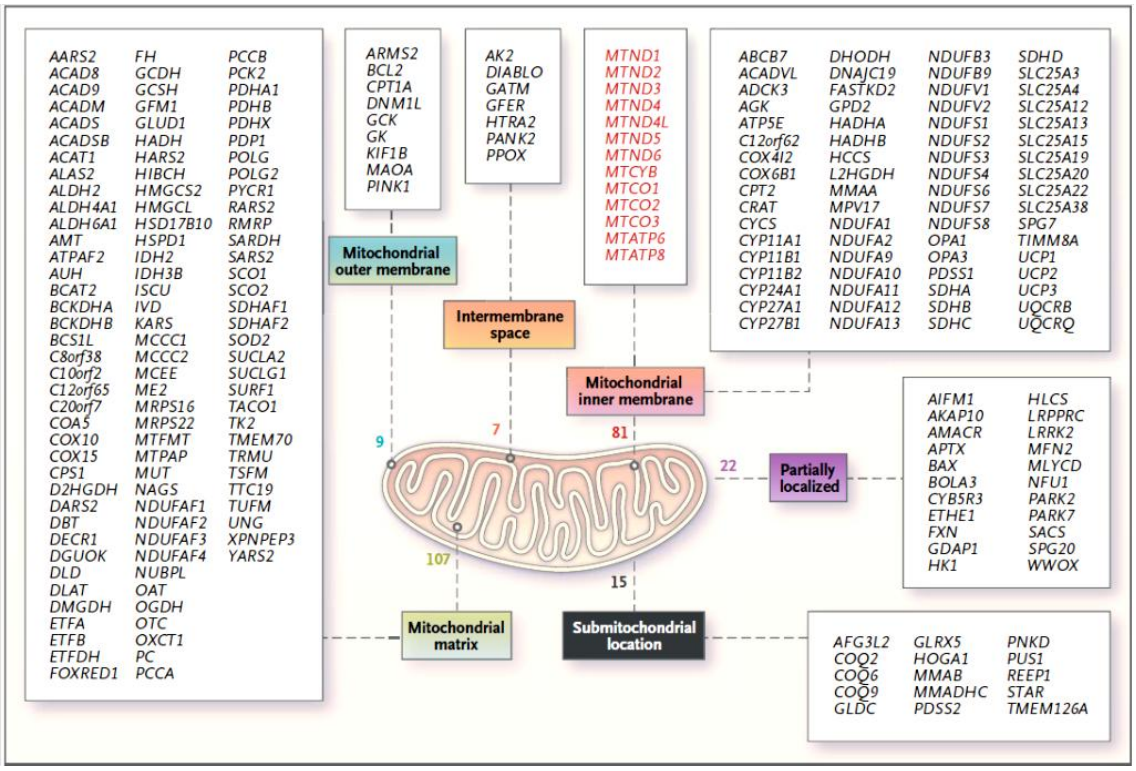

Figure 1.7. List of the mitochondrial (red) and nuclear (black) encoded genes whose mutation is associated with mitochondrial diseases in humans. Genes are grouped according to the predicted localization of the corresponding protein: mitochondrial matrix (107 genes), outer membrane (9 genes), inner membrane (81 genes) and intermembrane space (7 genes). In the figure are also reported genes coding for proteins of unknown localization (15 genes) and gene products that partially localize to mitochondria (22 genes).

Other patients have been identified with a reduced activity of complex I due to mutations in NDUFS1, NDUFS4, NDUFS7, NDUSF8 genes, or with the impairment of complex II function because of mutations in SDHB, SDHC, SDHD genes that result in the formation of an unusual class of tumor known as paragangliamas (Astuti et al. 2001; Chew 2001; Douwes Dekker et al. 2003; Procaccio & Douglas C Wallace 2004; Raimundo et al. 2011).

Complex III-associated deficiencies are rather uncommon, but for example deletion of the nuclear gene UQCRB, encoding the human ubiquinone binding protein of complex III, has been reported in a consanguineous family.

A large number of patients carrying mutations in nuclear genes involved in mtDNA maintenance and replication have been identified. Mitochondrial DNA deletions can be caused by mutations in enzymes of the replication machinery as mtDNA polymerase or the elicase TWINKLE, or by mutations in enzymes involved in the regulation of the mitochondrial nucleotide pool as the thymidine phosphorylase or the adenine

nucleotide translocator (ANT1) (Kaukonen et al. 2000; Nishino et al. 2001). Among others mitochondrial disorders those due to a quantitative reduction of mtDNA copy number are worth mentioning, and are known as depletion syndromes.

The mtDNA depletion syndrome was first described by Moraes and coworkers as a fatal rare disorder, which shows up in early childhood, and which has been identified in several families (Moraes et al. 1991). The disorder is associated with an autosomal recessive trait that causes a severe depletion of mitochondrial DNA in specific tissues, most frequently liver, brain, heart and muscles.

A peculiar aspect is that no mutations in mitochondrial genes have been identified, otherwise the defect appears to reside on nuclear genes: the thymidine kinase 2 (TK2) and deoxyguanosine kinase (DGUOK) genes encode for proteins involved in a pathway responsible for the rescue of mitochondrial deoxyribonucleotides, the main source of precursors for mtDNA , while the POLG gene has a direct role in mtDNA replication.

1.3. MITOCHONDRIA AND AGEING

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Due to their central role in cellular metabolism, mitochondria are important determinants of lifespan and healthspan. Beyond mitochondrial disorders, impairment of mitochondrial function, by altering bioenergetics efficiency and decreasing stress resistance, has long been implicated in late-onset neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, Hungtington's disease and amyotrophic lateral sclerosis) and in the ageing process (Rourke et al. 2002). During the past two decades research has been focused on the understanding of molecular and genetic basis of ageing, and to which extent it correlates with mitochondrial dysfunction.

1.3.1. The mitochondrial theory of ageing

Age related mitochondrial decline has been reported in different organisms and it is a proven evidence that ageing is accompanied by a decline in mitochondrial respiratory activity, accumulation of somatic mutations and morphological alterations including enlargement, matrix vacuolization and abnormal *cristae* (Shigenaga et al. 1994). Despite the clear evidences which show a tight relationship between mitochondrial

dysfunction, ageing and neurodegenerative pathologies, the mechanisms behind these processes still remains largely unknown.

Undoubtedly mitochondrion is a key player in the ageing process, but the challenge is to clarify if the decline of mitochondrial functions is central to the process or just a strong contributor influencing the timing and the severity of the overall deterioration (Scheffler 2008). Among the multiple theories proposed to explain the ageing process the mitochondrial theory of ageing, proposed by Harman in 1972, is still the prevalent (Harman 1972).

According to the theory, ageing results from a continuous process of accumulation of damages originated by reactive oxygen species (ROS), the byproducts of cellular respiration. Flowing along the ETC complexes, electrons can prematurely react with molecular oxygen leading to the formation of superoxide, hydrogen peroxide and hydroxyl radicals (Bonawitz & Shadel 2007).

Mitochondria are the main site of ROS production, which in turn triggers out a vicious cycle: by inducing oxidative damage to mitochondrial macromolecules, including mtDNA, ROS lead to impaired OXPHOS function and even greater ROS production (Balaban et al. 2005).

The major ROS production depends on complex I and complex III, and once produced the superoxide anion could be furthermore reduced to hydroxyl radical or react with nitric oxide to form a highly oxidizing peroxynitrite anion (Andreyev et al. 2005). Eukaryotic cells have evolved defense mechanisms based on proteins such as superoxide dismutase (cytosolic Sod1p or *Cu/Zn*Sodp and mitochondrial Sod2p or *Mn*Sodp), catalase, glutathione reductase and peroxidases which convert the deleterious ROS into non-toxic molecules.

In particular the manganese-dependent superoxide dismutase, localized in mitochondrial matrix, catalyzes the dismutation of superoxide into molecular oxygen and hydrogen peroxide. The latter is converted into water through enzymatic reactions catalyzed by catalase and glutathione peroxidase in the cytosol (Andreyev et al. 2005).

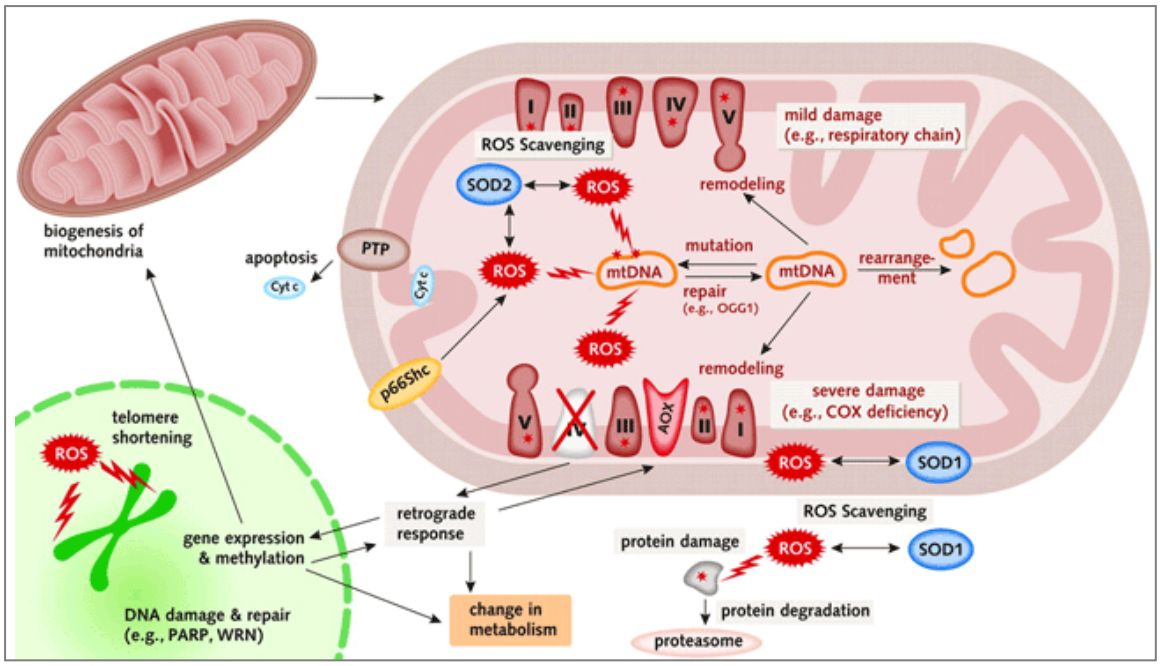


Figure 1.8. Molecular events related to normal and stress-induced senescence. ROS damage in part is rescued by scavenging systems. Once cellular components are damaged (red asterisks) they may be removed and replaced by newly synthesized proteins, if mtDNA is functional. If damage to mitochondria is too severe a retrograde response signals to the nucleus and induces the expression of additional genes that can rescue lost functions. Damage, however, also lead to the induction of apoptosis (from *EMBO reports* 3, 12, 1127–1132, 2002).

If superoxide or hydrogen peroxide production is too high and completely saturates the reduction capability of the antioxidant enzymes, the Fenton reaction may take place. The reaction involves hydrogen peroxide or superoxide as substrates and, in the presence of Fe^{2+}, leads to the formation of an hydroxyl radical, which is highly reactive and gives a permanent accumulation of oxidative damage (D C Wallace 1999).

$$Fe^{3+} + O_2^{-} \Rightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + OH + OH^{-}$$

Figure 1.9. Fenton reaction.

The efficiency of the defense systems normally decline with ageing, but although is commonly accepted the notion that oxidative damage accumulates with ageing, the establishment of a cause-effect relationship between the two remains controversial. In support of the mitochondrial theory of ageing it has been shown that overexpression of *MnSOD* in *Drosophila melanogaster* was able to extent lifespan in proportion to the enzyme increase (Parkes et al. 1998; J Sun & J Tower 1999; Jingtao Sun et al. 2002).

Moreover mice expressing a mitochondria-targeted catalase showed an increased lifespan in association with a reduction of ROS levels, oxidative damage and age-associated pathologies (Schriner et al. 2005).

However, any increase of lifespan was observed in mice overexpressing human catalase targeted to peroxisome or nucleus, suggesting that only mitochondrial ROS have a role in lifespan extension (Schriner et al. 2005; A Trifunovic & N-G Larsson 2008). In *Caenorabditis elegans* the treatment with small synthetic superoxide dismutase/catalase mimetic leads to a robust increase in life span, accompanied by a reduction of oxidative stress (Melov et al. 2000).

In addition, an increase in mutations and rearrangements on mtDNA correlates with ageing (Douglas C Wallace 2005; Bonawitz & Shadel 2007), and microarray profiles showed a decline in mitochondrial-related genes (involved in metabolism, DNA repair, catabolism and cellular transport) with ageing in worms, flies, mice and humans (McCarroll et al. 2004; Zahn et al. 2006). On the other hand, a large number of evidences appear to be in conflict with the free radical theory as determinant in ageing.

In contrast to what expected, the overexpression of the gene coding for mitochondrial ADP/ATP translocator (ANT) in *Drosophila* induces a strong reduction of ROS levels, without any increase in lifespan (Miwa et al. 2004). Knock-out mice for two of the main enzymes involved in detoxification (GPX1 and SOD2) were extremely sensitive to oxidative damage but their lifespan was unaffected (Holly Van Remmen et al. 2004), while in *Caenorabditis elegans* deletion of SOD2 extends lifespan increasing at the same time the ROS production (Van Raamsdonk & Hekimi 2009).

1.3.2. Mitohormesis

Apparently there is a strong contradiction between the findings reported above, according to which an increase in lifespan correlates with an increase in ROS amount and the postulates of the free radical theory.

The contradiction could be reconciled if we consider ROS not only acting as detrimental molecules but even as regulatory ones, by initiating cellular signaling that promote metabolic health and longevity. In fact, low levels of ROS production may exert downstream effects inducing stress defense mechanisms and ultimately extending lifespan, an adaptive response known as mitohormesis (Ristow & Schmeisser 2011). In

worms the exposure to various stress sources, such as heat, ROS and hypoxia extend lifespan (Cypser & T. E. Johnson 2002), while the immune cells for inflammatory response showed that the production of superoxide by NADPH oxidase is important to mediate the inflammasome activation (West et al. 2011).

In yeast the reduction of *TOR1* activity induces an increase of mitochondrial membrane potential, respiration rate and ROS production during growth phases, providing an adaptive signal that in stationary phase decreases ROS and increases lifespan (Y. Pan, E. A. Schroeder, et al. 2011). Moreover, in yeast cultures calorie restriction (reduced nutrients uptake) was shown to increase hydrogen peroxide levels in stationary phase, which in turn stimulates Sodp activity to inhibit superoxide anions accumulation and increase lifespan (Mesquita et al. 2010).

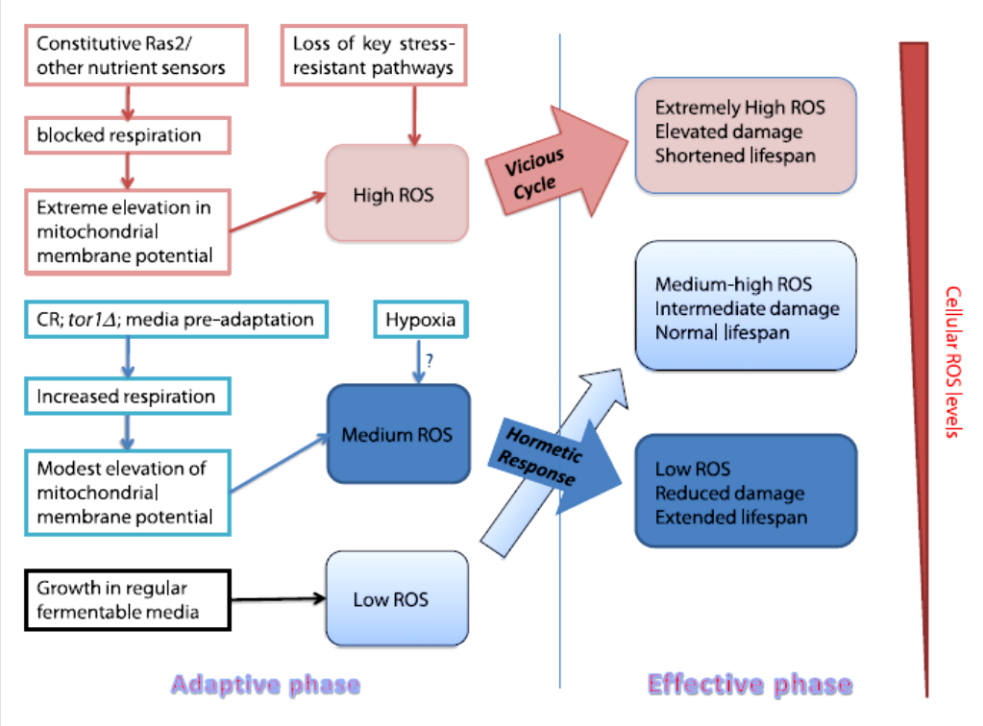


Figure 1.10. Possible readouts of ROS signaling on lifespan (from Pan, 2011).

Intriguingly, different hormetic ROS seems to exert their function in extending lifespan during different phases of yeast life; superoxide signal acts during exponential growth while peroxide takes action during stationary phase. Further investigations are needed to understand the molecular basis of that different behavior and whether or not, at

some point, they could converge on the same signaling pathways to influence lifespan (Y. Pan 2011).

1.3.3. Ageing and somatic mtDNA mutations

The notion that mitochondrial DNA integrity is strictly important to maintain proper mitochondrial function is commonly accepted. Ageing and age-associated diseases are accompanied by a progressive accumulation of mutations on mtDNA, despite a clear cause-effect relationship between the two still remains to be clarified (Douglas C Wallace 2005).

The first evidence of the involvement of mtDNA damage in ageing dates back in 1988, when electron microscopy studies on young, adult and senescent rats showed an increase of structural aberrations (deletions) on senescent rats mtDNA (Pikó et al. 1988).

A fast growing amount of evidences appeared in the next years, showing the increase of mtDNA rearrangements in different human tissues with ageing, followed: in cerebral tissue it has been reported an age-dependent increase of somatic mutations on mtDNA (Corral-Debrinski et al. 1992), as well as a decline in OXPHOS function correlated with ageing in skeletal muscle and liver (Trounce et al. 1989; Yen et al. 1989).

The observation that during ageing there is an accumulation of mutations does not clarify if mtDNA damage is a consequence of the ageing process or even the responsible.

A connection between the accumulation of mutations in mtDNA and ageing was first established by Trifunovic and coworkers: mice expressing a mitochondrial polymerase deficient in proof-reading activity showed a fivefold increase in point mutations on mtDNA, in addition to increased deletions.

The increase in mtDNA instability was correlated with reduced lifespan and premature appearance of ageing-related phenotypes such as weight loss, osteoporosis, heart disease and alopecia (Aleksandra Trifunovic et al. 2004). In contrast to the free radical theory no increased ROS production was observed in "mutator mice", despite of a severe respiratory chain dysfunction (Aleksandra Trifunovic et al. 2005).

A large body of literature claimed to refute the hypothesis of mtDNA mutations as the direct cause of ageing (Edgar et al. 2009; Kraytsberg et al. 2009), while other

evidences placed mtDNA deletions as the driving force behind the "mutator mice" shortened lifespan (Vermulst et al. 2008).

In yeast, lifespan extension matches with an increased genomic stability: base substitution, insertions/deletions or gross chromosomal rearrangements (GCRs) both increase in an age-dependent manner, unless the proto-oncogene *SCH9* is removed (Paola Fabrizio et al. 2004; Madia et al. 2008). It has been proven that *SCH9* promotes the accumulation of superoxide-dependent DNA damage, by activating the error-prone Rev1-Polζ DNA polymerase complex involved in DNA repair by translesion synthesis. (Madia et al. 2009).

1.4. MECHANISMS BEHIND LIFESPAN EXTENSION

1.4.1. Calorie restriction

The only non-genetic intervention known to slow ageing and the onset of age-related diseases in a wide range of species such as yeast, worms, flies and rodents is calorie restriction (CR). The concept was first introduced in 1935 and was based on the assumption that lower calorie uptake would delay the biological ageing process by reducing cellular metabolic rate (McCay et al. 1935; Ristow & Schmeisser 2011).

In contrast some recent findings support the notion that actually CR correlates with an increase in metabolic rate and ROS production both in worms and flies, inducing defense pathways essential for longevity (Ristow & Schmeisser 2011).

The precisely mechanisms acting upon CR are still elusive, but many of the effects of CR appear to be mediated by three highly conserved signaling pathway: the *TOR*, *SCH9* (AKT/S6K) and *RAS/cAMP/PKA* pathways integrate nutritional signals and others environmental cues to regulate cell growth and ageing, and are required for lifespan extension induced by CR (M. Wei et al. 2008).

In yeast calorie restriction promotes both replicative lifespan (RLS) and chronological lifespan (CLS), the two paradigms used to measure the timing of ageing. To confirm the partially overlapping mechanisms mediated by CR and nutritional signaling pathways, it has been shown that CR fails to further increase the RLS extension observed in both *SCH9* and *TOR1* null yeast mutants.

In yeast cultures calorie restriction is obtained by decreasing the glucose concentration in the media. Normally in the presence of high levels of glucose cells produce ethanol

by fermentation, with the result of lowering pH in the media and promote cell death. Switching non-dividing cell from ethanol-containing media to water mimics extreme starvation conditions and extends lifespan not only in a wild-type cell but even in a *sch9Δ* mutant, indicating that CR mediates lifespan extension in part by independent mechanisms (M. Wei et al. 2008).

As observed among eukaryotes, one possibility is that CR may act by inducing a metabolic reprogramming pushing mitochondrial biogenesis and increasing mitochondrial bioenergetics (Lin et al. 2002; R. M. Anderson & Weindruch 2007).

1.4.2. Longevity pathways

Many conserved regulatory pathways have been identified as mediators of longevity among different organisms. The high degree of conservation of those pathways, even in phylogenetically distant organisms, suggests that regulation of lifespan has evolved early during evolution as a mechanism used by ancestral microorganisms to afford long period of starvation (Valter D Longo et al. 2005; Kenyon 2005).

As pointed out before calorie restriction is one of the main interventions known to induce longevity in various organisms, from yeast to mammals. Genetic models to mimic CR have been identified and are mediated by reduction of the activity of glucose-signaling pathways, such as *RAS/cAMP/PKA*, *TORC1* and *SCH9* (Matt Kaeberlein 2010).

In presence of glucose the *RAS/PKA* pathway induces cell growth and proliferation by transcriptional activation of many downstream effectors. The response to *RAS/PKA* signaling in part depends on the ability to prevent the activation of stress response transcription factors Msn2/4 (Zaman et al. 2009).

The deletion of *RAS2* or *CYR1* in yeast doubles the lifespan and increases the stress resistance (both to heat and oxidative stress). The longevity phenotype has been demonstrated to be dependent on the activation of Mns2/4 and *SOD* (P Fabrizio et al. 2001; Paola Fabrizio & Valter D Longo 2003). Moreover a role of *RAS/PKA* pathway in the lifespan extension has been proven even in higher organisms, such as mice and mammals (Migliaccio et al. 1999; Yan et al. 2007; Enns et al. 2009).

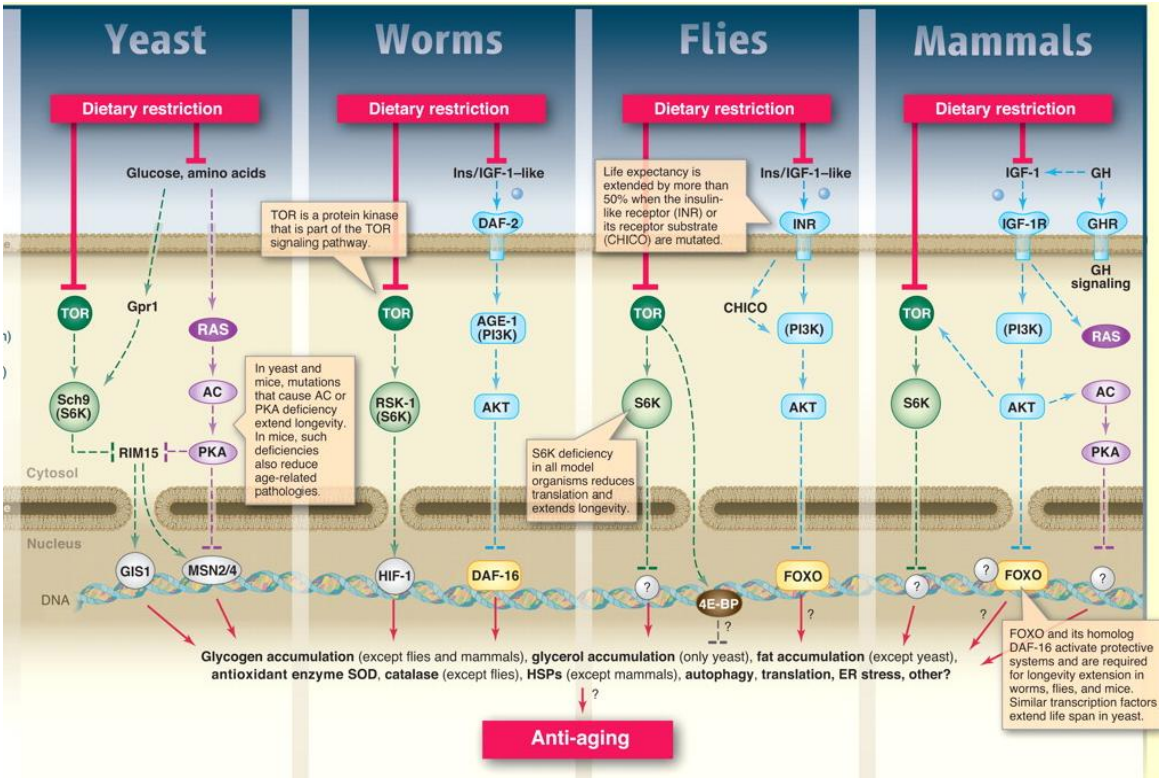


Figure 1.11. Mutations that reduce AKT/PKB, TOR e RAS pathways increase lifespan in different model organisms (from Fontana et al., 2010).

The *TOR* pathway is highly conserved among different organisms and stimulates cell growth by phosphorylating a large number of downstream effectors in response to nutrient availability and growth factors signals (Bonawitz et al. 2007). *TOR* is a serine/threonine kinase first identified in 90's following the discovery of rapamycin, an anti-fungal compound produced by *Streptomyces hygroscopicus* that binds and inhibits both Tor1/2p (Vézina et al. 1975; Zheng et al. 1995).

In all organisms investigated so far there is one Torp isoform that takes part in two distinct complexes, both nutrient responsive. The only exception is yeast, in which two partially redundant Tor kinases are present: Tor1/2p are part of TORC1 complex that positively regulate cell growth by activating transcription, translation and ribosome biogenesis and down-regulating autophagy, stress response and retrograde signaling; TORC2 complex on the other hand includes only Tor2p and is involved in cytoskeletal remodeling and body growth rate control (Bonawitz et al. 2007; Cybulski & Michael N Hall 2009). Tor2p carries out two essential functions: in TORC1, together with Tor1p, is required for translation initiation and G1 progression (Barbet et al. 1996), while in

TORC2 is required for signaling organization of the actin cytoskeleton during cell the cycle (Schmidt et al. 1996).

Only the first Tor2p function is inhibited by rapamycin and in addition, *TOR2* deletion is lethal. A large amount of literature definitely showed that mutation of *TOR* orthologs leads to increased lifespan in different organisms, such as yeast*, D. melanogaster, C. elegans* and mammals (Vellai et al. 2003; Kapahi et al. 2004; Matt Kaeberlein et al. 2005; Polak et al. 2008; Harrison et al. 2009), suggesting an evolutionary link between TOR pathway and ageing.

In yeast it has been shown that, in the presence of glucose, the inhibition of *TOR1* leads to an increase in respiratory activity sustained by an up-regulation of mitochondrial gene expression (Bonawitz et al. 2007). The increase of OXPHOS components, through the coordination of nuclear and mitochondrial genomes, allows to increase oxygen consumption early during growth, with the result of lowering ROS production and oxidative damage in ageing cultures (Y. Pan & Shadel 2009; Y. Pan, E. A. Schroeder, et al. 2011). A microarray analysis showed that in *tor1Δ* mutants a metabolic switch takes place, with the down-regulation of glycolytic enzymes and the up-regulation of TCA cycle components (Hardwick et al. 1999).

The latter evidences point out a new role of *TOR* in the regulation of mitochondrial metabolism, suggesting that enhanced respiration is associated to longevity. In fact, lifespan extension of a *tor1Δ* mutant is comparable to a wild-type cell in a respiratory deficient background (Bonawitz et al. 2007). Many of *TOR* readouts are mediated by the serine/threonine kinase *SCH9*, the yeast ortholog of human S6K. *SCH9* was first identified as suppressor of *PKA*, but more recently it has been clarified that they act in parallel pathways (Toda et al. 1988).

Actually there is an additive effect upon down-regulation of both pathways, and the *ras2Δsch9Δ* double mutant is one of the longest living.

SCH9 is involved in mediating glucose/nutrient-dependent response including stimulation of cell growth and glycolysis, inhibition of stress response and regulation of cell size according to environmental cues (Valter D. Longo 2003). The deletion of *SCH9* extends lifespan and increases the respiration rate. However there is a discrepancy on the ability of *SCH9* mutants to redirect the cell towards a respiratory metabolism because data in literature are quite controversial (Lavoie & Whiteway 2008; M. Wei et al. 2008; Y. Pan & Shadel 2009).

It has been however clearly demonstrated a role for *TOR1* and *SCH9* in ageing regulating mitochondrial function through a fine coordination of mitochondrial and nuclear genomes (Y. Pan 2011).

One possibility is that *TOR1* and *SCH9* may act activating a downstream effector to promote mitochondrial biogenesis and the metabolic switch. It has been shown that overexpression of *HAP4*, a transcription factor inducing genes involved in respiratory metabolism, is able to increase lifespan (Lin et al. 2002; Piper et al. 2006), but the physical interaction *SCH9* has still to be proven (Y. Pan 2011).

A new concept emerging in literature is that the lifespan extension seems to be mediated, at least in part, by a reduced cytosolic translation. Despite the exact mechanism behind this correlation still remains unclear, many evidences are known (Kennedy & Matt Kaeberlein 2009; Matt Kaeberlein & Kennedy 2011). Among these a growing number of translation-related proteins have been identified as determinant for the lifespan extension, including translation initiation factors, structural components of ribosomes, ribosomal RNA processing factors and ribosomal proteins (Kennedy & Matt Kaeberlein 2009).

Moreover, in mammals TORC1 directly controls the cytoplasmic translation through two downstream effectors: the phosphorylation of 4E-BP1 by TORC1 inhibits the binding protein and results in enhancing translation initiation, while the phosphorylation at the C-terminus residues of S6K activates the enzyme, which in turn promotes protein synthesis and ribosomal biogenesis (K. M. Hannan et al. 2003; Wullschleger et al. 2006; Matt Kaeberlein & Kennedy 2011). Reduced 4E-BP1 and S6K orthologs activities correlates with increased lifespan in many invertebrates (P Fabrizio et al. 2001; Kapahi et al. 2004; Matt Kaeberlein et al. 2005; Henderson et al. 2006; M. Hansen et al. 2007; K. Z. Pan et al. 2007).

In Drosophila a clear connection between *TOR1*, translation and calorie restriction has been reported. Upon calorie restriction 4E-BP1 protein is up-regulated and is required for lifespan extension and, at the same time, protein translation is addressed towards a specific pool of mitochondrial nuclear transcripts (and others), with simplest 5'UTRs. The proposed model is that upon *TOR1* inactivation the reduction of global translation rate might coincide with a change in translation profile, towards a setting compatible with lifespan extension (Zid et al. 2009).

A similar system of communication between *TOR1*, translation and lifespan has been reported in yeast long-lived strains with reduced 60S ribosomal subunit (Steffen et al.

2008), indicating that a change in the translation profile according to mRNA 5'UTRs complexity may represent a conserved mechanism to modulate lifespan extension according to metabolic requires (Kennedy & Matt Kaeberlein 2009).

Moreover *SCH9* promotes ribosome biogenesis and maintenance of efficient translation initiation, the reason why it has been proposed as a master regulator of protein synthesis. In fact *SCH9* regulates the activities of the three nuclear RNA polymerase (Woiwode et al. 2008) by phosphorylating downstream effectors or by a direct binding, as occurs for RNA polimerase I. In this way *SCH9* integrates metabolic signals coordinating expression, assembly and activity of the protein synthesis machinery (Huber et al. 2009).

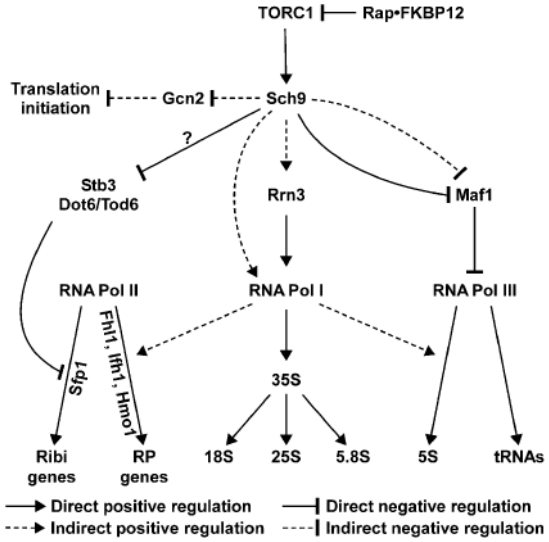


Figure 1.12. Regulatory steps and factors involved in ribosomes biogenesis and protein synthesis under SCH9 control (from Huber et al., 2009).

Similarly to *RAS2*, the *SCH9* null mutant shows increased resistance to heat and oxidative stress, in addition to extended lifespan (M. Wei et al. 2008). Under normal conditions the *TOR/SCH9* and *RAS/PKA* pathways prevent the nuclear localization of the transcription factor Rim15p, that promotes G0 phase entry (Pedruzzi et al. 2003; Cameroni et al. 2004). Upon down-regulation of these pathways or calorie restriction the transcription factor translocates in the nucleus, where through Mns2/4p and Gis1p the stress response is activated.

The lifespan extension of *SCH9* and *RAS2* null mutants similarly depend on *RIM15*, while *GIS1* deletion reverts *Δras2* lifespan to a lesser extent than *Δsch9*. The latter evidence suggests that the two major pathways *TOR/SCH9* and *RAS/PKA* regulates the

longevity in a *RIM15*-dependent manner, but leading to different readouts in terms of gene transcription (M. Wei et al. 2008). In addition, under CR the lifespan extension of *SCH9* and *RAS2* null mutants only partially depend on *RIM15*, indicating that others pathways promote stress resistance and survival exist (Wei et al. 2008).

RIM15 function contributes to lifespan extension in strains with reduced *TORC1* signaling and much of the lifespan-extending effect of *sch9Δ* can be attributed to *RIM15*-dependent stress response activation.

On the other hand, *SCH9* deletion can extend lifespan independently of *RIM15* as well as *sch9Δ* can increase stress response by directly inducing Mns4p nuclear localization, without *RIM15* contribution (Y. Pan, E. a Schroeder, et al. 2011).

It is worth of note that some functions under *SCH9* control are regulated independently of *TORC1*; many others sites of phosphorylation are available on *SCH9* C-terminus even if the upstream effectors, as well as many of the downstream one, still remains unknown.

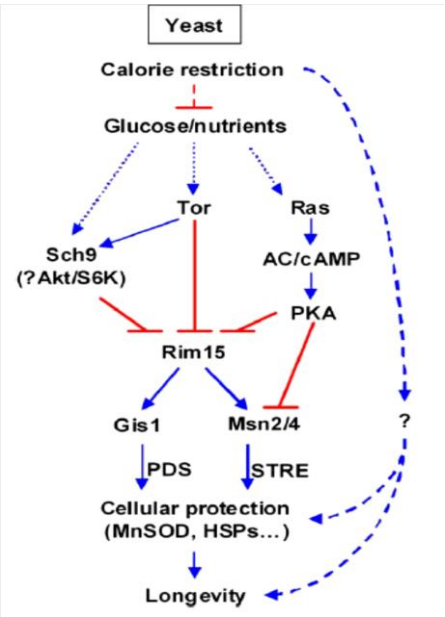


Figure 1.13. Longevity regulatory pathways. In yeast, nutrient-sensing pathways controlled by *Sch9*, *Tor1*, and *Ras* converge on the protein kinase Rim15. In turn, the stress response TF Msn2, Msn4, and Gis1 activate stress response genes and enhance cellular protection, which leads to lifespan extension. Although a major portion of the effect of CR on longevity appears to be mediated by the down-regulation of the Ras and Tor-Sch9 pathways and consequent activation of the Rim15-controlled Msn2/4 and Gis1 protection system, additional mediators are involved (from Wei et al., 2008).

Another important process involved in the quality control of cellular components and regulated by *TORC1* is autophagy. *TOR1* negatively regulates autophagy by direct phosphorylation of the autophagy related genes (*ATG*) or indirectly by interfering with

their expression at a transcriptional or translational level (C. He & Klionsky 2009). Reduced *TOR* signal leads to increased autophagy in many organisms (Noda & Ohsumi 1998; Chang et al. 2009), and it has been demonstrated that yeast long-lived strains require a functional autophagy system (Alvers, Fishwick, et al. 2009; Alvers, Wood, et al. 2009).

In addition, autophagy is negatively regulated even by *SCH9* and *RAS2*; hyperactivation of *SCH9* or constitutive activity of *RAS2* leads to suppression of autophagy, even if *TOR1* function is down-regulated. Moreover, the common douwnstream targets of *SCH9* and *RAS2*, the transcription factors Mns2/4 and Rim15p, are required to induce autophagy upon inactivation of the two nutrient-signaling pathways, but not for activation upon inhibition of *TOR1*. These data indicate that the activities of *TOR1*, *SCH9*, *RAS2* act in parallel to coordinate the autophagy process (Sampaio-Marques et al. 2011).

1.5. YEAST AS A MODEL ORGANISM

The yeast *Saccharomyces cerevisiae* is one of the most intensively studied model organisms to investigate the molecular and genetics basis of higher eukaryotes (Botstein 1991). Despite its simplicity, yeast shares many cellular activities with humans such as RNA transcription and translation, DNA replication, recombination and repair, proteins trafficking and metabolism (Françoise Foury & Kucej 2002). Another advantage of using yeast is the possibility to directly address the regulation of mitochondrial biogenesis, taking advantage of its behavior as a facultative aerobe/anaerobe.

Yeast metabolism is regulated in accordance to carbon sources and oxygen availability. When grown in the presence of glucose, yeast uses glycolysis to produce ATP, while respiration is almost completely suppressed. When glucose is exhausted there is a rapid metabolic shift from fermentation toward respiration, with the induction of all the genes encoding for subunits responsible for oxidative phosphorylation. Although glucose is the preferred source, yeast is able to use other carbon sources that are not fermentable as glycerol, ethanol, acetate and lactate.

All these metabolites require a proper functioning mitochondria, and are commonly exploited in mitochondria-related research to investigate mitochondrial dysfunctions.

The peculiar aspect of yeast to be a facultative aerobe renders *S. cerevisiae* the only eukaryotes able to survive even in the absence of respiratory activity. Yeast mutants with impairment in OXPHOS function are known as respiratory-deficient mutants (RD) and are unable to grow in the presence of oxidative carbon sources and dependent on the presence of fermentable substrates.

The RD mutants are distinguishable from a respiratory competent strain for their morphology and physiology. Actually they give rise to small colonies and this is the reason why they are commonly known as *petite* mutants.

The small size of *petite* mutants depend on their inability to metabolize the ethanol produced in culture by glucose fermentation and this consequently results in a slow replication rate. *Petite* colonies arise from mutations on genes for OXPHOS components encoded both by nuclear or mitochondrial genome. In the latter case it is possible to distinguish ρ^- mutants with gross deletions on mtDNA, and ρ^0 mutants with a complete loss of mtDNA (Dujon 1981; A Tzagoloff & Dieckmann 1990).

1.5.1. *Saccharomyces cerevisiae* as a model for ageing

Almost all of the molecular pathways that regulate mitochondrial functions and the ageing process are well conserved between yeast and higher eukaryotes. In addition, the relatively short lifespan of yeast cells and the easy ways to measure it render yeast extremely convenient for ageing studies. Two models have been established to evaluate yeast cellular ageing: the replicative lifespan (RLS) and the chronological lifespan (CLS).

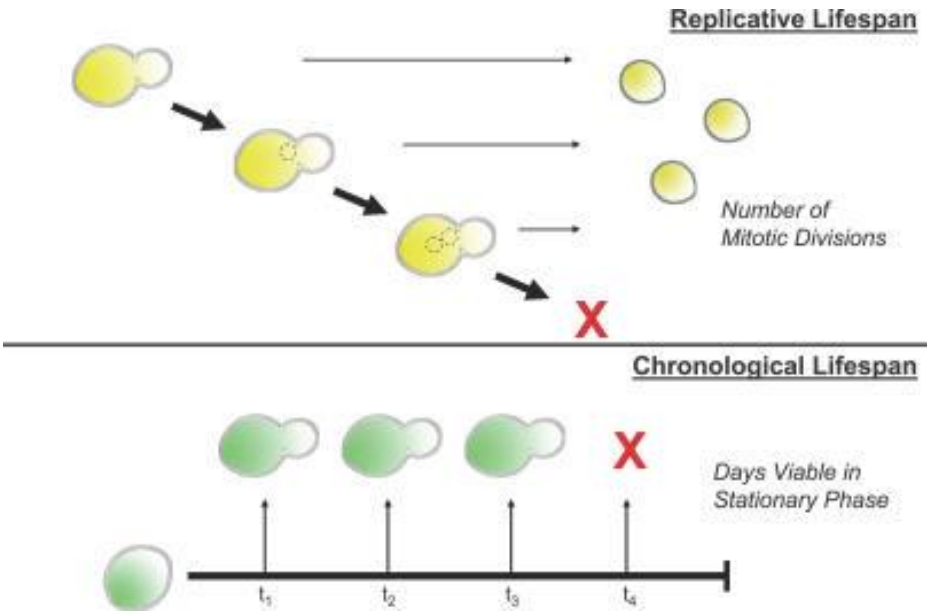


Figure 1.14. Models for measure ageing in yeast (from Kaeberlein et al., 2007).

RLS is defined as the number of daughter cells produced by a single mother cell before senescence, and it has been proposed as a model for ageing in mitotically active cells. Sir2, a NAD^+-dependent histone deacetylase, has a key role in the regulation of RLS by controlling several physiologically important functions such as maintaining DNA integrity, silencing of telomeres and rDNA and ageing (Bitterman et al. 2003). Overexpression of Sir2 strongly increases RLS, while its deletion shortens the lifespan upon 50% (M Kaeberlein et al. 1999).

CLS is a model of ageing of post-mitotic cells and is defined as how long yeast can survive in culture in a non-dividing state (Matt Kaeberlein 2010). Longevity interventions like calorie restriction and reduction of nutrient signaling pathways have been involved in the regulation of both RLS and CLS, but some of the mechanisms behind the two ageing paradigms seem to be different.

It has been shown that the impairment of mitochondrial respiration only impacts CLS, while it has no any influence on RLS (Woo & Poyton n.d.). Our interest focused on the understanding of the role of mitochondrial dysfunction in ageing and on verifying a possible correlation between lifespan extension and increased stability of mitochondrial DNA. For this reason the work presented in this dissertation is focused only on the CLS model.

CHAPTER II

Results and discussion (part I)

2.1. IMPACT OF MUTATIONS IMPAIRING MITOCHONDRIAL FUNCTION ON YEAST LIFESPAN

In the past decades the understanding of the molecular mechanisms behind became a big challenge, since ageing represents one of the major risks for many of the primary diseases affecting modern societies such as cardiovascular diseases, neurodegenerative disorders and cancer. Biological ageing is a complex multifactor process that converges in a progressive declining ability to respond to stress, damage and imbalanced homeostasis.

Mitochondria play a central role in ageing and cellular death in many organisms, from yeast to humans. According to the mitochondrial theory of ageing the energetic decline and the production of ROS are the primary cause behind the ageing process, inducing damage to mitochondrial macromolecules and overwhelming stress resistance and repair systems (Harman 1972).

Respiratory capacity is essential for lifespan extension as well as integrity of mitochondrial structure and function (D. L. Smith et al. 2007; Aerts et al. 2009).

As pointed out by Barrientos, complementary roles of mitochondrial respiration capability and ROS signaling are involved in promoting cell survival. Respiration influences CLS mostly by altering the stress resistance response needed during stationary phase and the metabolism of storage nutrients (Barrientos 2012).

Yeast strains with high level of respiration and ROS production show increased lifespan, while strains with low respiration and ROS have shorter CLS and may take advantage from additional external ROS or a pulse of ROS due to inhibition of TOR pathway (Y. Pan, E. A. Schroeder, et al. 2011; Ocampo et al. 2012).

To investigate the role of mitochondrial function in yeast chronological lifespan (CLS) we analyzed *S. cerevisiae* strains impaired in different processes essential for mitochondrial function and biogenesis. In particular were analyzed mutants compromised in cellular respiration, integrity and stability of mitochondrial DNA, ADP/ATP transport and assembly of respiratory complexes.

All those mutants are known to destabilize mitochondrial function and to be associated with many human mitochondrial pathologies. Chronological lifespan is a measure of the survival time of non-dividing cell. Yeast cells are usually aged in media containing 2% of glucose; under these conditions yeast rapidly grows by fermentation of pyruvate to ethanol, while cellular respiration is completely repressed in a glucose-dependent manner.

Once glucose has been exhausted a switch to a respiratory metabolism take place, cell growth slows and depends entirely on the ethanol accumulated in the media. Chronological lifespan is measured when yeast cells enter in a non-dividing state.

2.1.1. Mutants that compromise cellular respiration

Among the multiple mutations know to compromise mitochondrial function, through an impairment of electron transport chain components, we focused our attention on mutants in succinate dehydrogenase (SDH) subunits and complex III assembly factor BCS1.

Succinate dehydrogenase localizes to the inner mitochondrial membrane and catalyzes the conversion of succinate to fumarate and the reduction of ubiquinone to ubiquinol. This enzyme is the second complex of the electron transport chain (ETC) and the only one in Krebs cycle directly linked to oxidative phosphorylation.

The succinate dehydrogenase consists of four subunits: in yeast Sdh1p and Sdh2p form the catalytic core, facing mitochondrial matrix while Sdh3p and Sdh4p are two integral membrane proteins that anchors the complex to inner mitochondrial membrane (Lemire & Oyedotun 2002).

Impairment of complex II function because of mutations in SDHB, SDHC, SDHD human genes results in the formation of an unusual class of tumor known as paragangliamas (Astuti et al. 2001; Chew 2001; Douwes Dekker et al. 2003; Procaccio & Douglas C Wallace 2004; Raimundo et al. 2011).

Deletions or point mutations in genes coding for SDH subunits have both been extensively studied in our laboratory, showing that dysfunction of SDH leads to a drastic loss of succinate dehydrogenase activity accompanied by inability to grow on non-fermentable carbon sources and almost a ~50% reduction of the respiration rate (Goffrini et al. 2009; Panizza et al. 2012).

The residual respiratory activity demonstrates that the OXPHOS phenotype is imputable to the loss of SDH activity, impairing tricarboxylic acid cycle (TCA). Moreover the spectra profile of *SDH* mutants revealed a reduction in the absorption peak corresponding to cytochrome aa_3, probably in relationship with a structural role of SDH in the stabilization of the others respiratory complexes.

SdhΔ mutants show even higher hypersensitivity to oxidative damage, increased ROS and a pronounced mtDNA instability (Goffrini et al. 2009; Panizza et al. 2012).

We analyzed the CLS of *SDH1, SDH2, SDH4 and SDH5* null mutants in the BY4741 background, whereas deletion of *SDH3* is lethal in this genetic contest.

SDH5 encodes for a soluble protein localized to the mitochondrial matrix required for the assembly of FAD cofactor with the subunit Sdh1p; deletion of *SDH5* leads to a partial assembly of an instable complex II and a strong reduction of SDH activity, without compromising the other TCA or ETC enzymes (Hao et al. 2009).

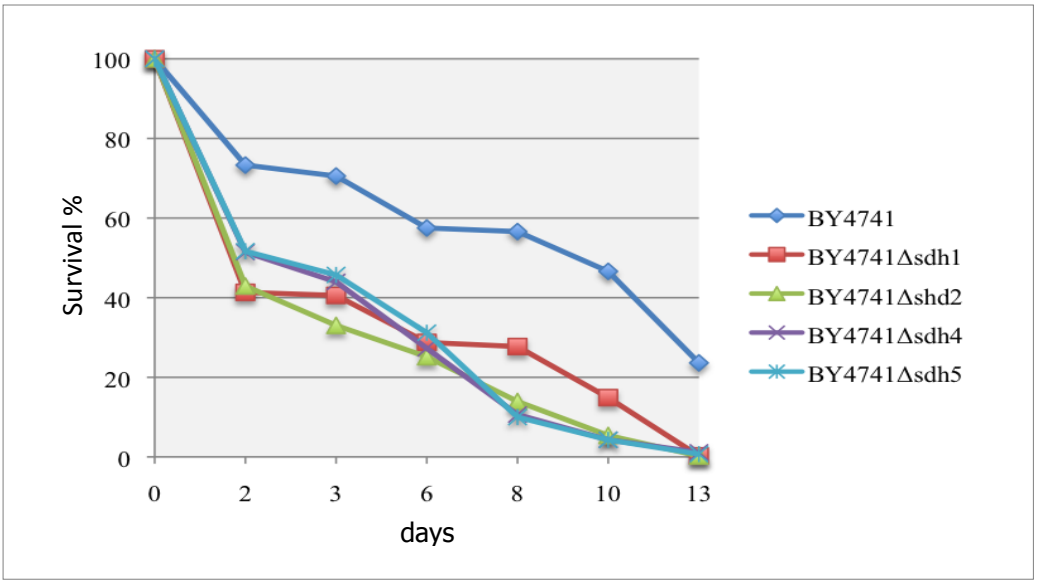


Figure 2.1. CLS of *sdh1Δ , sdh2Δ , sdh4Δ , sdh5Δ* strains in BY4741 background. The results are the mean of three independent experiments.

The measure of CLS shows that deletion of any of SDH subunits strongly reduces lifespan compared to wild-type strain, without appreciable differences between catalytic or structural subunits (Fig. 2.1). As expected, impairment of mitochondrial function correlates with premature ageing, probably dependent on the accumulation of oxidative damage in *sdhΔ* strains.

The reduced lifespan phenotype observed seems not due to defects of respiration, that in *SDH* mutants is not completely compromised but only reduced. As recent findings clarify, yeast cells have a large reserve respiratory capacity to sustain CLS, as respiration only shorten lifespan when reduced below a ~40% of wild-type threshold. Strains that respire above the threshold during exponential growth, adjust their

metabolic rate after diauxic shift at similar levels to that of wild-type strains, on the contrary strains seriously compromised that respire below the respiratory threshold in early phases show lower respiration rate in the stationary phase and very short CLS (Barrientos 2012).

The other mutant we took into account is that devoid of the mitochondrial protein encoded by the gene *BCS1*, involved in the assembly of the respiratory chain complex III.

The protein is directly required for the assembly of the Rieske Fe-S protein, one of the catalytic cores, into cytochrome bc_1 (Cruciat et al. 1999). It has been shown that in *S. cerevisiae* the absence of Bcs1p leads to a complete absence of growth on non-fermentable carbon source such as ethanol, as well as a complete loss or respiratory capability.

The ectopically expression of the human gene *hBCS1* is able to rescue the OXPHOS phenotype, while no differences with the null mutant were observed when the strain is complemented with the pathological allele *hBCS1L*^{G129R} (Tuppen et al. 2010).

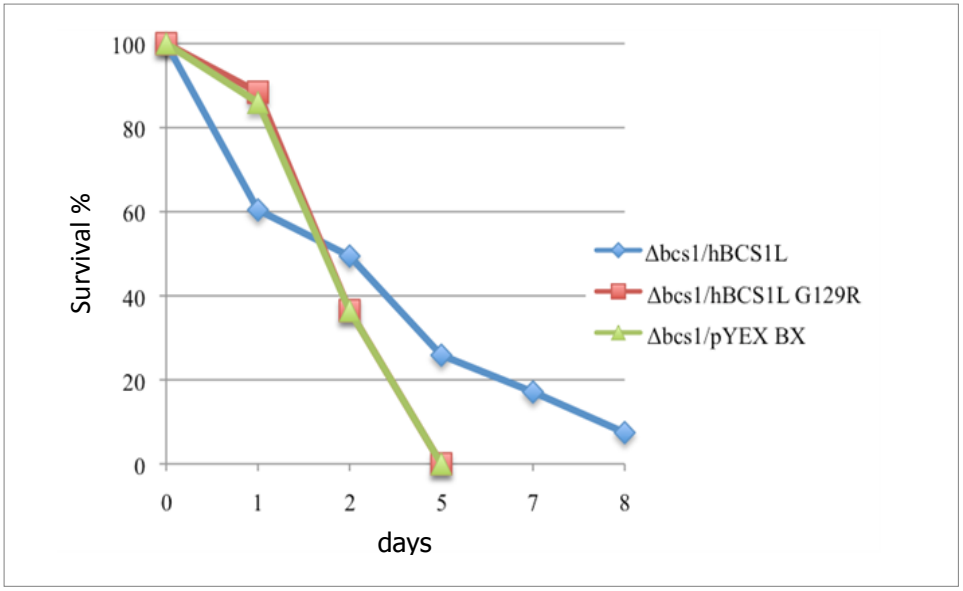


Figure 2.2. The strain W303-1A *bcs1Δ* was transformed with pYEX plasmid carrying the wild-type human BCS1L (*hBCS1L*), the mutant allele *hBCS1L*^{G129R} or the empty vector. CLS was performed growing the yeast cells in YNB DO-URA supplemented with 2% of glucose. After cells reached the stationary phase lifespan measure was performed by CFU assay, on three independent yeast cultures.

We measured the CLS of both the null mutant strain (W303-1A *bcs1Δ*) and the strains complemented with the human wild-type or pathological allele.

CLS analysis showed a reduction of lifespan for both the null mutant and the pathological allele, interestingly at the same extent, compared to the wild-type strain (Fig.2.2). This result indicates that the point-mutation is functionally equivalent to the complete absence of the gene, impairing in the same way complex III assembly and lifespan.

2.1.2. Impairment of mitochondrial function by ADP/ATP carrier mutants

The ADP/ATP carrier has a central role in energetic metabolism by importing ADP in to mitochondrial matrix and exporting ATP in cytosol. Different mutations impairing the ADP/ATP carrier (ANT) have been identified in humans and are responsible for adult or later-onset degenerative disease, autosomal dominant Progressive External Ophthalmoplegia (adPEO).

Beside its role as carrier, ANT is also believed to be part of the mitochondrial permeability transition pore (MPTP) and could play a role in mitochondrion-mediated apoptosis.

As well as in human cells, *S. cerevisiae* has three genes (*AAC1, AAC2, AAC3*) coding for different isoforms of the carrier (Adrian et al. 1986; Kolarov et al. 1990).

AAC1 and *AAC3* are not essential for growth on non-fermentable carbon sources and moreover their deletion does not impair the ADP/ATP transport (Drgon et al. 1992).

AAC2 encodes for the most important of those carriers and is required for growth on non-fermentable carbon sources, such as ethanol, glycerol and lactate.

In this work we analyzed the CLS of the *AAC2* null mutant and one recessive pathological mutation $AAC2^{A137D}$, in the WB-12 genetic background carrying a double knock-out *aac1Δaac2Δ* (Palmieri et al. 2005).

As shown in the graph (Fig.2.3), the absence of the carrier severely compromises the CLS in both null and pathological mutants compared to wild-type WB-12 yeast cells. Interestingly the point mutation shows a lifespan reduction at the same extent as the null strain, despite some of the phenotypes induced by $AAC2^{A137D}$ mutant are more severe.

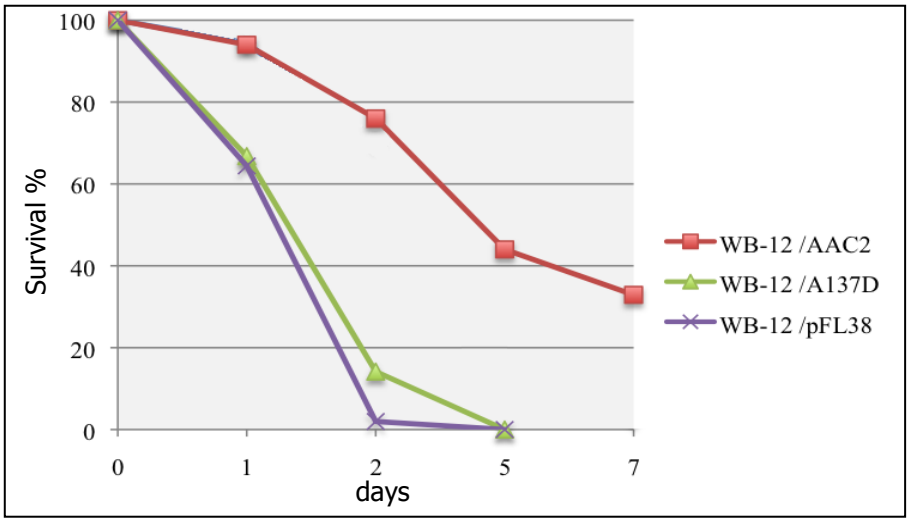


Figure 2.3. CLS of the strain WB-12 (*aac1Δaac2Δ)* transformed with the plasmid pFL38 or with pFL38 carrying the pathological allele *AAC2*^{A137D} or the *AAC2* wild-type gene. Cells were grown in YNB DO-URA media containing 2% glucose. The result of four biological replicates are shown.

As reported in literature, *AAC2* deletion leads to a complete loss of oxidative growth accompanied by a mild reduction of respiratory activity and respiratory complexes amount; on the contrary in the case of the point mutation the OXPHOS phenotype correlates with a complete loss of respiration and cytochromes b and aa_3, but not of cytochrome c (Palmieri et al. 2005).

It is worthy of note that both cytochromes b and aa_3 are encoded by mtDNA while cytochrome c by nuclear DNA, supporting the idea that mtDNA instability may be involved in the pathogenicity of the *AAC2*^{A137D} mutation.

These results indicate that a defective protein can cause more damage that the absence of the protein itself. In fact the ATP uptake is impaired in both the null and pathological mutant, in which the carrier is present but catalytically inactive, and the reason behind differences in respiration rate and cytochromes profile need to be clarified.

In addition increased oxidative damage and subsequent mitochondrial DNA instability was observed in the *AAC2*^{A137D} mutant and authors suggest that loss of ADP/ATP translocation activity inhibits the ATP synthase, thereby blocking the proton influx into the matrix (Palmieri et al. 2005).

As a consequence, the electrochemical gradient can increase up to a level at which respiration-linked proton pumping and electron flow are both stalled, resulting in increase of ROS and mtDNA damage (Palmieri et al. 2005).

In any case those mutants are a clear demonstration of the importance of proper functioning mitochondria to promote lifespan and support the notion of a key role of respiration and ROS as determinants of ageing.

Moreover Chen *et al*. observed that the *AAC2*^{A128P} point mutation correlates with membrane depolarization (inducing defects on mitochondrial biogenesis and mtDNA stability) and shortened replicative lifespan, and that genetic interventions that reduce cytosolic protein synthesis suppress the mitochondrial degeneration of the mutant. They suggest that decreased cytosolic synthesis rate may alleviate mitochondria from unassembled protein stress and reduces the proton-leakage across inner mitochondrial membrane (IMM) during ageing (X. Wang et al. 2008). This finding provides a link between protein homeostasis, cellular bioenergetics and mitochondrial maintenance during ageing.

2.1.3. Mutants leading to defects on mitochondrial DNA stability

Lifespan extension depends on genetic or non-genetic interventions that improve mitochondrial bioenergetics efficiency and increase stress resistance. For example down-regulation of TORC1 signaling pathway exerts a beneficial effect on lifespan by maintaining the robustness of mitochondrial function and stimulating mitochondrial respiration. As pointed out before the presence of a proper respiratory capacity is essential for lifespan extension.

Treatment of yeast cells with ethidium bromide, a specific mtDNA intercalating agent, causes a pronounced instability and subsequent complete loss of mitochondrial DNA. The complete absence of mtDNA leads to an impairment of respiratory complexes assembly and as a consequence loss of respiration activity.

We compared the CLS of a wild-type strain and ρ^0 mutant in BY4741 genetic background (Fig.2.4) and, as expected, the mutant lifespan is strongly affected by the mitochondrial dysfunction resulting from the absence of mitochondrial information.

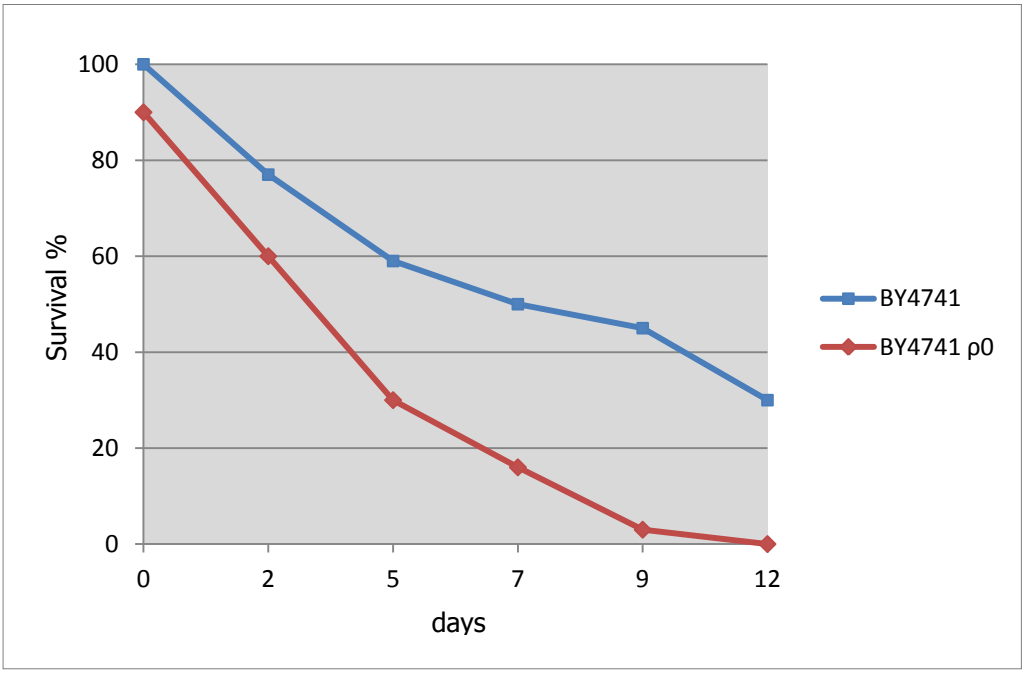


Figure 2.4. Lifespan of BY4741 and BY4741 ρ^0 strains in YPD media. The graph indicates the results from three independent experiments.

Instability of mtDNA may even depend on defects in the only one mitochondrial DNA polymerase found in eukaryotes (pol γ), which is responsible for recombination, replication and repair of mitochondrial DNA.

Over two hundred mutations in *POLG* have been identified in patients with mitochondrial disorders, including progressive external ophthalmoplegia and ataxia-neuropathy syndrome (http://tools.niehs.nih.gov/polg/) (Copeland 2012). In yeast the mitochondrial DNA polymerase γ is encoded by the nuclear gene *MIP1* and, conversely to the human ortholog, consists of one subunit (F Foury 1989).

Deletion of *MIP1* induces instability and loss of mtDNA followed by impairment of the assembly of complex III and IV (whose components are in part encoded by mitochondrial DNA) and complete absence of cellular respiration (Isaya et al. 1994; Baruffini et al. 2006).

We analyzed the impact on CLS of two different *MIP1* mutations, whose phenotypes have been previously studied in our laboratory: the first one is *MIP1*^{Y757C} , a dominant mutation in the polymerase domain corresponding to the human POLG^{Y955C} mutation, while the second is a recessive mutation *MIP1*^{G224A} in the exonuclease domain, corresponding to the human POLG^{g2684}. The two mutations both increase point

mutations and deletion on mtDNA: the point mutations increases at the same extent for the two mutants, while deletions appear to accumulate at higher rate in the *MIP1*^{Y757C} mutant (Baruffini et al. 2006).

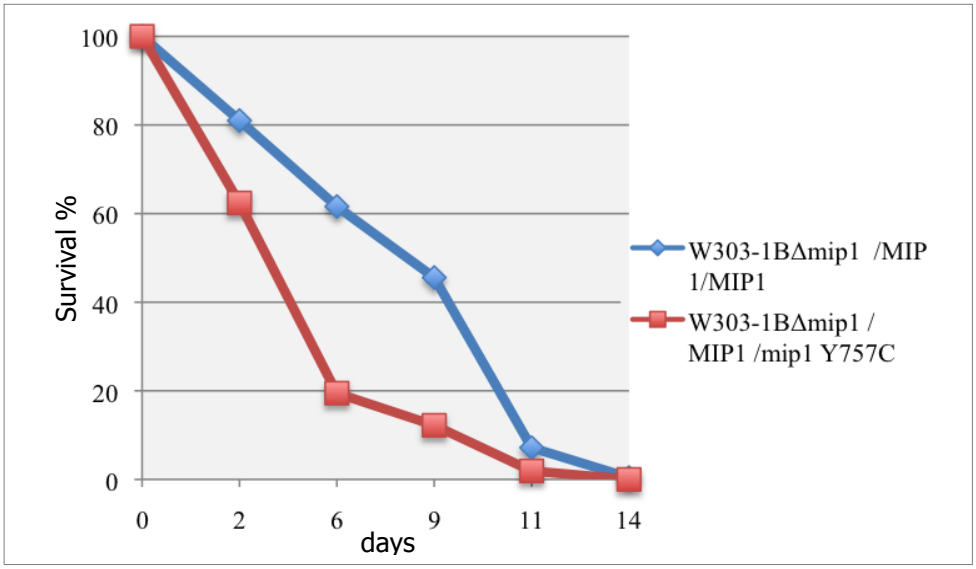
It was shown that in yeast the *MIP1*^{Y757C} mutation in heterozygosity determines a strong increase of oxidative damage on mtDNA (Stuart et al. 2006), and in mouse the correspondent mutation (*Y955C*) induces a three-fold increase in 8-oxodeoxiguanosine content in mitochondrial genome (Lewis et al. 2007).

Oxidative stress-induced defects are often turned into point mutations by error prone repair systems and as the last evidences suggest, this seems to be likely the reason behind point mutation increase in *MIP1*^{Y757C} mutant strains.

Moreover it has been shown that the high *petite* frequency does not depend on the increase in point mutations but on the increased replicative stall due to the reduction of the enzyme catalytic activity (Graziewicz et al. 2004).

In any case in the *MIP1*^{Y757C} mutant the high rate of mitochondrial DNA large-scale rearrangements is the main and primary cause behind the genomic instability observed. In our opinion the different inclination of the *MIP1*^{G224A} , *MIP1*^{Y757C} mutants, towards single nucleotide or deletion defects on mtDNA, was particularly interesting to address the question whether mitochondrial dysfunction induced-ageing correlates with a specific type of damage on mtDNA.

A)



B)

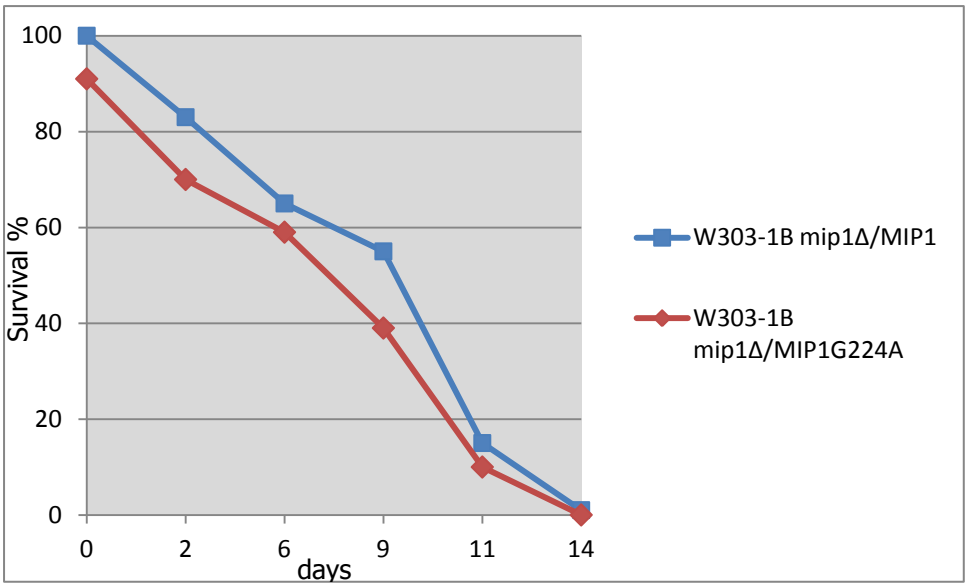


Figure 2.5. CLS measure in YNB with the amino acids for the auxotrophy and glucose 2%. A) W303-1B *mip1Δ/MIP1/MIP1* and W303-1B *mip1Δ/MIP1/MIP1*^{Y757C} strains. B) W303-1B *mip1Δ/MIP1* and W303-1B *mip1Δ/MIP1*^{G224A} strains.

The measure of the CLS (Fig.2.5) revealed that the *MIP1*^{G224A} mutation does not impact lifespan of yeast cells compared to wild-type, while the dominant mutation *MIP1*^{Y757C} affects survival rate in a pronounced manner. This result suggests that large-scale rearrangements on mtDNA play a major role in lifespan regulation and underlines a tight relationship between mtDNA deletions and ageing.

It is noteworthy that a progressive accumulation of point mutations has been shown with ageing in many organisms, but probably substantial amount of single mutations has to be reached before to severely compromise mitochondrial function, and it is more plausible that other types of mutations, like deletions, are more relevant for the ageing process.

CHAPTER III

Results and discussion (part II)

3.1. ROLE OF MITOCHONDRIAL DNA DELETIONS IN AGEING

Mutation on mitochondrial DNA, both deletions and point mutations, normally accumulate in an age-dependent manner in different organisms (Cortopassi & Arnheim 1990; Chomyn & Giuseppe Attardi 2003; Tońska et al. 2009).

Studies in muscle fibers from rhesus monkey having abnormalities in their ETC, revealed the presence of large deletions in mtDNA, suggesting that alterations in mitochondrial DNA (particularly deletions) lead to abnormalities in ETC function (Mao et al. 2012).

Specifically, a 4997 bp deletion, flanked by direct repeats at its junctions, has been found as the most common deletion in ageing humans (Phadnis et al. 2005). This deletion was found to be common in Kearns-Sayre syndrome and chronic external ophthalmoplegia patients.

At least 66% of mtDNA deletions have direct repeats at their junctions, implying that direct-repeat-mediated deletions give rise to these excisions. Deletions on mitochondrial DNA are generally due to errors during replication, but recent findings support the notion that large-scale rearrangements may occur even during the repair of damaged mtDNA (Mao et al. 2012).

The largest number of mitochondrial DNA deletions originate as a response to a break in double-stranded mtDNA: the frequency at which they occur in mtDNA is higher than that of nuclear genome, probably depending on factors such as the proximity of mtDNA to the primary site of ROS production and the absence of protective histones.

In humans, several evidences have implicated direct-repeat-mediated recombination in age-related instability of mitochondrial DNA (Chomyn & Giuseppe Attardi 2003; Phadnis et al. 2005).

In yeast many proteins having activities consistent with a role in recombination have been identified; among those the protein encoded by the*MHR1* gene promotes pairing of single-strand DNA with homologous double-strand DNA in vitro, to form heteroduplex joints, which are necessary intermediates of homologous recombination.

Despite its involvement, *MHR1* is not essential for recombination suggesting the presence of another pairing protein (Ling & Shibata 2002).

The gene product of *NUC1* was instead proposed to be required for initiation of strand invasion (Zassenhaus & Denniger 1994).

3.1.1. Accumulation of mutations during ageing

Integrity of mitochondrial structure and function has been proven to be fundamental for cell health. A decline in mitochondrial function has been observed in many ageing-associated diseases, and loss of respiratory activity curtails lifespan in different model organisms.

Previously in this dissertation we analyzed *S. cerevisiae* mutants compromised in various key mitochondrial functions and we observed that, at different extent, they were negatively affected in chronological lifespan.

Ageing organisms show a progressive accumulation of mutations in their genomes. In particular, due to the tight relationship between mitochondrial functioning and lifespan, it is a common belief that mutations in mtDNA might play an important role in the ageing process by giving rise to cells with a decreased oxidative metabolism.

Many evidences in higher organisms support the existence of a direct link between large-scale rearrangements on mtDNA and ageing or age-associated pathologies. Inoue and coworkers generated a mouse model carrying mutations on mitochondrial genome (K. Inoue et al. 2000).

The mitochondrial-transgenic mice had various level (5-90%) of large deletions on mtDNA and showed the typical mitochondrial disease phenotypes with body weight loss, systemic ischemia, hearing loss and male infertility (Inoue et al. 2000; Nakada et al. 2001; Kazuto Nakada et al. 2006).

Moreover in those mice was observed a strong correlation between the seriousness of the pathological phenotype and the level of deleted mitochondrial DNA (Dogan & A Trifunovic 2011).

3.1.2. May longevity mutations reduce the rate of mtDNA mutability?

As previously described, we observed that a mitochondrial polymerase mutant, that mainly increases deletions on mtDNA, was responsible for a pronounced decrease in lifespan extension. In order to clarify the relationship between ageing and mitochondria we decided to investigate whether increased lifespan correlates with a decrease in the accumulation of large-scale rearrangements on mtDNA.

To address the question whether longevity interventions can suppress the ageing-dependent accumulation of deletions on mtDNA we took in consideration eight different longevity mutants reported in literature (M. Wei et al. 2008). The genes

whose deletion is responsible for increased lifespan are involved in many cellular functions:

- ***REI1***: encodes for a cytoplasmic pre-60S factor required for the correct recycling of shuttling factors Alb1, Arx1 and Tif6 at the end of the ribosomal large subunits biogenesis. Is even involved in bud growth in the mitotic signaling network.
- ***RPL6B***: encodes for a N-terminal acetylated protein which is part of the ribosomal 60S subunit. The gene has a paralog (*RPL6A*) originated form a duplication event.
- ***RPL31A***: encodes for a ribosomal 60S component; loss of both Rpl31p and Rpl39p (another ribosomal 60S subunit protein) confers lethality.
- ***TMA19***: encodes for a protein that associates with ribosomes; the correspondent GFP-fusion protein normally localizes to the cytosol but under oxidative stress relocates to the mitochondrial outer membrane.
- ***RPD3***: encodes for an histone deacetylase that regulates transcription, silencing and other processes by influencing chromatin remodeling. Rpd3p forms at least two different complexes with distinct functions and components. The recruitment of the protein to the subtelomeric region is regulated by interaction with the arginine methyltransferase Hmt1p.
- ***GPR1***: encodes for a plasma membrane G protein coupled receptor that integrates nutritional signals to modulate cell death by the PKA pathway.
- ***TOR1***: encodes for a subunit of TORC1, a complex that controls cellular growth in response to nutrients by regulating transcription, translation, ribosome biogenesis, nutrient transport and autophagy.
- ***SCH9***: encodes for an AGC family protein kinase that regulate ribosome biogenesis, translation initiation and entry in G0 phase. Sch9p integrates nutritional and stress signals to regulate lifespan and the activation of osmo-stress response genes.

It is noteworthy that most of the selected genes, whose deletion is known to increase lifespan, have a direct/indirect role in the assembly or functioning of the protein synthesis machinery. A large number of studies showed that reduced or altered protein translation can promote longevity in invertebrate model organisms, and lifespan

extending mutations have been identified in a growing number of genes that code for translation-related proteins (Matt Kaeberlein & Kennedy 2011).

All the longevity mutants have been analyzed in the *S. cerevisiae* haploid BY4147 genetic context of the Euroscarf knock-out collection, with the only exception of the *SCH9* null mutant that was deleted in our laboratory by one-step gene disruption.

To evaluate the amount of large-scale rearrangements in mitochondrial DNA of the longevity mutants, the spontaneous frequency at which respiratory-deficient colonies (*petite*) arise was evaluated.

Mutations in genes encoding for mitochondrial proteins can increase the mitochondrial mutability; *S. cerevisiae* has the unique property to produce cells with homoplasmic deleted mtDNA molecules, referred as *petite* mutants.

Yeast *petite* colonies are compromised at different extent in their mitochondrial DNA, which in turn results in loss of respiratory activity and the complete dependence on fermentative metabolism. The petite induction assay was performed at 28 °C in YP or SC media as described in the material and methods section.

The *petite* colonies were counted after 6 days of growth and the data reported below are the mean of five independent experiments.

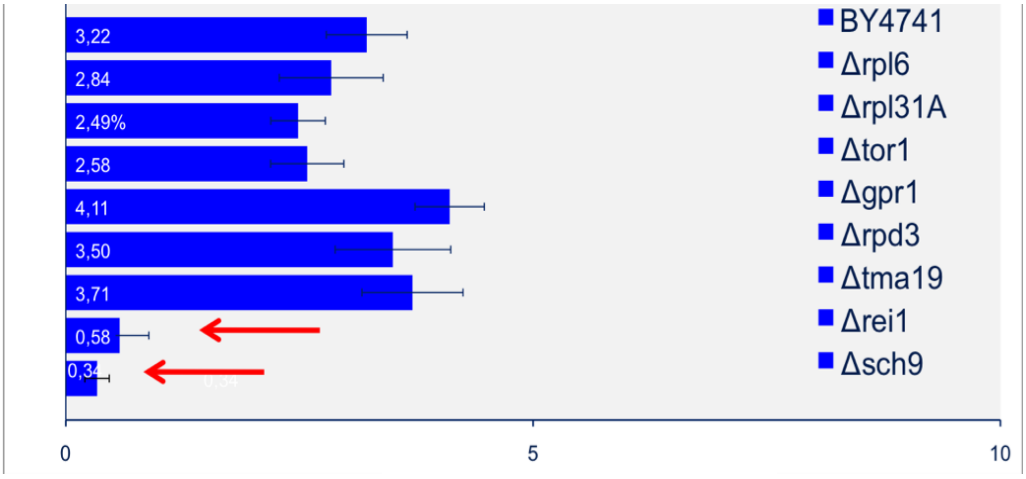


Figure 3.1. Effect of longevity mutations on mtDNA extended mutability. It is indicated the percentage of respiratory deficient colonies (*petite*) in the several longevity mutants.

As shown in figure 3.1 all the longevity mutants but two (*Δsch9, Δrei1*) have a *petite* frequency comparable or greater than wild-type strain. The substantial reduction of

spontaneous *petite* colonies in *Δsch9* and *Δrei1* mutants is indicative of a reduction in the amount of large-scale rearrangements in mitochondrial DNA, thus leading to a major stability of the organelle genome and mitochondrial function. The same results are confirmed even in minimal complete medium, albeit the percentage of *petite* colonies is quite superior for all the strains (data not show).

The evidence that only two of the eight longevity mutations analyzed were able to increase mitochondrial DNA stability is an interesting result, indicating that maintenance of mtDNA is only one of the actors involved in the regulation of the ageing process and that different mechanisms cooperate to regulate lifespan extension in yeast.

It is particularly interesting to observe that *SCH9* and *TOR1* mutants, which are known to be part of the same signaling pathway, behave differently: the reduced petite colonies frequency in the *sch9Δ* mutant, but not in the *tor1Δ* null mutant, is a quite remarkable result.

Sch9p and Tor1p are part of the same signaling pathway that integrates nutritional cues to stimulate cell growth, and Sch9p is a downstream effector whose function is regulated in a *TOR1* dependent manner (Y. Pan & Shadel 2009).

Actually Sch9p has many sites of phosphorylation in the C-terminal region and only few of them are direct targets of *TOR1* kinase, thus indicating that others unknown factors regulate Sch9p function.

The evidence that *tor1Δ* null mutant does not reduce mitochondrial mutability suggests that the increase of mitochondrial DNA stability observed in *sch9Δ* mutant is a peculiar feature of *SCH9* itself, and does not depend on the down-regulation of *TOR1* pathway.

3.1.3. Effect of the *sch9Δ* and *rei1Δ* mutations on induced mitochondrial DNA mutability

Once observed a strong reduction of the spontaneous mutability of mtDNA in *sch9Δ* and *rei1Δ* mutants, we wanted to investigate whether the two longevity mutations were able to increase mitochondrial DNA stability even in "high mutator" backgrounds, in which mutability increase is induced by different interventions.

We first considered a genetic background in which the presence of a dominant mutation in the polymerase domain of *MIP1* gene, encoding for the unique

mitochondrial DNA polymerase, was able to induce a strong increase in mtDNA mutability.

The wild-type, *sch9Δ, rei1Δ, rpl6BΔ* and *rpl31AΔ* strains were transformed with pFL38 plasmid containing the $MIP1^{Y757C}$ allele, previously produced (Baruffini et al. 2006). The *rpl6BΔ* and *rpl31AΔ* mutant strains were unable to reduce the spontaneous mitochondrial mutability and thus have been used in this experiment as a kind of negative controls.

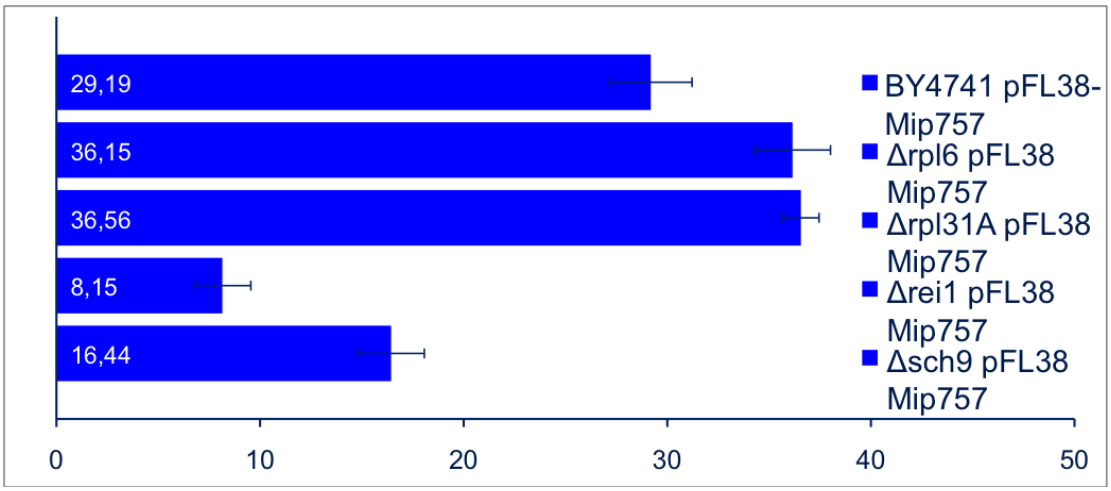


Figure 3.2. The percentage of RD cells in wild-type strain and longevity mutants after the introduction of a dominant pathological allele in mitochondrial DNA polymerase. Cells are grown in YNB DO without uracil.

The graph shows that *per se* the introduction of the pathological allele determine a tenfold (from 3% to 30%) increase of the petite frequency in the wild-type strain. The two longevity mutations *sch9Δ* and *rei1Δ* are able to maintain mtDNA stability even in this "highly mutator" background induced by an altered mitochondrial DNA polymerase. To investigate whether the longevity mutations were able to reduce the *petite* frequency independently of what is liable for it, we analyzed the effect of the two mutations in a second "high mutator" genetic condition due to deletion of *SYM1* gene.

SYM1 is a nuclear gene that encodes for a small mitochondrial membrane protein induced by heat shock (Trott & Morano 2004), whose absence leads to an OXPHOS defective phenotype only at 37 °C as well as a strong increase in mtDNA instability (around 90%) and a 40% reduction of SDH activity (Dallabona et al. 2010).

The double knock-out mutant *sch9Δsym1Δ* was obtained by replacing *SYM1* ORF with a KanMX4 cassette, coding a resistant determinant for geneticin (G418), in a *sch9Δ*

strain. The *rei1Δsym1Δ* mutant was obtained by crossing the haploid BY4741*rei1Δ* and BY4742*sym1Δ* strains, obtained by replacing the wild-type ORF with the KanMX4 cassette. After sporulation and tetrads analysis, one non-parental tetrad carrying two spores able to growth in geneticin and two spores sensitive to geneticin were identified, and the spores *rei1Δ sym1Δ* were isolated.

BY4741*Δrei1* *x* BY4742*Δsym1*

Kan^R Kan^R

Parental		Non parental		Tetratype	
sym1 REI1	+	*SYM1 REI1*	–	*sym1 REI1*	+
sym1 REI1	+	*SYM1 REI1*	–	*SYM1 rei1*	+
SYM1 rei1	+	***sym1 rei1***	+	*SYM1 REI1*	+
SYM1 rei1	+	***sym1 rei1***	+	*sym1 rei1*	+

Figure 3.3. Tetrad analysis: is reported the genotype of the parental, non-parental tetrads and the tetratype and phenotype of growth in presence of geneticin (+ growth, - absence of growth).

The *petite* assay was performed in more permissive conditions to allow OXPHOS defective *sym1Δ* cells to proliferate. *Rei1Δsym1Δ* and *sch9Δsym1Δ* were pre-grown at 28 °C in YPE in order to counterselect the *petite* cells and then inoculated and maintained four hours at 28 °C in YP supplemented with 2% glucose. Then 2% ethanol was added and the cultures were shifted at 37 °C and plated after 24 and 48 hours of growth.

As the graphs show deletion of *SCH9* and *REI1* is able to drastically reduce the high mitochondrial DNA instability normally observed in a *SYM1* mutant, both at 24 and 48 hours of growth. According to us this result is really impressive if we consider that the *petite* frequency ranges from the 90% of the *sym1Δ* to the 8% of the double mutants. As a consequence we can affirm that *sch9Δ* and *rei1Δ* mutants act as suppressors of conditions that alter mtDNA stability, regardless of whether it origininates from mutations in mitochondrial DNA polymerase or *SYM1*. The beneficial effect of *sch9Δ* and *rei1Δ* mutations on mtDNA stability seems to be imputable to a general

mechanism of protection/repair rather than a mutation-specific suppression, as the observed rescue affects genes having different role in mitochondrial genome maintenance.

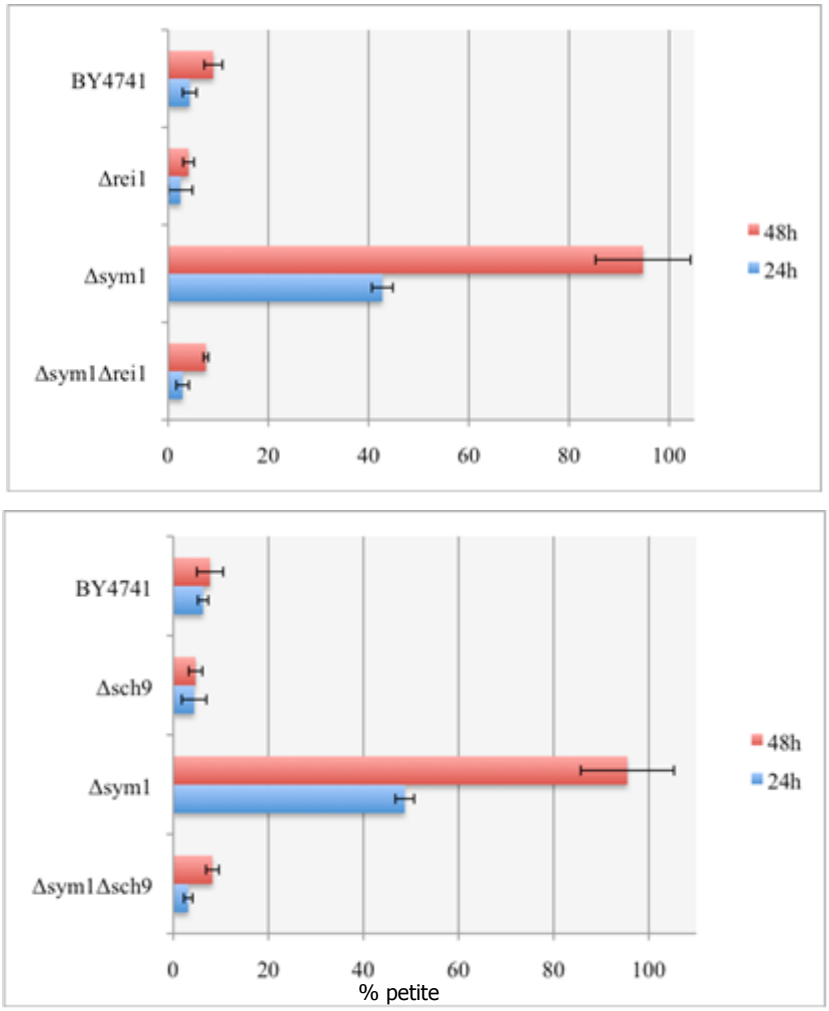


Figure 3.4. Analysis of mtDNA stability was performed at 37 °C in YP supplemented with 2% glucose and then shifted in 2% ethanol. The values reported are the mean of six independent experiments.

Another phenotype associated with deletion of *SYM1* is the reduction of succinate dehydrogenase activity (Dallabona et al. 2010). We observed a reduction of deletions on mtDNA in a *rei1Δsym1Δ* and *sch9Δsym1Δ* mutant and we wanted to address the question whether this phenotype correlates with an improvement of SDH activity. The SDH assay was performed only for *sch9Δsym1Δ* strain and wt, *sch9Δ, sym1Δ* as controls.

Cell were grown in YPD at 28 °C until they reached the exponential phase and then were transferred in stress conditions, with the addition of ethanol and growth at 37 °C,

for 13 hours. Mitochondria were isolated and quantified before the assay was performed, as described in details in the material and methods section.

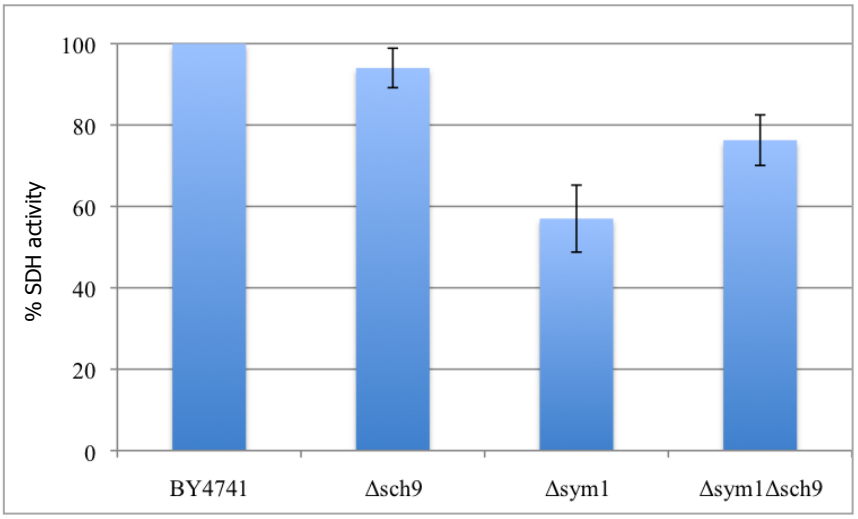


Figure 3.5. SDH assay: the results are expressed as nmols of substrate consumed *per* min *per* mg of proteins. The values are the mean of four independent experiments.

As indicated in the histogram the *sch9Δsym1Δ* double mutant has an intermediate phenotype compared to the *sch9Δ* and *sym1Δ* single mutants. Thus deletion of *SCH9* in a *sym1Δ* background is sufficient to increase SDH activity, but it is not obvious to affirm whether the improvement is a consequence of the reduced mtDNA mutability observed in *sch9Δ* mutant or depends on a general improvement of mitochondrial performances due to *SCH9* deletion.

However recent findings (Dallabona personal communication) proved that in a *sym1Δ* mutant the decrease of SDH activity and the instability of mitochondrial DNA depend on different events. In fact the overexpression of the four SDH subunits in a *sym1Δ* mutant was able to restore the SDH activity, without changing the rate of accumulation of large-scale rearrangements on mtDNA. This result underlined that in *sym1Δ* mutant the SDH activity and *petite* phenotypes result from different defects and suggest that the improvement of SDH activity observed in *sch9Δsym1Δ* double mutant is not due to the increase of mtDNA stability, but probably to a general well-being of *sch9Δ* mutant.

3.1.4. Evaluation of *sch9Δ* and *rei1Δ* mitochondrial mutability during ageing

Ageing associates with a progressive accumulation of mutations on DNA, as observed among different organisms. Previously in this dissertation we demonstrated that deletion of *SCH9* and *REI1* genes in *S. cerevisiae* leads to a reduced accumulation of deletions on mitochondrial DNA.

To gain better insight into the connection between the longevity phenotype and the reduced mtDNA mutability of *sch9Δ* and *rei1Δ* mutants, we decided to clarify how and whether the increased mtDNA stability observed in the two mutant strains was changing during ageing.

Cells from wt, *sch9Δ* and *rei1Δ* strains were pre-grown in YPD and thereafter plated; seven colonies for each strain were grown in YPE to counterselect the *petite* cells and the same amount of cells was inoculated in YPD and aged. After the 3 or 6 days of incubation cells were plated on a typical non *petite* counter-selection assay medium and incubated for seven days at 28 °C , as reported in the materials and methods section.

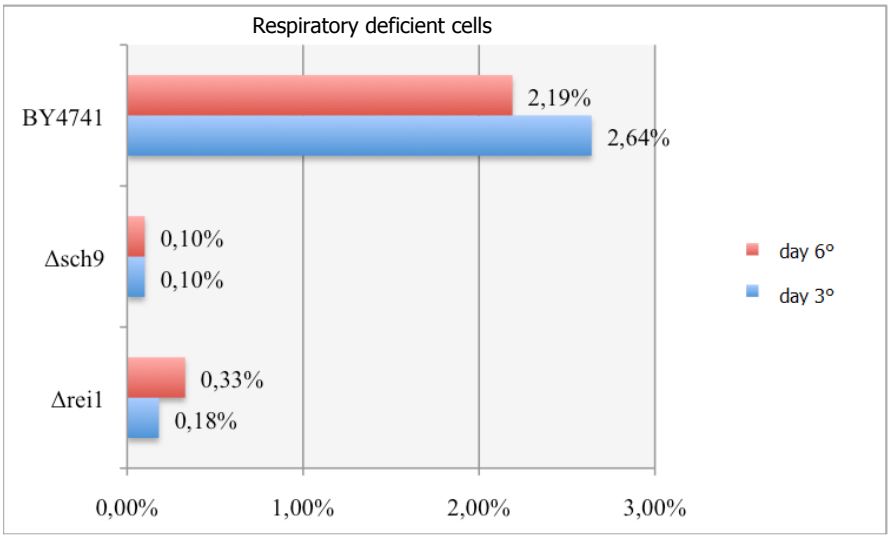


Figure 3.6. *Petite* frequency in aged cells. The values are obtained after 3 or 6 days of cell growth.

As previously observed the *sch9Δ* and *rei1Δ* mutants maintain *petite* frequency at lower levels than wt cells, and this ability is held even during ageing. Despite the intriguing scenario in which *SCH9* and *REI1* are directly involved in mtDNA stability, the results so far obtained might hide a different origin: one possibility is that in *sch9Δ* and *rei1Δ* mutants *petite* cells may die before than wild-type *petite*s or that their division time is higher in both the mutants strains.

In order to assess these hypothesis and evaluate the *petite* cells fitness we treated wt, *sch9Δ* and *rei1Δ* cells with ethidium bromide to obtain the correspondent ρ^0 mutants. A competition growth analysis was performed by setting up YPD cultures in which mixed BY4741ρ^+ and BY4741ρ^0, *sch9Δ*ρ^+ and *sch9Δ*ρ^0, *rei1Δ*ρ^+ and *rei1Δ*ρ^0 cells were inoculated at the same concentration and grown for 24 or 48 hours (see materials and methods).

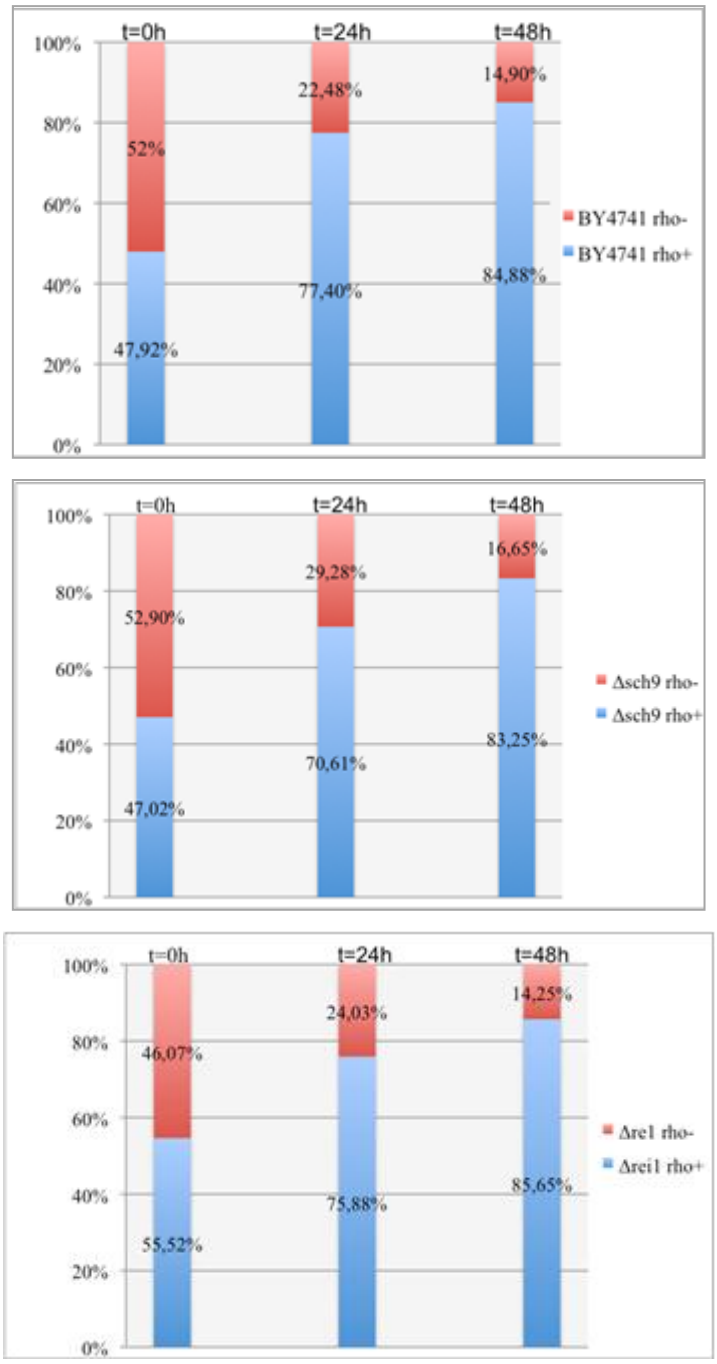


Figure 3.7. Fitness analysis of ρ^0 mutants in wild-type, *sch9Δ* and *rei1Δ* cells. Strains were plated immediately after their preparation, or after 24 and 48 hours. The results are the mean of tree independent experiments.

It is well known that respiratory deficient cells have a shorter lifespan compared to wild-type cells; the data in figure 3.7 show that the decrease in the amount of ρ^0 cells is equivalent between the *sch9Δ* and *rei1Δ* mutants and wt, both after 24 or 48 hours of growth. Therefore the results suggest that the reduced mtDNA instability observed in the two longevity mutants does not depend on a lower fitness of their *petite* cells.
To test whether a difference in the cell division time was the reason behind the low petite frequency, we analyzed the growth curves of ρ^0 and ρ^+ cells for the two mutants.

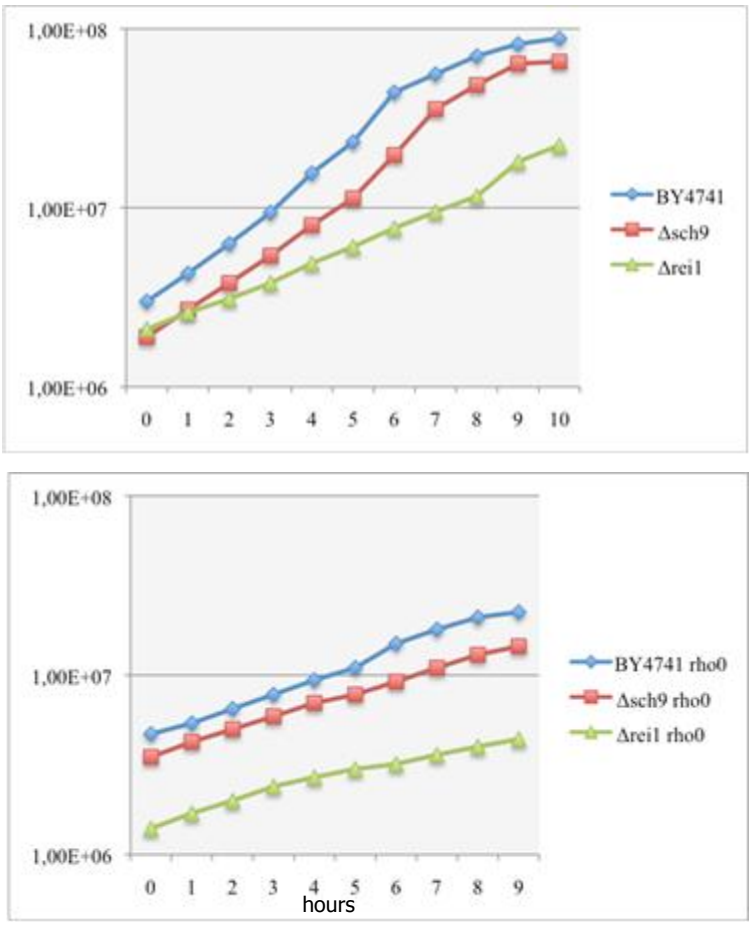


Figure 3.8. Growth curves of ρ^0 and ρ^+ cells. Cells were grown in YPD at 28 °C until they reached 0.2-0.4 OD at 600 nm. Cellular concentration was then measured every hour up to plateau.

Per se deletion of *SCH9* and *REI1* alters the division time of respiratory competent cells: *sch9Δ* ρ^+ cells have a slight increase in the division time compared to wt ρ^+ cells while in *rei1Δ* ρ^+ it doubles. A lower growth rate was observed in many others longevity mutants suggesting that it might be one factors involved in lifespan regulation.

If we consider the respiratory deficient cells we observe that their division time is increased compared to the correspondent ρ^+ cells, but the difference between ρ^+ and ρ^0 cells is quite similar for all the strains. Thus we suggest that the reduced *petite* frequency observed in *sch9Δ* and *rei1Δ* mutants during ageing is not imputable to an increase death rate or division time but depends on the involvement of *SCH9* and *REI1* in one of the many processes that regulate mtDNA stability.

3.1.5. Reduced mtDNA mutability mainly depends on *SCH9* deletion

Sch9p is a serine/threonine kinase that stimulates cell growth and proliferation in response to nutritional signals. Sch9p is a downstream effector of *TOR1*, but many others upstream regulators exist and still remain to be identified.

We have been able to identify an increased mtDNA stability phenotype associated to *SCH9* but not *TOR1* deletion, and we wondered whether this property was imputable to *SCH9* itself or might depend on one of the effectors that mediate and regulate *SCH9* signal.

First of all we focused our attention on *RAS2*, which takes part in a pathway that works in parallel to *SCH9* to promote cell growth and inhibit stress response activation. *SCH9* and *RAS2/PKA* pathways are tight in communication: Sch9p directly regulates PKA and *SCH9* deletion enhances PKA activity (A. Zhang et al. 2011).

Moreover it has been show that *RAS2* and *SCH9* deletions have an additive effect on CLS and that the *ras2Δsch9Δ* is one of the longest living yeast strains (Valter D. Longo 2003). Based on the evidence that *SCH9* and *RAS2* cooperate to extend CLS we investigated whether the same assumption was true for mtDNA stability.

As shown in the graph below, *RAS2* deletion alone does not impact the frequency at which petite arise compared to wt, suggesting that mtDNA stability is specifically under *SCH9* control. However the evidence that *ras2Δsch9Δ* mutant accumulates respiratory deficient cells at lower rate than *sch9Δ* is an intriguing and not obvious observation. The result is consistent with *SCH9* and *RAS2* being part of different pathways, but also suggests that down-regulation of all the signals that prevent stress response activation might be one of the mechanisms to control mtDNA integrity.

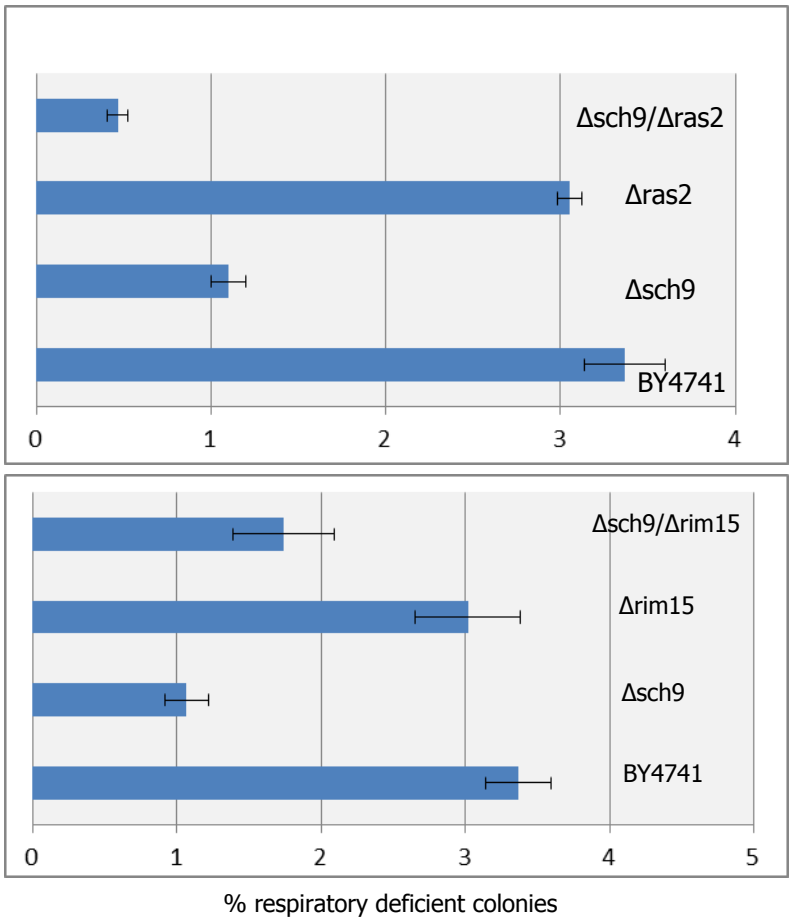


Figure 3.9. Mutability strains in YP at 28 °C. Results are the mean of four independent experiments.

Many of the readouts of TOR1/SCH9 pathway down-regulation depend on Rim15p, a kinase that induces the stress response transcription factors (Mns2/4, Gis1) and contributes to increase yeast lifespan. Deletion of *RIM15* curtails CLS, however *SCH9* deletion is able to extend CLS independently of *RIM15*.

It has been shown that many but not all of the CLS extending effect of a *sch9Δ* strain rely on *RIM15* activation. To mention one case, *SCH9* deletion can induce stress response in a *RIM15*-independent manner by directly inducing Msn4 nuclear localization (Y. Pan, E. A. Schroeder, et al. 2011).

We tested whether the low petite phenotype of the *sch9Δ* strain required the presence of a functional active Rim15p. As shown in the graph, *per se* deletion of *RIM15* does not influence *petite* frequency compared to a wt strain, however it reduces the beneficial effect of *SCH9* deletion on *petite* mutability: the *sch9Δrim15Δ* mutant has a frequency or respiratory deficient cells halfway between the two single knock-out mutants. This result suggests that mtDNA stability upon *SCH9* deletion partially

depends on *RIM15*, and that the same determinants of lifespan extension are in part involved in enhancing of mtDNA protection thus proving a link between mtDNA stability and CLS.

We next addressed whether the increased mtDNA integrity in a *sch9Δ* strain was imputable to a greater availability of dNTP, a well-known limiting factor in mtDNA stability and copy number. The ribonucleotide reductase RNR is responsible for the rate-limiting step of the dNTP synthesis and is a downstream target of the Mec1/Rad53p kinases, thus implicating this DNA damage checkpoint in mtDNA stability (Lebedeva & Shadel 2007).

The *SML1* gene encodes for an inhibitor that represses RNR activity, and whose deletion leads to increased levels of dNTPs. Moreover *SML1* deletion is able to rescue the high frequency of *petite* mutants induced by mutations on *MIP1*, while its overexpression increases mtDNA instability perhaps by limiting mtDNA repair capacity (Baruffini et al. 2006; Lebedeva & Shadel 2007).

As expected *per se* the absence of the inhibitor is able to reduce the petite levels at least at the same extent of the *sch9Δ* strain. The double knock-out mutant shows an additive amelioration of mtDNA stability compared to the two single mutants suggesting that *SCH9* and *SML1* are involved in different pathways that control mtDNA integrity.

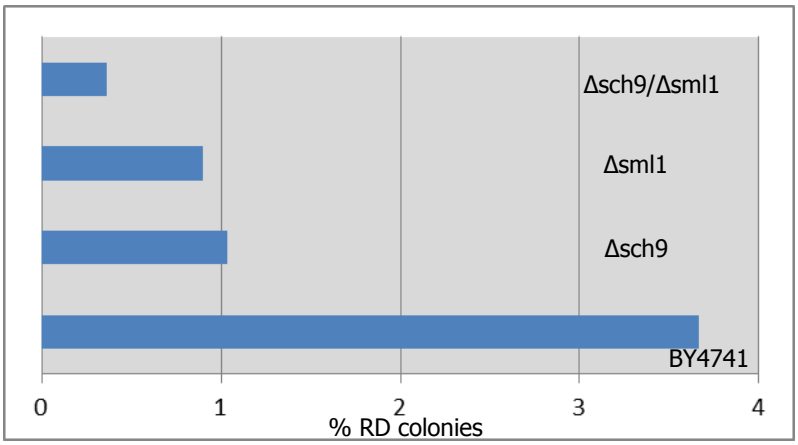


Figure 3.10. Mutability of different strains in YP at 28 °C. Results are the mean of four independent experiments.

3.1.6. *SCH9* and *REI1* deletion only affects mitochondrial DNA large-scale rearrangements

Ageing is accompanied by a progressive decline in mitochondrial function that at least in part depends on the accumulation of both point mutations and large deletions on mitochondrial DNA.

We demonstrated that among the longevity mutants we analyzed, only two were able to drastically decrease the frequency at which *petite* mutants arise.

In order to address the question whether in *sch9Δ* and *rei1Δ* strains the reduction of large-scale rearrangements on mtDNA was accompanied by a reduced accumulation either of point mutations, we determinate the frequency of mutants resistant to erythromycin (Ery^R).

Resistance to erythromycin is acquired through mutations in the mitochondrial 21S rRNA gene, either substitutions at positions, or G or A insertions in the gene sequence. Mutations conferring erythromycin resistance are always localized in the mtDNA, result from all types of nucleotide substitutions and do not alter the mitochondrial function (Baruffini et al. 2010).

Strain	Frequency Ery^R x 10^{-7}
BY4741	4,66 ± 2,32
Δsch9	2,37 ± 0,89
Δrei1	1,89 ± 0,43

Table 3. Frequency of Ery^R mutants in longevity mutants and wild-type strain.

We observed that there is no difference in the rate of accumulation of point mutations between the two mutants and the wt strain, suggesting that *SCH9* and *REI1* functions impact only mechanisms behind large-scale rearrangements.

3.2. ROLE OF *SCH9* AND *REI1* IN PROTEIN TRANSLATION AND RIBOSOMAL BIOGENESIS

Both *SCH9* and *REI1* genes have been previously reported as determinants for cytosolic protein synthesis, as their deletion negatively affect translation rate. Recent finding in many different organisms have shown that reduced cytosolic protein translation directly extends lifespan (X. Wang et al. 2008).

Chen *et al*. found that reduced cytosolic protein synthesis promotes mitochondrial maintenance and suggest that longevity interventions not only delay the onset of ageing associated phenotypes, but even improve increased stress resistance and mitochondrial bioenergetics rate (X. Wang et al. 2008).

As previously described in details the hypothesis is that reduced cytosolic protein synthesis might allow to redirect the translation machinery towards a specific change in the set of proteins expressed, leading to alterations in the expression profile which may benefit cell survival. Furthermore *SCH9* has been identified as a central node in ribosome biogenesis, since directly regulates the activity of all three RNA polymerase and integrates environmental cues to regulate expression, assembly and function of the protein synthesis machinery (Huber et al. 2009). Also *REI1* is involved in ribosome biogenesis and function: beside its role in bud growth *REI1* has been identified as a component of the ribosome RiBi regulon, a set of co-regulated genes that encode for accessory factors involved in ribosome biogenesis.

In particular *REI1* is a cytoplasmic pre-60S factor required for the correct recycling of shuttling factors Alb1, Arx1 and Tif6 at the final steps of the ribosomal large subunit biogenesis. Ribosome biogenesis is the primary occupation of growing cells, and represents at least the 50% of total transcription, and ribosome synthesis is regulated by the rate of transcription of ribosomal RNA and ribosomal proteins (Jorgensen et al. 2004).

Cytoplasmic ribosomes consist of two subunits: the large 60S subunit is the catalytic core and is made by 25S, 5.8S and 5S rRNAs and many ribosomal proteins, while the small 40S subunit consists only of 18S rRNA associated with many ribosomal proteins (RP). To ensure the proper transcription, processing and assembly of the 78 RP and rRNAs that constitute a ribosome, a large number of accessory factors (RiBi) are required (Huber et al. 2011). The RiBi regulon includes not only genes involved in the assembly and modification of rRNAs and RP but even subunits of RNA polymerase I and III, tRNA synthase and translation factors (Fatica & Tollervey 2002; Jorgensen et al. 2002).

To limit energetic dissipation under stress or starvation conditions, the synthesis of ribosomal components and factors involved in their biogenesis need to be tightly regulated. This regulation is in part dependent on the nutritional signaling pathways TOR/SCH9 and RAS/PKA.

In particular it has been shown that sch9p by phosphorylating the transcriptional repressors Stb3, Dot6 and Tod6 is able to antagonize their ability to inhibit RiBi genes transcription (Huber et al. 2011). Downregulation of RiBi genes transcription severely impairs ribosome synthesis, leading to an accumulation of precursors components and lowering the cytoplasmic synthesis rate.

There is a tight relationship between cytosolic protein synthesis and mitochondrial function, as many of the mitochondrial proteins involved in mtDNA replication and maintenance (among those the mitochondrial DNA polymerase), in mitochondrial transcription and translation or proteins involved in key mitochondrial metabolic pathways such as the Krebs cycle, are encoded by nuclear genes. Mitochondrial respiration itself requires a coordination between nuclear and mitochondrial genomes as many of the components and assembly factors, required for a proper respiratory capability, are encoded in the nucleus.

Thus it is not unexpected that regulation of RiBi genes expression has an impact on mitochondrial function. Considering that many longevity mutations have shown reduced cytosolic protein synthesis, we suggest that probably lowering the translational rate is one of the mechanism to improve mitochondrial function by enhancing quality control of respiratory complexes assembly or by reducing the occurrence of mutations on mitochondrial DNA or both.

To assess whether the decrease in cytoplasmic translation rate was accompanied by a downregulation of ribosome components transcription we performed a Northern blot analysis in longevity mutants *sch9Δ* and *rei1Δ*. We decided to investigate the transcription levels only for the two rRNAs 25S and 5S and the reason is that 25S, 18S and 5.8S are transcribed by RNA polymerase I as a single transcription unit, whereas 5S rRNA is transcribed independently by RNA polymerase III.

Total RNA were extracted from *sch9Δ* and *rei1Δ* strains, quantified, separated by electrophoresis in a formaldehyde gel and then transferred to a nylon membrane, through a capillarity blotting system. Thereafter the membrane was incubated overnight with probes labeled with P^{32} complementary to 25S and 5S rRNAs and signals were detected by autoradiography (see materials and methods section).

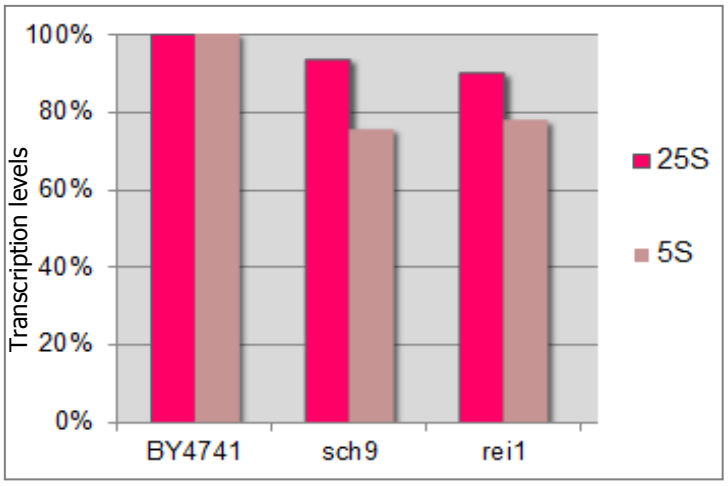


Figure 3.11. Northern blot to test the expression levels of 25S and 5S rRNA in longevity mutants. The analysis was performed in exponential growth phase of yeast cells and the transcription signal from *sch9Δ* and *rei1Δ* strains is normalized to wild-type.

As reported in Fig.3.11 both strains show a reduction in the transcripts amount, suggesting that reduced cytosolic protein synthesis rate is regulated also at transcriptional levels in *sch9Δ* and *rei1Δ* mutants. The results show a major reduction of transcription relative to 5S rRNA, suggesting that transcription regulation primarily or mainly impacts RNA polymerase III. This is an interesting data considering that RNA polymerase III is responsible also for tRNA genes transcription, whose reduction impinges directly on translation efficiency.

SCH9 and *REI1* are directly involved in the proper functioning of the cytosolic translation machinery. On the basis of the regulatory role exerted by *SCH9* on RiBi regulon transcription and the notion that *REI1* belongs to the RiBi genes class, we investigated whether deletion of other genes involved in ribosome biogenesis was able to increase the stability of mitochondrial DNA.

In yeast at least 200 genes are grouped into the RiBi regulon: we first screened the list by taking into account the genes whose deletion was not lethal, and among those we selected the genes that were available in our Euroscarf knock-out collection. Finally we selected seven RiBi genes holding as much as possible different functions in ribosome biogenesis. The genes we took into account for our analysis are listed below:

- ***NOP6***: encodes for a protein required for 40S ribosomal subunit biogenesis. Deletion of NOP6 leads to a reduced 18S amount because of the inhibition of the rRNA precursor processing and increased glycogen accumulation.

- ***RRP8***: encodes a nucleolar protein involved in rRNA processing by pre-rRNA cleavage at site A2. The protein is also involved in telomere maintenance and deletion of the gene lead to increased telomere length and decreased cell size.
- ***SLX9***: encodes for a protein that associate with the 90S pre-ribosome and 43S small ribosomal subunit precursor. Deletion of the gene leads to increased 20S pre-rRNA accumulation, decrease of the mature 18S rRNA, increased heat sensitivity and decreased nuclear export and exponential growth rate.
- ***ARX1***: encodes for a protein involved in the biogenesis of ribosomal large subunit biogenesis. Arx1p is a shuttling factor associated with the ribosomal export complex that translocates the pre-60S complex from the nucleus to the cytoplasm. In addition the protein interacts directly with Alb1 and is responsible for Tif6 recycling defects in absence of Rei1.
- ***NSR1***: encodes for a nucleolar protein that binds nuclear localization sequences and is required for pre-rRNA processing and ribosome biogenesis. Deletion of the gene leads to a reduction in the synthesis of the 40S subunit and is associated with decreased oxidative stress resistance and extended lifespan.
- ***MRT4***: encodes a protein that localizes to the nucleolus and is involved in mRNA turnover and ribosome assembly. The deletion of the gene delays the accumulation of the mature 25S and as a consequence decreases the amount of 60S subunit.
- ***PUF6***: encodes for a protein that co-sediments with the 60S ribosomal subunit and is required for its biogenesis.

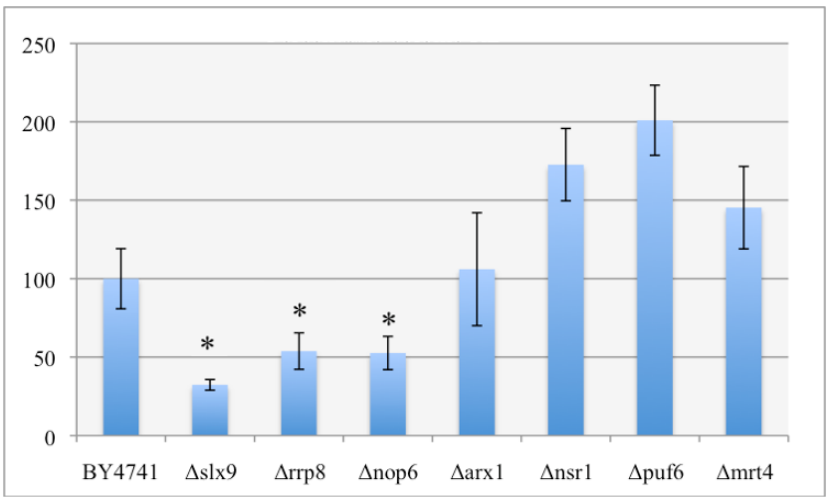


Figure 3.12. Percentage of respiratory deficient cells in various mutants involved in ribosomal biogenesis. The value of a wild-type strain has been referred as 100% and the mutants are compared to that value.

We measured the *petite* frequency of yeast strains deleted in those RiBi genes by a *petite* assay in YP medium at 28 °C. Among the genes we analyzed three were able to strongly reduce the accumulation of large-scale rearrangements on mitochondrial DNA. This result is really promising if we consider that almost half of the mutants analyzed show an increased mtDNA stability and suggests again a tight connection between cytoplasmic protein synthesis and mitochondrial function. Altogether the results obtained suggest that there are others genetic conditions that that impact cytoplasmic translation rate and reduce mitochondrial DNA instability and it will be relevant to determine whether deletion of the three RiBi genes even positively influences lifespan extension.

3.3. *SCH9* AND *REI1* PROMOTE LIFESPAN EXTENSION AND MITOCHONDRIAL DNA STABILITY BY DIFFERENT MECHANISMS

Part of the work described in this section was carried out under the supervision of Prof. Gerald Shadel at Yale University School of Medicine department of Pathology, during the six months I spent in his laboratory.

3.3.1. *Sch9Δ* and *rei1Δ* mutants differently modulate mitochondrial function

As reported from many studies in different model organisms, lifespan extension correlates with an efficient respiratory activity and in yeast it has been proven that strains with the highest respiratory rate are the longest living (Bonawitz et al. 2006). Deletion of *TOR1*, impacting one of the key nutritional signaling pathways involved in lifespan extension, almost doubles oxygen consumption compared to a wild-type strain and the increased respiration rate is required for full lifespan extension.

In fact it has been shown that in cells unable to respire there is no difference in lifespan between a *tor1Δ* mutant and a wt strain (Bonawitz et al. 2007). To determine whether increased respiratory rate was a property shared by our long-lived mutants *sch9Δ* and *rei1Δ* we recorded oxygen consumption on whole cells, as described in material and methods section.

The measure of respiration rate is a convenient method to evaluate the real functionality of mitochondrial respiratory complexes.

Strain	Respitatory activity
BY4741	31,6 ± 1,8
Δsch9	42,32 ± 3,14
Δrei1	58,81 ± 3,82

Figure 3.13. Respiratory rate of strains grown until early stationary phase in YP supplemented with low glucose (0.6%). The values are expressed as nmol O_2*min^{-1}*(mg dry weight)$^{-1}$ and standard deviation of four biological replicates normalized to wild-type is shown.

Both the mutants show an increase in their respiratory ability compared to wild-type.
In principle the increased respiratory activity we observed might depend on either an higher mitochondrial content per cell or on an increased amount of respiratory complexes in the *sch9Δ* and *rei1Δ* mutants. To evaluate this hypothesis we determined the mtDNA copy number in *sch9Δ* and *rei1Δ* null mutant, in accordance to the evidence that usually the amount of mitochondrial DNA correlates with the mitochondrial abundance.
Mitochondrial DNA copy number was determined by RT-PCR and measured as the ratio of the mitochondrial gene target *COX1* relative to the nuclear gene target *ACT1*.

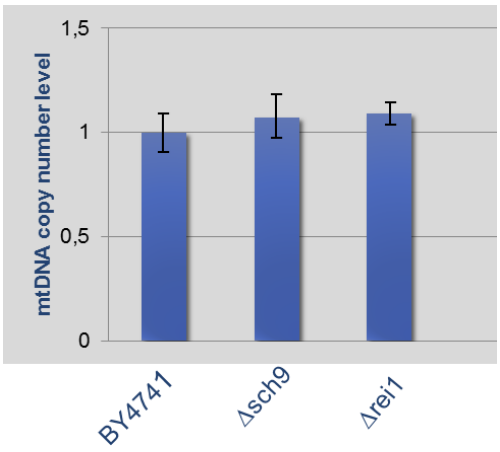


Figure 3.14. Mitochondrial DNA copy number in wild-type and longevity mutants strains determined by qRT-PCR.

No significant differences in mtDNA amount were found suggesting that the increased respiration rate is imputable to higher OXPHOS complexes density rather than an increase in mitochondrial biogenesis.

Shadel and coworkers previously demonstrated that downregulation of TORC1 pathway induces high respiration rate through upregulation of the OXPHOS machinery, that results from an increased mitochondrial gene expression.

They suggest the TORC1 normally is required to repress mitochondrial gene expression and respiration in the presence of glucose and that in a *tor1Δ* strain derepression of respiration is implicated in lifespan extension (Y. Pan & Shadel 2009).

Moreover they found that during early growth stages the increased respiration coupled with ATP production induces an increase in mitochondrial membrane potential and as a consequence a ROS production. The elevated levels of ROS during growth act as an adaptive mitochondrial signal that promote longevity in stationary phase by reducing both membrane potential and ROS amount (Y. Pan, E. a Schroeder, et al. 2011).

We decided to evaluate whether the increased respiration of the *sch9Δ* and *rei1Δ* longevity mutants also in our genetic background was accompanied by changes in mitochondrial properties and the same adaptive response observed by Pan *et al.*.

We measured mitochondrial membrane potential and ROS production during exponential and stationary growth phases by FACS analysis. Cells were grown both in YPD or SC medium and then incubated with different dyes: $DiOC_6$ was used to measure mitochondrial membrane potential, while DHE to measure ROS content.

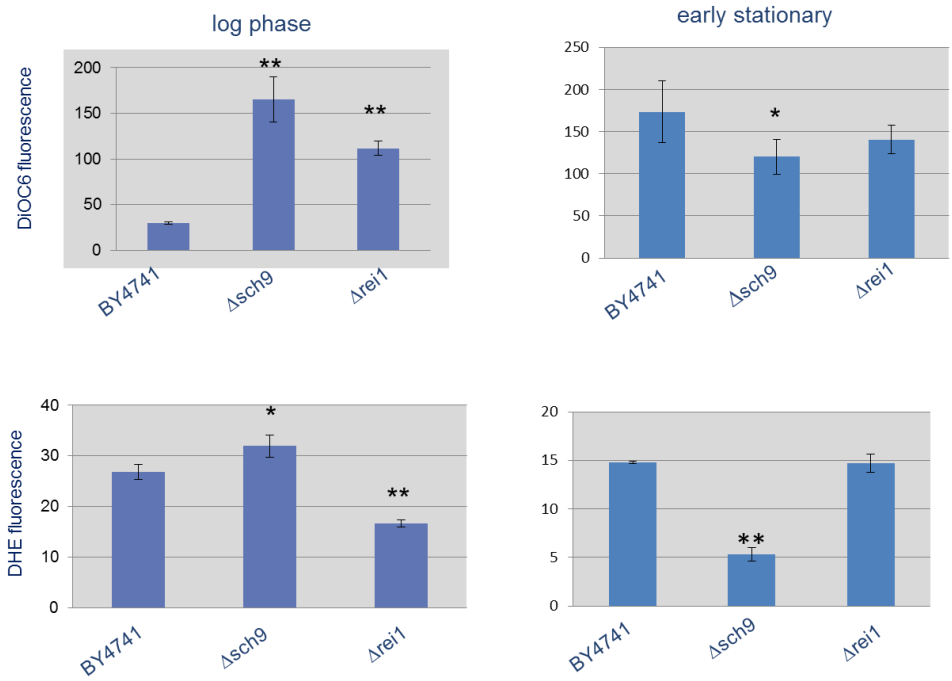


Figure 3.15. FACS analysis of wild-type and SCH9 and REI1 null strains stained with $DiOC_6$ or DHE. The values are the mean of six independent biological replicates.

During exponential growth both *sch9Δ* and *rei1Δ* mutants show an increased mitochondrial membrane potential compared to wild-type strain, that in turn is significantly lowered during stationary phase only in the *sch9Δ* null mutant.

Moreover the high mitochondrial membrane potential of the *sch9Δ* mutant during early stages of growth leads to an increase in ROS production, that is considerably reduced during stationary phase.

Contrariwise the *rei1Δ* mutant has a quite constant membrane potential during all the stages of growth, while ROS accumulate with ageing.

This result suggests that only deletion of *SCH9* leads to an increase of ROS production early during growth to promote an adaptive signal that stimulates lifespan extension and reduces oxidative damage in stationary cells.

Our hypothesis is that the pulse of ROS during exponential growth was able to induce a reorganization of gene expression activating a stress response cell program that lowers ROS accumulation in stationary phase, protects yeast cells from accumulation of mutations on mitochondrial DNA and as final result promotes longevity.

Therefore SDS-PAGE was performed to evaluate the levels of the mitochondrial superoxide dismutase *SOD2* in the *sch9Δ* and *rei1Δ* null mutants. Mitochondria were

extracted as described in materials and methods section and quantified by Bradford assay.

40 µg of mitochondria for each strain were separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane.

Western Blot with Anti-Sod2p was performed and immunoblot for the mitochondrial outer membrane protein porin was used to show equivalent protein loads per lane (Fig.3.16).

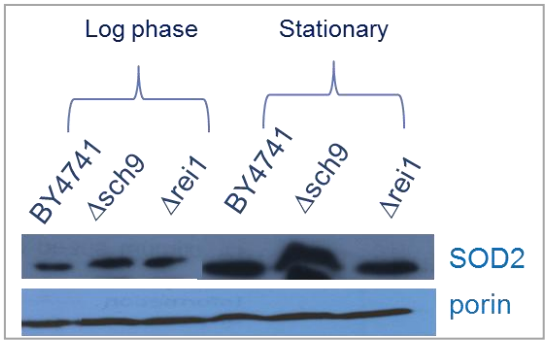


Figure 3.16. SDS-PAGE Western Blot of *sch9Δ* and *rei1Δ* mutants and BY4741 in exponential or stationary growth phase.

In agreement with our hypothesis, the ROS adaptive signal observed in the *sch9Δ* null mutant induces an increase in the amount of Sod2p, which is mild during exponential growth and relevant during stationary phase.

The different amount of the protein during the two growth phases could be explained as the induction of a rapid response to the ROS pulse initiated early during cell growth that is finally accomplished in stationary phase.

The increased amount of Sod2p suggests that activation of stress response genes in *sch9Δ* null mutant is crucial to prevent mitochondrial damage and dysfunction and to promote longevity.

In the case of *rei1Δ*, the Western Blot reveals a mild increase in Sod2p levels during exponential growth compared to wt, which is completely lose in stationary phase.

This result suggests once more that the mechanism by which deletion of *REI1* positively influences mtDNA stability and lifespan is completely different from that of *SCH9*.

It is noteworthy to mention that *SCH9* is part of a well-known pathway involved in the regulation of stress response genes, while *REI1* encodes for a protein involved in ribosome biogenesis and its role in lifespan extension is still elusive.

On the other hand many genes encoding for proteins involved in protein translation machinery assembly or regulation have been actually identified as determinants for longevity, probably because they have different functions beside their primary role in protein synthesis.

To prove that the increase of ROS in *sch9Δ* strain during exponential growth was acting exclusively as a signal and did not induce oxidative damage inside the cell, we performed an aconitase activity assay. Aconitase is a mitochondrial enzyme that isomerizes citrate to isocitrate, a key intermediate of the Krebs cycle.

Measure of aconitase activity can be used as an intracellular sensor of oxidative damage since the enzyme can be reversibly inactivated through the oxidation of its Fe-S cluster.

In general measure of aconitase activity during different stages of CLS is indicative of superoxide levels in ageing yeast (Paola Fabrizio & Valter D Longo 2003). Cells were grown in YPD at 28 °C until late exponential growth was reached (OD 1) and the assay was performed as described in the material and methods.

We observed no significant differences between the *sch9Δ* null mutant and the wild-type strain, indicating that the pulse of ROS is not responsible for induction of oxidative damage (Fig.3.17). Moreover we performed a Dot Blot assay on total DNA from *sch9Δ* null mutant and its parental strain BY4741 to measure the level of 8-oxoguanosine, the major modification on DNA induced by oxidative damage.

Total cellular DNA extracted from the mutant and wild-type strain and treated with proteinase K and RNAse to eliminate any contamination, was quantified at nanodrop and aliquots of 5-10 µg were diluted in TE buffer.

The mixture was then applied directly on a nylon membrane as a dot and after membrane was dried one hour at 55° C. From that moment the nylon membrane was treated as a normal Western Blot membrane and incubated with Anti-8-oxoguanosine antibody.

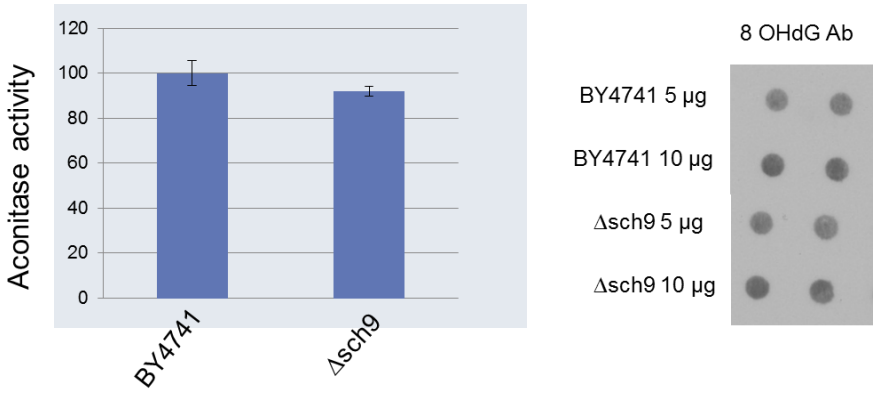


Figure 3.17. Aconitase activity assay on mitochondrial and Dot Blot assay on total DNA from sch9Δ and wild-type strains during exponential growth. Dot Blot signal was normalized to the amount of membrane-bound DNA measured as intensity of the dot after staining with methylene blue.

As shown in figure 3.17 the amount of 8-oxoguanosine is similar between *sch9Δ* mutant and the wild-type strain, confirming that the increase of ROS does not induce oxidative damage but only activate stress defense systems to promote longevity.

As previously discussed both *sch9Δ* and *rei1Δ* strains showed an increased respiratory rate compared to a wt that was not imputable to an increased mitochondrial biogenesis.

In order to investigate whether higher OXPHOS complexes density was responsible for the increased respiratory activity we performed a Western Blot analysis on whole cell extracts from *sch9Δ* and *rei1Δ* strains.

The same amount of total cell extract for each strain was loaded on a 15% polyacrilamide gel and transferred to a PVDF membrane. The latter was then incubated with Anti-Cox2p, Anti-Cox3p and Anti-Cox4p antibodies, while the immunoblot for porin was used to normalize samples loading.

Cox2p and Cox3p are subunits of cytochrome oxidase complex encoded by mitochondrial DNA while Cox4p is encoded by nuclear DNA.

In agreement with the increased respiration activity we observe an increase in the steady-state levels of cytochrome oxidase subunits Cox2p and Cox3p and a mild increase in Cox4p, respectively encoded by mtDNA and nuclear genome, but no increase in porin level was observed (Fig.3.18). The increase in OXPHOS components density was observed only in *sch9Δ* mutant both in YPD, YPGal or SD, but not for *rei1Δ* null strain (data not show).

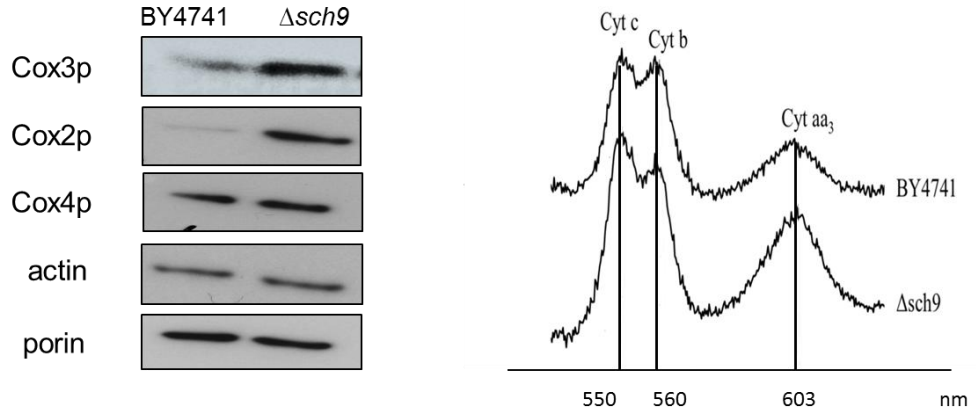


Figure 3.18. On the left Western Blot on whole cell extracts from sch9Δ and BY4741 in exponential grow phase. On the right cytochromes spectra profile on intact cells in early stationary phase.

This result suggests that deletion of *SCH9* is able to up-regulate OXPHOS expression to improve mitochondrial respiration without any effect on mitochondrial biogenesis. In fact no differences in actin:porin ratio (normally used as indicator of mitochondrial content) was observed confirming data relative to mtDNA copy number.

Moreover the cytochromes spectra profile, which is an index of the structural integrity of the respiratory chain complexes, showed an increase in all three cytochromes content in *sch9Δ* cells and particularly prominent in the case of cytochrome aa_3. This result furthermore support the Western Blot result and the idea that increased respiratory rate is sustained by an up-regulation of respiratory subunits.

Consistent with the results discussed above, we performed a mitochondrial translation assay using a standard *in vivo* labeling approach (described in details in materials and methods) to measure mitochondrial proteins synthesis rate, expected to be increased. Late exponential phase cells grown at 28 °C from *sch9Δ* and its parental strain BY4741 were treated with cycloheximide to inhibit cytoplasmic translation and then incubated with S^{35}-labeled methionine to measure the incorporation rate in proteins encoded by mtDNA.

After 15' of incubation with the S^{35}-labeled methionine (pulse), cells were incubated for 30' or 60' with unlabeled methionine (chase) in order to evaluate also mtDNA encoded proteins stability over the time.

Loading control is performed by staining of the dried gel with Comassie blue (data not shown, see materials and methods).

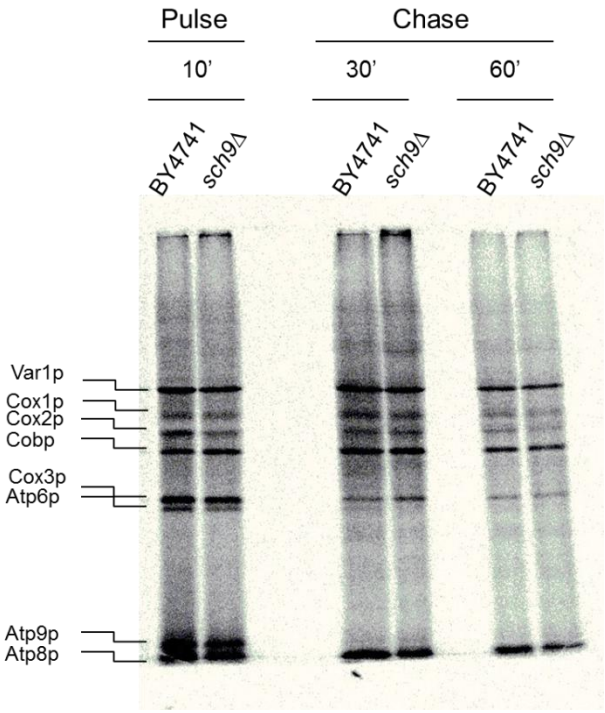


Figure 3.19. MPS analysis. An equivalent of 0.6 OD of cells grown until late exponential growth was labeled for 10 minutes with S^{35}-labeled methionine (pulse), and later incubated for 30' or 60' with unlabeled methionine (chase). Total proteins were extracted and precipitated with TCA and then resolved in SDS-PAGE 17.5%. Autoradiography of MPS analysis for about 15 hrs is shown.

No difference either in translation rate (pulse) or proteins stability (chase) was observed between the wild-type and *sch9Δ* null strain. This result seems contradictory considering the increased amount of mtDNA-encoded COX components observed by Western blot analysis. We speculate that the different division time between *SCH9* and *sch9Δ* null cells might be the reason behind this result. Indeed, as previously described in this dissertation *sch9Δ* cells grow slower than wild-type cells to such an extent that during the 10 minutes of incubation with S^{35}-labeled methionine the translation rate of the mutant strain is *per se* lower than that of wild-type cells.

Therefore, it seems reasonable that longer incubation time with S^{35}-labeled methionine could be necessary to picture the real amount of mitochondrial protein translation in *sch9Δ* null strain. Normally in a wild-type strain after 20 minutes of incubation with S^{35}-labeled methionine no more radioactivity is incorporated and the steady-state level of neo- synthesized proteins is reached.

We speculate that incubation of wild-type and *sch9Δ* cells for at least 30 minutes will allow us to eliminate differences imputable to delayed growth rate of *sch9Δ* mutant.

3.3.2. Induction of nucleoids components is responsible for increased mtDNA stability in *REI1* null mutant

Both nuclear and mitochondrial DNA are constantly exposed to exogenous and endogenous agents that induce damage and instability. ROS, generated inside of mitochondria, are the primary source of oxidative damage in the cell and a responsible for many lesions on DNA such as single/double strand breaks, abasic site formation and accumulation of oxidized bases.

Accumulation of DNA damage leads to impairment of DNA replication and transcription, chromosomal rearrangements and general genomic instability (Gredilla et al. 2010). Mitochondrial DNA is particularly susceptible to oxidative damage because of its proximity to the main site of ROS production, the absence of a nucleosome-like structure to protect mtDNA and the exiguity of mtDNA damage response pathways compared to the ones present in the nucleus (Rourke et al. 2002).

In fact, the steady-state levels of oxidative induced lesions observed in mtDNA can be several-fold higher than those in nDNA (Barja & Herrero 2000; Hamilton et al. 2001). DNA damage is believed to play an important role in the ageing process and the functional decline observed in many tissues during ageing has been associate in particular to mtDNA integrity (Gredilla et al. 2010).

Recently many evidences suggest that many of the nuclear repair mechanisms actively function even in mitochondria, stressing their importance in maintenance of genome integrity. One example is given by the nucleotide excision repair (NER) system that repairs lesions such as alkylation or oxidation induced by ROS and are systematically shuttled between mitochondria and nucleus depending on where the site of damage is localized.

Mitochondrial DNA is protected from oxidative damage by the packing in a structure known as nucleoid, which is found to be associated with the inner mitochondrial membrane.

In yeast nucleoid is made up by at least 22 different proteins involved directly in mtDNA maintenance or even in metabolic pathways. The latter characteristic renders nucleoid a dynamic structure that is able to change in accordance to metabolic cues and regulate mtDNA maintenance depending on the nutritional conditions (for more details see 1.1.3 section).

Mitochondrial DNA stability relies on the activity of 24 proteins involved in nucleoid organization and 35 proteins involved in mitochondrial genome maintenance. We investigated whether the reduced content of large-scale rearrangements observed in our longevity mutants *sch9Δ* and *rei1Δ* was dependent on increased gene expression of the components responsible for mtDNA stability.

Among the two lists of gene involved in nucleoid or mtDNA maintenance we focused our attention on three of them that belong to both categories, *ABF2*, *ACO1* and *ILV5*. Ilvp and Aco1p are metabolic enzymes respectively involved in aminoacids biosynthesis and Krebs cycle while Abf2p is involved directly in mtDNA stability and recombination. A Northern Blot analysis was performed on total RNA from *sch9Δ* and *rei1Δ* mutants and their parental strain.

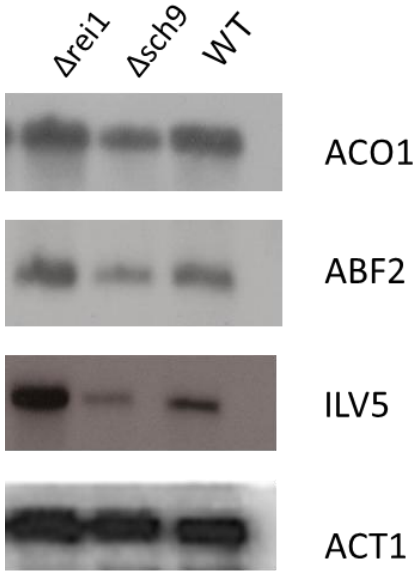


Figure 3.20. Northern Blot assay to identify the expression levels of genes involved in mtDNA stability.

As shown in figure 3.20 only the *rei1Δ* null mutant shows an increase in the amount of transcription of the target genes. In particular the increased is prominent for *ILV5* and less pronounced for *ACO1* and *ABF2*.

This result suggests that the reduced instability observed in *rei1Δ* mutant is mediated by an increased protection of mtDNA from oxidative damage due to a more efficient packing in a nucleoid structure.

The strong induction of *ILV5* is quite an interesting result because in general upon aminoacids starvation Ilv5p mediates redistribution of mtDNA molecules into new nucleoid structure (without net DNA synthesis), increasing nucleoids number and

improving the probability that daughter cells inherit a full complement of mtDNA (Kucej et al. 2008).

To gain a better understanding of the link between *REI1* deletion and *ILV5* overexpression one possibility is to investigate whether *rei1Δ* null mutant is able to mimic aminoacids starvation conditions to increase *ILV5* expression.

Aco1p and Abf2p are physically able to bind mtDNA and it was shown that Aco1p is essential for mtDNA maintenance even in the presence of Abf2, suggesting that the proteins have separate functions (Chen & Butow 2005). Abf2p abundance regulates nucleoid compaction and mtDNA recombination while Aco1p directly controls replication, recombination and repair of mitochondrial genome.

Furthermore it was shown that Aco1p expression is induced by the heme activator protein (HAP) system which is most active in cells with pronounced respiratory activity, which is the case of *rei1Δ* mutant, and under these conditions mtDNA transcription and recombination were induced (X. J. Chen et al. 2007).

It will be interesting to evaluate whether the increased gene expression of *ABF2*, *ACO1* and *ILV5* corresponds to an increased content of the correspondent proteins in *REI1* null cells.

Overall these results suggest that reduced mtDNA rearrangement and increased respiratory activity upon *REI1* deletion depend on a completely different mechanism from the adaptive response observed in *sch9Δ* mutant, and appear to rely on a direct stabilization of mitochondrial DNA through overexpression of nucleoid components.

CHAPTER IV

Functional role of HCCS deficiency in the development of microphthalmia with linear skin defects syndrome

4.1. DOMINANT MICROPHTHALMIA WITH LINEAR SKIN DEFECTS SYNDROME

The work described below originates from a collaboration between our research team and the group of Prof. Brunella Franco at the Telethon Institute of Genetics and Medicine (TIGEM) of Naples. We performed analysis in the yeast *S. cerevisiae* while our coworkers used the medaka fish as model organism to get a better understanding of the biochemical functional effects of *HCCS* deficiency and its pathological mutations in the development of MLS syndrome. The work has been published and it is available in the issue 1, volume 5 of EMBO Molecular Medicine Journal (http://onlinelibrary.wiley.com/doi/10.1002/emmm.201201739/pdf).

4.1.1. Clinical feature of MLS patients

Microphthalmia with linear skin defects syndrome (MLS), also known as MIDAS (microphthalmia, dermal aplasia, and sclerocornea), is a rare X-linked dominant neurodevelopmental disorder associated with in utero males lethality. Affected females at birth show anophthalmia or unilateral/bilateral microphthalmia and linear skin defects, which are limited to the face and neck.

Additional features that characterize the syndrome are sclerocornea, chorioretinal abnormalities, infantile seizures, congenital heart defect and mental retardation (Wimplinger et al. 2006). The disorder is caused by deletions that results in segmental monosomy in the Xp22 chromosomal region.

Three different genes are present in the critical region such as *MID1*, *HCCS* and *ARHGADP6*: *MID1* was found to be mutated in patients affected by Opitz G/BB syndrome whereas no diseases where previously associated to mutations in the others two genes. Wimplinger and coworkers provided for the first time the evidence of *HCCS* complete or partial deletion and/or loss of function point mutations in patients affected by MLS, thus suggesting its involvement in the appearance of the disease (Wimplinger et al. 2006).

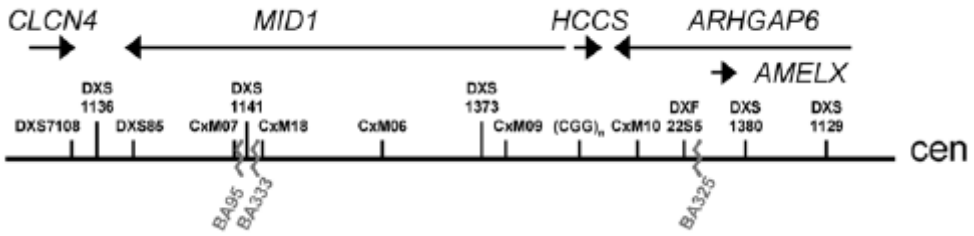


Figure 4.1. Schematic representation of the minimal critical region for MLS (from Wimplinger et al., 2006).

HCCS is an highly conserved gene, ubiquitously expressed, that encodes for a mitochondrial holocytochrome *c*–type synthase, also known as "heme lyase". The enzyme catalyzes the covalent attachment of heme to both apocytochrome *c* and *c*1, leading to the mature forms, which are necessary for proper functioning of the mitochondrial respiratory chain (Prakash et al. 2002). In addition to its role in oxidative phosphorylation (OXPHOS), cytochrome *c* is important to mediate caspase-dependent cell death (apoptosis), in response to a variety of death-promoting stimuli.

MLS has an incidence of 1/5000 newborn and a high intra and inter-familiar variability has been reported, although it does not correlate with the extent of the deletion in the Xp22 chromosomal region (Wimplinger et al. 2007).

A few patients for example show only the ocular defects without any skin lesion while others present only dermal defect. The variability observed among patients was suggested to depend on the influence that the pattern of X inactivation may play in the development of the various clinical manifestations that characterize MLS (Wimplinger et al. 2007).

Heterozygous loss of function mutations in the *HCCS* gene have been reported in patients with MLS phenotype but normal karyotype. Our coworkers have previously reported two different mutations in two female patients from the same family, a heterozygous c.589C=>T (p.R197X) nonsense mutation in exon 6, as well as a heterozygous c.649C0>T (p.R217C) missense mutation in exon 7 of the *HCCS* gene.

The patient with the R197X mutation shows the classical MLS phenotype, while the sister with the R217C mutation presented bilateral microphthalmia and sclerocornea, but no linear skin defects.

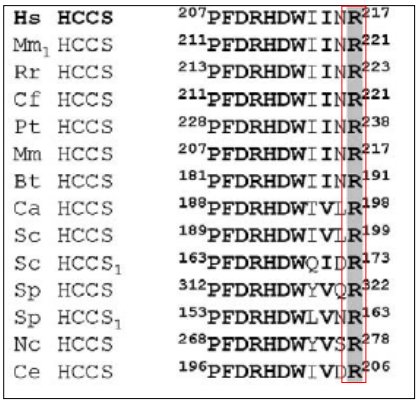


Figure 4.2. Alignment of heme lyases protein sequence between 207-217 position among various species. The region of the protein aligned is given in figure and conserved amino acids are shown in bold while the arginine at position 217 is shaded in grey and included in the red box.

The missense mutation impacts an highly conserved arginine residue at position 217 of the *HCCS* protein, suggesting an important role of this amino acid in heme lyase function. In contrast, the nonsense mutation found in the other patient causes a premature stop located 21 nt upstream of the last splicing-generated exon-exon junction, which is responsible for the presence of an *HCCS* protein lacking 72 C-terminal amino acids (D197–268) (Wimplinger et al. 2006).

Moreover another heterozygous missense mutation c.475G=>A/p.E159K was found in a single female patient with bilateral microphthalmia and sclerocornea in both eyes. The E159K mutation converts an highly conserved glutamate residue to lysine, in a region which is responsible for the import of the heme lyase into the intermembrane space of mitochondria.

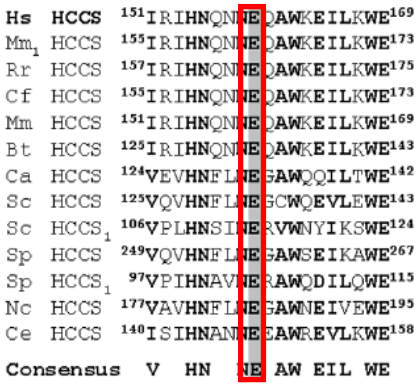


Figure 4.3. Alignment of heme lyases protein sequence between 151-169 amino acids, among various species. The conserved amino acids are shown in bold while the glutamate at position 159 is shaded in grey and included in the red box.

In heme lyases the targeting sequence consists of about 60 amino acids residues which constitute two highly conserved motifs, from residues 151-169 and 190-216 of human *HCCS*. Experiments carried out in human cells showed that *HCCS E159K* mutation does not impair the translocation of the mutated heme lyase to mitochondria (Wimplinger et al. 2007). Similarly the *HCCS R217C* protein was found to be localized into mitochondrial while the truncated form (*HCCS D197–268*) was diffusely dispersed in the cell, suggesting that the last 72 C-terminal amino acids residues are important for mitochondrial targeting (Wimplinger et al. 2006).

Unlike higher eukaryotes, which hold only one heme lyase responsible for the maturation of both cytochrome c and c_1, in the yeast *Saccharomyces cerevisiae* two different heme lyases are present: the enzyme encoded by the gene *CYC3* is strictly specific toward cytochrome c, while the enzyme encoded by the gene *CYT2* is active toward cytochrome c_1. Due to their essential role in electron transfer chain it is not surprising that the absence of the mature forms of both cytochrome c and c_1 lead to a complete loss of the respiratory capability (Bernard et al. 2003).

As previously described the strain B8025 deleted in *CYC3* is unable to grow on non-fermentable carbon sources indicating a severely depleted respiratory function (Dumont et al., 1987). While ectopic expression of human *HCCS* wild-type is able to restore respiratory function, expression of *HCCS* mutants associated with MLS (*HCCS E159K*; *HCCS R217C*) did not complement the function (Wimplinger et al., 2006; Wimplinger et al., 2007).

Beside its primary role in OXPHOS, cytochrome c is a key factor in the apoptotic response and deficiency of the mature isoform could lead to a severe impairment in the apoptosis process, which in turn is important for a proper neuronal development. In fact yeast strains lacking Cyc3p were shown to be more resistant to cell death while accumulation in the cytosol of cytochrome c has been shown to inhibit the caspase-dependent cell death in eukaryote cells (Ludovico et al. 2002; A. G. Martin 2002). Thus, as suggested by Wimplinger and coworkers, the functional nullisomy of *HCCS* may alter the balance between apoptosis and necrosis pushing cells toward a caspase-independent cell death.

Necrotic cell death is usually accompanied by loss of mitochondrial membrane potential, lowering of ATP intracellular concentrations, ROS production and bears the danger of inflammatory reactions leading to damage to neighboring cells, which might be the reason behind the eye malformations and the other symptoms found in MLS-

affected patients (Wimplinger et al. 2007). Loss of *HCCS* function in addition to the OXPHOS impairment, typically found in mitochondrial disorders due to dysfunction of components and/or assembly factors of the mitochondrial respiratory chain, is also responsible for a severe developmental disorder with eyes and skin defect probably imputable to its second function in apoptosome organization (Wimplinger et al. 2006). In fact it is well-known that during central nervous system (CNS) and eyes embryogenesis development depends on a fine regulation of apoptotic events, that represents an important mechanism to regulate the size of cell populations.

4.1.2. Cytochrome c synthesis and import in yeast

Cytochrome c is a soluble protein localized in the outer surface of the IMM where it mediates the electron transfer between complex III and complex IV (Mayer et al. 1995). Cytochrome c is also involved in the cellular death pathway in vertebrates, by signaling mitochondrial status (Bernard et al., 2003; Lim et al., 2002).

Like most mitochondrial proteins, cytochrome c is encoded in the nucleus, synthesized in the cytoplasm and then translocated into mitochondria (Dumont et al. 1988). Cytochrome c is synthesized as apocytochrome c without any amino-terminal region for correct localization, and its maturation requires the addiction of the heme group, covalently attached via thioether linkages to two cysteine residues of the protein (Sherman & Stewart 1973; Dumont et al. 1988).

The covalent attachment of prosthetic group to form the holo-cytochrome c is catalized by the enzyme cytochrome c heme lyase (Basile et al. 1980; Dumont et al. 1988). In yeast the enzyme is encoded by the nuclear gene *CYC3* and then transferred in the mitochondrion, where it is found associated with the outer surface of the mitochondrial membrane.

Cytochrome c import requires Tom40 the protease-resistant channel part of the TOM complex, Tom 22 and cytochrome c heme lyase acting as a trans-side receptor (Mayer et al. 1995; Diekert et al. 2001; Wiedemann et al. 2003).The binding of cytochrome c to the enzyme and the folding of the holoprotein are the driving forces responsible for the apocytochrome import into intermembrane space (Enosawa & Ohashi 1986; Dumont et al. 1991).

It was proposed that apocytochome c enters into mitochondria crossing the outer membrane (OMM) and then interacts with heme lyase; the enzyme, localized in the outer face of inner mitochondrial membrane (IMM), might play a role in mitochondrial

cytochrome c accumulation acting as a trap and pushing cytochrome c towards conformational changes and import into mitochondrial matrix (Dumont et al. 1991).
It was shown that heme lyase mutants could not accumulate cytochrome c to wild-type levels (Dumont et al. 1988; Nargang et al. 1988).
As previously reported, apocytochrome c can be reversibly imported into mitochondria, suggesting that the early steps of translocation do not depend on heme attachment (Hakvoort et al. 1990; Dumont et al. 1991).

4.1.3. Effects of *HCCS* deficiency on mitochondrial functions in yeast

Due to their role in energy metabolism and in progression through apoptotic pathway mitochondria have long been known to be critical for cell survival. As described above mutations that affect *HCCS* negatively impact OXPHOS function and caspase-dependent apoptosis, leading to a severe and complex syndrome (MLS) that, beside the common features of mitochondrial disorders, shows symptoms such as neurodevelopmental defects, microphathalmia, sclerocornea and areas of aplastic skin. To get a better insight into the contribution of pathogenic *HCCS* mutations in the development of MLS and define *HCCS* biochemical function and its effect on OXPHOS, we analyzed the effect of *HCCS* deficiency on mitochondrial functions using yeast as a model organism.
For this purpose we performed *HCCS* complementation studies in the yeast *Saccharomyces cerevisiae* using a strain deficient for the *HCCS* orthologue, *CYC3.* In particular the B-8025 strain is deleted also in *CYC7*, which encodes for cytochrome c_1 to eliminate any residual respiratory activity due to the conversion of apocytochrome c_1 in the correspondent holoform by the other heme lyase Cyt2p.
As previously described, the strain B-8025 deleted in *CYC3* is unable to grow on non-fermentable carbon sources indicating a severely depleted respiratory function (Dumont et al., 1987). Transformation of B-8025-*cyc3Δ* with the human *HCCS* restored mitochondrial respiration and oxidative growth, unlike three forms bearing mutations identified in MLS patients. These include two missense mutations (*E159K; R217C*) involving two highly conserved amino acids and a non-sense mutation (*D197-268*) resulting in a *HCCS*-protein lacking 72 c-terminal amino acids that are required for mitochondrial targeting (Wimplinger et al. 2006; Wimplinger et al. 2007).

In particular we observed the thermo-sensitive behavior of *HCCSE159K* mutation. In fact when grown at the permissive temperature of 28 °C the mutant *HCCSE159K* showed a remarkable ability to grow on non-fermentable carbon sources, as well as wild-type *HCCS* strain, while did not complement the function at 33 °C. Moreover the ability to grow in non-fermentable carbon sources showed by the strain complemented with the human wild-type allele appears to be slightly improved when cells are grown at 33 °C (Fig.4.4).

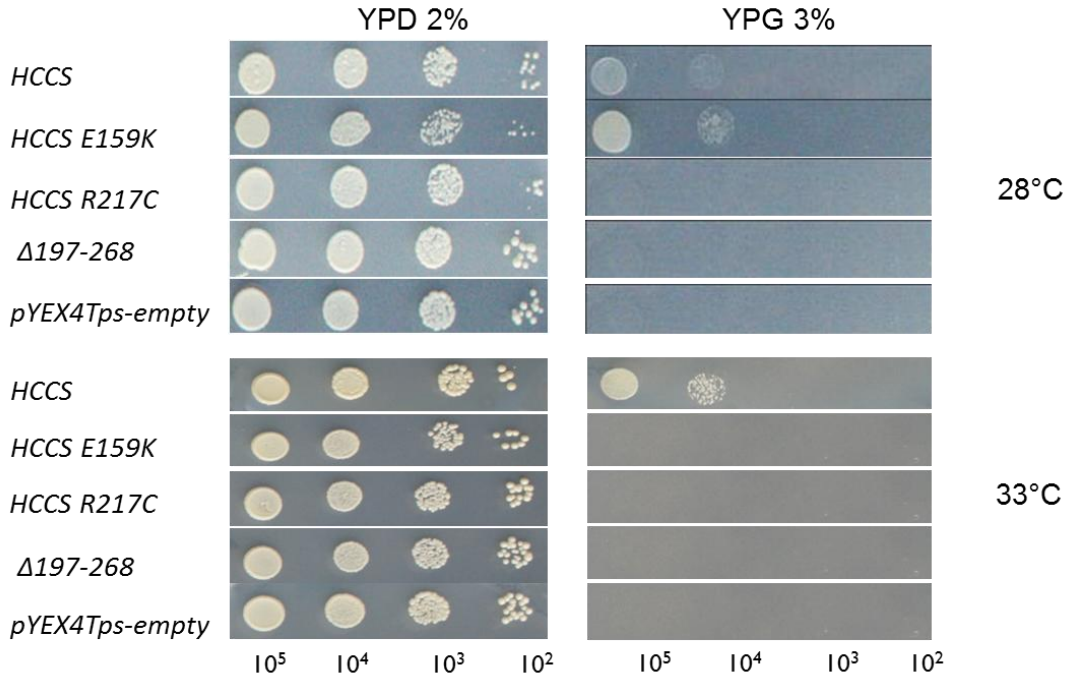
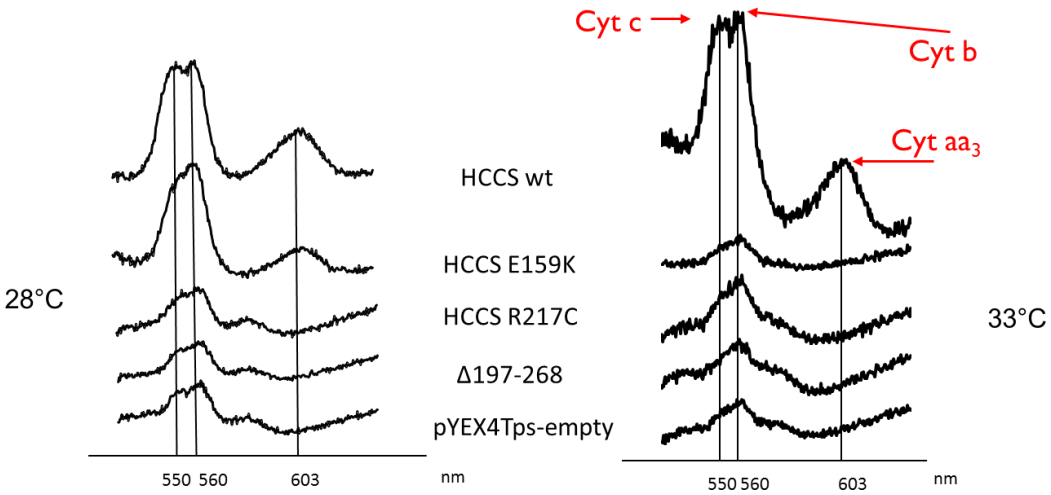


Figure 4.4. Spot assay at 28 °C and 33 °C in YNB DO-URA and LEU medium supplemented with copper to induce expression of the GST-*HCCS*-fusion proteins. Cells at different concentrations were plated on glucose and glycerol containing plates after serial dilutions.

In order to assess the structural integrity of the mitochondrial respiratory chain complexes we analyzed the content of mitochondrial cytochromes by measuring their absorption spectra. In the B-8025 strain (*cyc3Δ*) transformed with wild-type *HCCS* human gene the spectra profile was indistinguishable from the strain carrying the *CYC3* wild-type yeast gene (data not shown). On the contrary in the *hccs* null strain we observed a pronounced reduction of the absorption peak corresponding to cytochrome c, but also to cytochrome aa_3, a component of complex IV. This indicates the loss of the structural integrity of complex IV that requires the presence of the folded and mature holo-Cytc (Pearce & Sherman 1995).

similar behavior was displayed by the *cyc3Δ* strain transformed with the *HCCS* mutant alleles and pYEX empty vector (Fig.4.5). The disappearance of cytochrome aa3 has been reported previously as a secondary effect due to the absence of holo-cytochrome c (Barrientos *et al.*, 2003; Bernard *et al.*, 2003). The observed reduction of the cytochromes content parallelized with a reduction in the respiratory activity indicating that the mutated HCCS alleles identified in MLS patients are pathogenic via impaired OXPHOS (Fig.4.5).

Once again both spectra profiles and respiratory activities show the thermo-sensitive behavior of *HCCSE159K* mutation: the mutant has a mild phenotype at the permissive temperature of 28 °C while it is remarkably compromised when grown at 33 °C, at the same extent of the *cyc3Δ* strain.



	Respiration[a]
Hccs	14,02± 4,2
HccsE159K	8,56± 2,1
HccsR217C	< 2
HccsΔ197-268	< 1
Null mutant	< 1

	Respiration[a]
Hccs	30,1± 2,8
HccsE159K	2,4± 1,1
HccsR217C	< 1
HccsΔ197-268	< 1
Null mutant	< 1

Figure 4.5. Cytochrome profiles and oxygen consumption of B-8025 strain transformed with human wild-type *HCCS*, the mutants *HCCS E159K*, *HCCS R217C*, *HCCS Δ197-268*, and the empty vector grown in YNB DO-URA and LEU supplemented with glucose 0.6% at 28 °C and 33 °C. Cells were grown until glucose was exhausted to promote the expression of respiratory chain components. The values are expressed as nmol O_2*min^{-1}*(mg dry weight)$^{-1}$ and standard deviation of four biological replicates normalized to wild-type is shown.

Beside the primary role in the attachment of heme to cytochrome c, heme lyase is also responsible for the apo-cytochrome import into intermembrane space. The binding of cytochrome c to heme lyase and the folding of the holo-protein are the driving forces behind the import, with Hccsp acting as a trans-side receptor (Mayer et al. 1995; Diekert et al. 2001; Wiedemann et al. 2003). It has been reported that mitochondria from *cyc3Δ* strains were essentially incapable of importing newly synthesized apo-cytochrome c and that less than 5% of either *CYC1* or *CYC7* gene product could be found into mitochondria isolated from those strains (Dumont et al. 1988).

We wondered whether *S. cerevisiae* strains carrying either wt *HCCS* or *HCCS* mutant alleles were able to properly import cytochrome c into mitochondria. Western blotting were performed on mitochondria with intact outer membrane, using antibody anti yeast cytochrome c, to assay the presence/absence of the protein inside mitochondria. Of course we did not take into account the truncated form (*HCCS D197–268*) of the heme lyase which was found to be impaired in mitochondrial import. In agreement with previous studies, western blotting analysis showed the absence of cytochrome c in the cytoplasmic fraction (data not shown) and a great reduction of cytochrome c content in mitochondria from the null yeast strain B-8025 (Fig.4.6). We observed that mitochondria from strain B-8025 transformed with wild-type *HCCS* contains levels of cytochrome c similar to those present in wild-type strain B-7553 (control parental strain, data not shown), while strains transformed with the mutated alleles (*HCCS E159K*, *HCCS R217C*) show an amount of cytochrome c that is significantly reduced compared to that observed in wild-type, but greater than that of null strain.

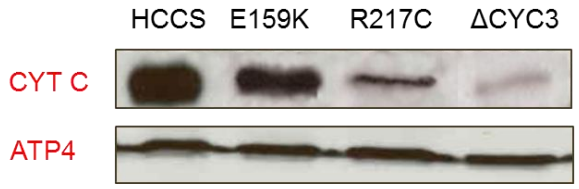


Figure 4.6. Western Blot on mitochondria with intact outer mitochondrial membrane of B-8025 strain transformed with wild-type *HCCS* and the mutated alleles *HCCS E159K*, *HCCS R217C* or the empty vector, grown in YNB DO-URA and LEU media. 40 µg of mitochondria were loaded and Anti-ATP4 antibody is used to normalize the loading.

In particular mutant *HCCS E159K* shows a signal more intense then strain transformed with the *HCCS R217C* allele, despite the absorption spectra profile are similarly compromised. This result indicates that whilst both mutant *HCCS* proteins fail to attach

the heme moiety into apo-Cytc, the *R217C* protein prevents translocation of Cytc, whereas the *E159K* is permissive, suggesting that the import of the heme-less apo-Cytc into mitochondria requires the presence of the *HCCS* protein even if not catalytically active. These data point to an additional role of *HCCS* as an apo-Cytc chaperone; the differences between *E159K* and *R217C* mutants consist in their ability to physically bind, chaperone and drive apo-Cytc to the inner mitochondrial membrane. The different accumulation of cytochrome c between *HCCS E159K* and *HCCS R217C* mutants should be ascribed to a different structural conformation between the two heme lyase proteins, possibly modifying their membrane interaction. These results also indicate that *HCCS* directly controls the import of cytochrome c into mitochondria independently from its heme lyase activity, suggesting that *HCCS* binding to apo-cytochrome c, rather than heme attachment, is the critical step for cytochrome c import into mitochondria, as previously proposed (Dumont et al. 1991).

Respiratory chain dysfunction can lead to an increased production of ROS. When electrons transfer along the respiratory chain is inhibited downstream of complex III, electrons coming from succinate oxidation can by reverse transported from complex II to complex I, leading to ROS generation (Lambert & Brand 2004; St-Pierre et al. 2002). ROS production can also increase as a consequence of low respiratory rates, increasing the electrons stall along the ETC and the possibility to prematurely escape from respiratory chain complexes (Korshunov et al. 1997), or in pathological situations associated to mitochondrial respiratory chain defects (Douglas C Wallace 2005).

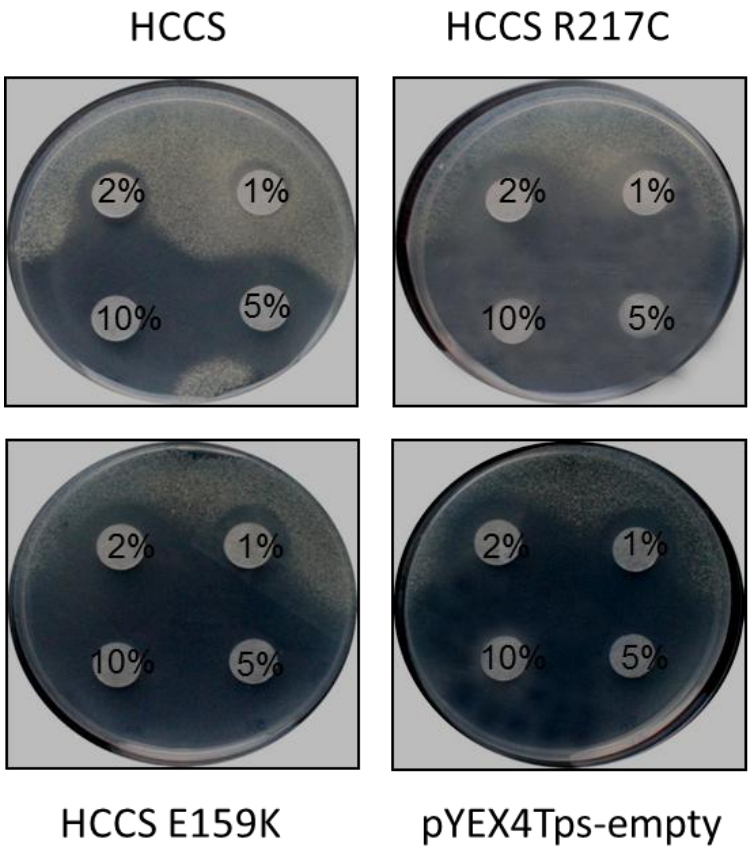
In yeast, strains that produce elevated level of ROS display an enhanced sensitivity to free radical inducers, because the imposition of an additional exogenous stress overwhelms the detoxifying defense mechanisms of the cell. To evaluate the sensitivity of *hccsΔ* mutants to hydrogen peroxide a filter-paper disk method was performed with four different concentrations of H_2O_2. 10e7 cells of each strain were plated on YNB+DO- URA and LEU glu 2% as described in the material and methods section. 10 µl of H_2O_2 (1%, 2%, 5% and 10%) were adsorbed on sterile Whatman disks (diameter 9 mm) placed in the middle of the plate. After three days of growth at 33 °C the plates were photographed as shown in fig.4.8. The strains analysed were the B-8025 transformed with wild-type *HCCS* and the mutated alleles *HCCS E159K*, *HCCS R217C* or the empty vector.

Accordingly, H_2O_2-induced growth inhibition was markedly increased in the B-8025-*cyc3Δ* strain defective for heme lyase function as well as the *hccs* pathological alleles

compared to the wild-type strain, indicating that HCCS deficiency leads to an increase of ROS production.

Since increased ROS production can affect chronological life span (CLS) we tested the effect of *hccs* pathological mutations on the survival rates of chronologically aged cells. Viability was measured over time by plating serial dilutions of the cultures onto YPD plates and counting colony forming units (CFU) after three days of growth. As shown in fig.4.7 both the strains carrying the *hccs* pathological alleles showed a significant decrease in CLS as well as the B-8025-*cyc3Δ* null mutant, indicating that loss of functional *HCCS* is deleterious for cell survival.

A)



B)

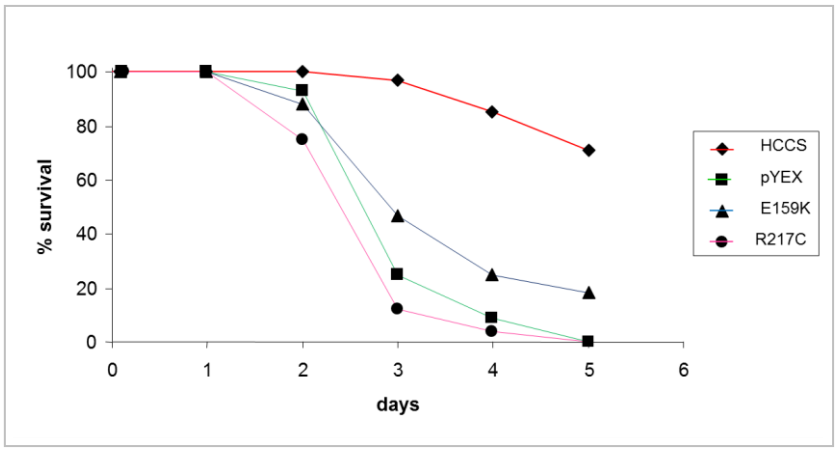


Figure 4.7. A) Paper disk assay with four different H_2O_2 concentrations. B) CLS of the B-8025 strain transformed with the wt *HCCS* or the pathological alleles in YNB+DO- URA and LEU supplemented with 2% glucose. When cells reach the stationary phase their survival is measured.

To gain insight into the role of *HCCS* in the development of MLS syndrome our coworkers decided to investigate a vertebrate animal model.

HCCS inactivation results in embryonic steam (ES) cells lethality in mouse preventing the possibility to use this model organism to study MLS features. For this reason another valuable model was generated, by *hccs* knock-out in medaka fish. *Hccs*-deficient medaka embryos presented the phenotypes observed in MLS patients and confirmed the results obtained in yeast: the embryos showed significant reduction of complex III activity and Cytc amount, the presence of abnormal mitochondria with disorganization of the *cristae* in morphants retinas, and an increase in ROS content which is responsible for caspase-9 activation in the mitochondria. *Hccs*-deficient medaka embryos showed that microphthalmic and microcephalic phenotype is due to the mitochondrial caspase-9 activation that occurs in an apoptosome-independent manner, and as a consequence of mitochondrial respiratory dysfunction and ROS increase.

In conclusion we found that in addition to controlling holo-cytochrome c synthesis, *HCCS* has a key role during eye and brain development by controlling the activation of a non-canonical pathway leading to cell death in an apoptosome-independent manner, *via* the activation and release of caspase-9 (Indrieri et al. 2012).

Despite *HCCS* is ubiquitously expressed and required to maintain cellular respiration the phenotypes observed in MLS patients are restricted to specific tissues, suggesting that heme lyase dosage and function may be critical in particular organs.

MATERIALS AND METHODS

5.1. Yeast strains

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

STRAIN	GENOTYPE	ORIGINE
BY4741	*Mata his3-1 leu2-0 met15-0 ura3-0*	*Euroscarf* collection*;* *Brachmann et al., 1998*
BY4741 *Δsch9*	*Mata his3-1 leu2-0 met15-0 ura3-0 sch9::KAN^R*	*The present work*
BY4741 *Δrei1*	*Mata his3-1 leu2-0 met15-0 ura3-0 rei1::KAN^R*	*Collezione Euroscarf;* *Brachmann et al., 1998*
BY4741 *Δtor1*	*Mata his3-1 leu2-0 met15-0 ura3-0 tor1::KAN^R*	
BY4741 *Δgpr1*	*Mata his3-1 leu2-0 met15-0 ura3-0 gpr1::KAN^R*	
BY4741 *Δtma19*	*Mata his3-1 leu2-0 met15-0 ura3-0 tma19::KAN^R*	
BY4741 *Δrpl6*	*Mata his3-1 leu2-0 met15-0 ura3-0 rpl6::KAN^R*	
BY4741 *Δrpl31A*	*Mata his3-1 leu2-0 met15-0 ura3-0 rpl31A::KAN^R*	
BY4741 *Δsdh1*	*Mata his3-1 leu2-0 met15-0 ura3-0 sdh1::KAN^R*	
BY4741 *Δsdh2*	*Mata his3-1 leu2-0 met15-0 ura3-0 sdh2::KAN^R*	

BY4741 *Δsdh4*	*Mata his3-1 leu2-0 met15-0 ura3-0 sdh4::KAN^R*	
BY4741 *Δsdh5*	*Mata his3-1 leu2-0 met15-0 ura3-0 sdh5::KAN^R*	
BY4741 *Δnop6*	*Mata his3-1 leu2-0 met15-0 ura3-0 nop6::KAN^R*	
BY4741 *Δrrp8*	*Mata his3-1 leu2-0 met15-0 ura3-0 rrp8::KAN^R*	
BY4741 *Δslx9*	*Mata his3-1 leu2-0 met15-0 ura3-0 slx9::KAN^R*	
BY4741 *Δarx1*	*Mata his3-1 leu2-0 met15-0 ura3-0 arx1::KAN^R*	
BY4741 *Δnsr1*	*Mata his3-1 leu2-0 met15-0 ura3-0 nsr1::KAN^R*	
BY4741 *Δpuf6*	*Mata his3-1 leu2-0 met15-0 ura3-0 puf6::KAN^R*	
BY4741 *Δmrt4*	*Mata his3-1 leu2-0 met15-0 ura3-0 mrt4::KAN^R*	
B-7553	MATα *can1-100 CYC1 cyc7::CYH2 cyh2 his3-Δ1 leu2-3,112 trp1-289*	*Wimplinger et al., 2006*
B-8025	MATα *can1-100 CYC1 cyc3-Δcyc7-Δ::CYH2 cyh2 his3-Δ1 leu2-3,112 trp1-289*	*Wimplinger et al., 2006*
W303 *Δmip1*	*Mata ade2-1 leu2-3 ura3-1 trp1-1 his3-11 can1-100 sml1::HIS3/ Mata ade2-1 leu2-3 ura3-1 trp1-1 his3-11 can1-100 mip1::KAN^R*	*Baruffini et al., 2006*

WB-12	*MATa ade2-1 trp1-1 ura3-1 can1-100 aac1::LEU2 aac2::HIS3*	*Hashimoto, 1999*
W303-1B *Δbcs1*	*Mata ade2-1 leu2-3 ura3-1 trp1-1 his3-11 can1-100 bcs1::KAN^R*	*Tuppen H. et al., 2010*

5.2. Media and growth conditions

For yeast the following media were used:

- YP (1% peptone, 0.5% yeast extract)
- YPA (2% peptone, 1% yeast extract, 7.5mg/ml adenine)
- YNB (YNB ForMedium™ w/o aminoacids w/o NH4SO4 1,9 g/L, NH4SO4 5 g/L). Minimum media was enriched with drop-out powder (Kaiser *et al.* 1994).
- SD (YNB ForMedium™ w/o aminoacids w/o NH4SO4 6,7 g/L).
- YPAGE-Ery (2% peptone, 1% yeast extract, 40 mg/L adenine, 25 mM K-phosphate buffer pH 6.5, 3g/L erythromycin (SIGMA), 3% glycerol, 2% ethanol).

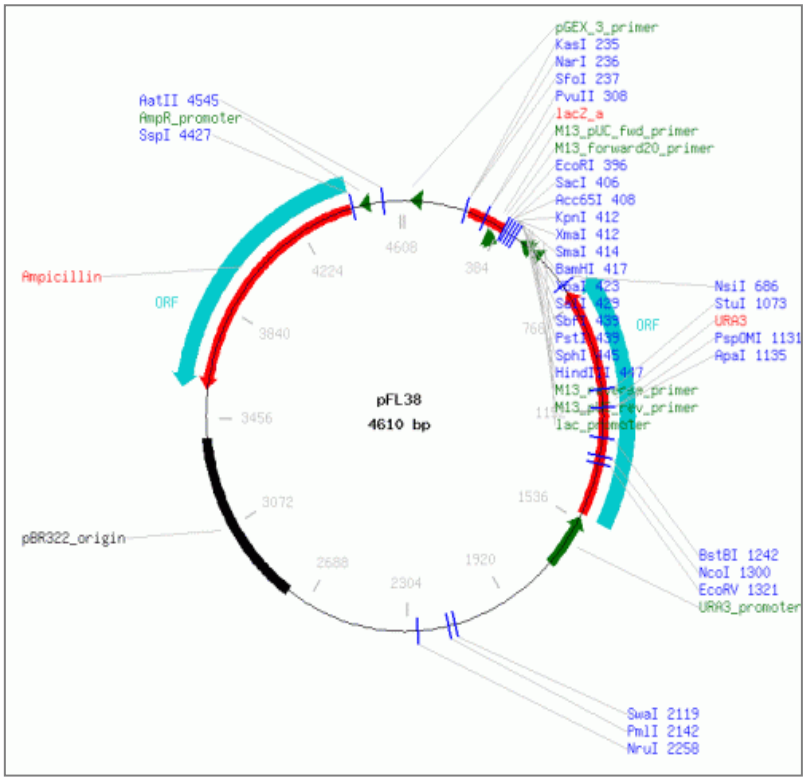
If necessary singles aminoacids could be excluded from complete drop-out to maintain selective pressure. As solidifying agent agar ForMedium™ 2% was added. Carbon sources were added at final concentration of 2% if not specified differently.

The following sources were used: Glucose (D), Ethanol (E), Glycerol (G), Galactose (Gal), Lactate (L). *S. cerevisiae* was cultured at 28°C, in constant shacking 120 rpm if liquid media was used. To induce heat stress, cultures were incubated at 37°C in thermostat or water bath.

For *E. coli* LB media was used (1% bacto tryptone Difco™, 0.5% yeast extract Difco™, 0.5% NaCl, pH 7.2-7.5). Agar 2% and ampicillin (Sigma-Aldrich®) 100mg/ml were added if needed.

For α-complementation selection 80µl of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Xgal) 2% (dissolved in dimethilformamide) and 40µl isopropyl-beta-D-thiogalactopyranoside (IPTG) 23,8 mg/ml were added. Cultures were incubated at 37°C in constant shacking if necessary.

5.3. Plasmids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EcoRI 396
SacI 406
Acc65I 408
KpnI 412
XmaI 412
SmaI 414
BamHI 417
NsiI 686
StuI 1073
URA3
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AmpR_promoter
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ORF
pFL38
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EcoRV 1321
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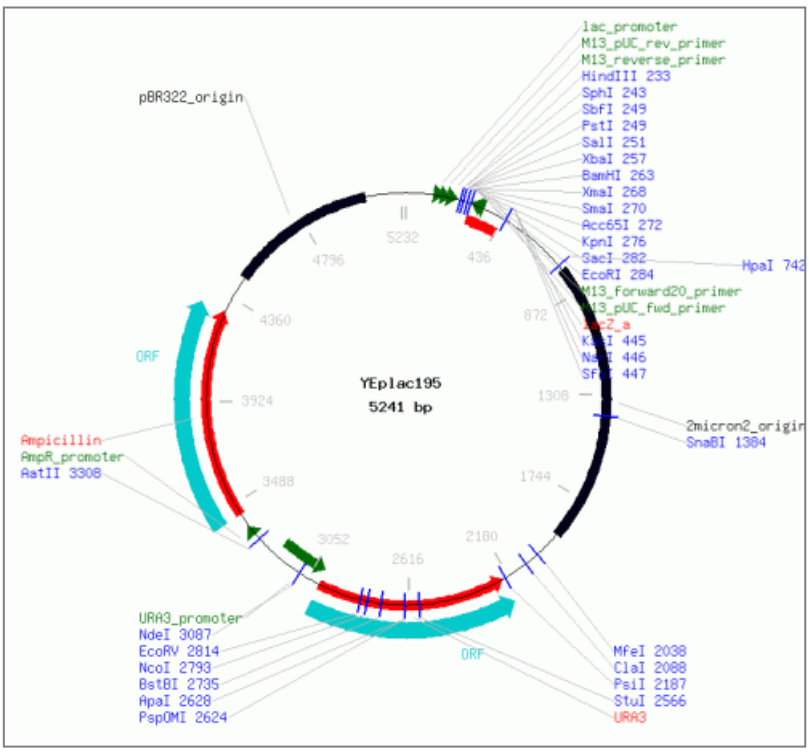
NruI 2258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SphI 243
SbfI 249
PstI 249
SalI 251
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BamHI 263
XmaI 268
SmaI 270
Acc65I 272
KpnI 276
SacI 282
EcoRI 284
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M13_pUC_fwd_primer
lacZ_a
KasI 445
NarI 446
SfoI 447
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2micron2_origin
SnaBI 1384
Ampicillin
AmpR_promoter
AatII 3308
ORF
YEplac195
5241 bp
URA3_promoter
NdeI 3087
EcoRV 2814
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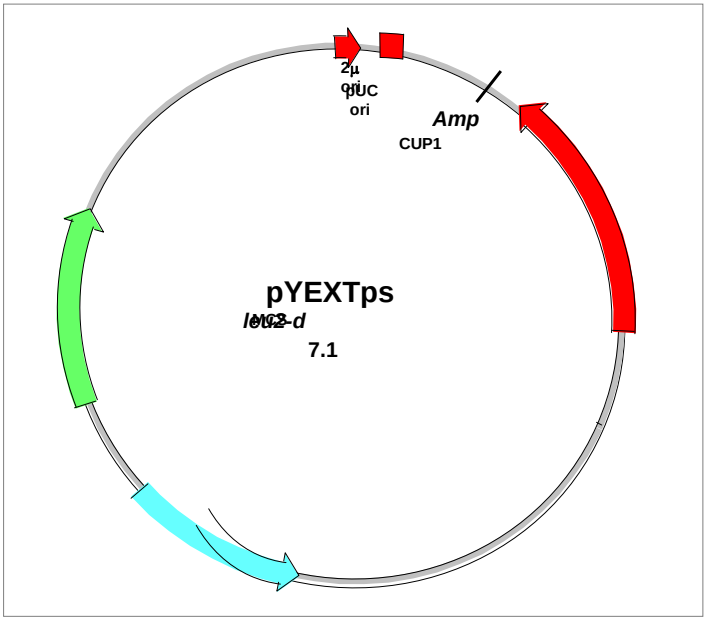

5.4. Nucleic Acids manipulation

All the manipulations were carried out with standard techniques (Maniatis *et al.* 1982). Plasmid DNA was extracted from *E. coli* with Wizard® Plus SV Minipreps, Wizard® Plus Minipreps (©Promega) or following standard procedures (Maniatis *et al.* 1982). DNA recovery from agarose gel and purification of PCR products were carried out with Wizard® SV Gel and PCR Clean-up System (©Promega) commercial kit.

Genomic DNA from *S. cerevisiae* was extracted as previously described (Hoffman *et al.* 1987; Looke *et al.* 2011).

Enzymatic manipulations (restriction, ligation, dephosphorylation) were carried out following manufacturer indications (New England Biolabs® *Inc.* NEB, Invitrogen™).

Sequencing was performed with external services (©Eurofins-MWG for Europe, Genewiz® *Inc.* for United States).

5.5. Primers

NAME	OLIGO SEQUENCE 5'-3'
FwSch9Kan	CGTCGAATCAGGATACTGGATTTAGCTCTCAACACCAACATCCCGTACGCTGCAG GTCGAC
RvSch9Kan	GGGATAAAGGAACCAGGTTCCATAAAGTACGAGTTTTGTAGGATCGATGAATTCGAGCTCG
FwSch9Up	CGCCGTCGACGATAGACCTCTACAGCTTTTAC
RvSch9Low	GGGCGAATTCCGACTTTGGGAAATCACAGC
FwRei1Up	CGCCGGATCCACTCTCATTCCCGATATTCCC
RvRei1Low	GGCCAAGCTTGCTGTATATTTCCTTTGCTGTA
K2	GAAAGAAGAACCTCAGTGGC
SYM-HYGF	TCTTTTGTTATTACTATAGGAAAGATAGAATTGATATTAAAACAGCGTACGCTGCAGGTCGAC
SYM-HYGR	GCTAGTTGAATTCCAAATATTTTCAAAATCAGGATTTCTTATCCAATCGATGAATTCGAGCTCG
FwACT1mtDNA	GTATGTGTAAAGCCGGTTTTG
RvACT1mtDNA	CATGATACCTTGGTGTCTTGG
FwCOX1mtDNA	CTACAGATACAGCATTTCCAAGA
RvCOX1mtDNA	GTGCCTGAATAGATGATAATGGT
FwSc5S	GGTTGCGGCCATATCTACCAG
RvSc5S	GATTGCAGCACCTGAGTTTCG
FwSc25S	GCCAGCTGGGACTGAGGACT
RvSc25S	GGATTCCCTTTCGATGGTGGCC
FwACT1_probe	GGTTCTGGTATGTGTAAAGCCGGTTTTGC
RvACT1_probe	GAAACACTTGTGGTGAACGATAGATGGACC
FwABF2_probe	GCCTATTAACTAGATCTTTCC
RvABF2_probe	CCTTAATGCTTTGATCCAAGG
FwACO1_probe	GGTATCACAACTGTCAAAGG
RvACO1_probe	CCTCTGTATTTCAACCATGG
FwILV5_probe	CCAGATTGATCTGCAACTCC
RvILV5_probe	GGTACCAGTCCAAAGCACC

5.6. Polymerase Chain Reaction

All the reactions were performed following manufacturer indications. For analytical purpose *Taq* DNA polymerase 2X master mix (NEB) or GoTaq® DNA polymerase (©Promega) were used. Preparative reactions (for gene cloning, cassette transfer, sequencing) were performed with a high fidelity polymerase. KOD HiFi DNA polymerase was used (Novagen®).

5.7. mtDNA copy number analysis by qRT-PCR

The relative amount of mtDNA and nuclear DNA was determined using a quantitative real-time PCR strategy. Separate PCR reactions with SYBR Green and standard PCR primers are used to quantify the amplification signals from the mtDNA target and nuclear target.

The yeast strains to be analyzed were grown in 5 ml of liquid YPG medium to an optical density (at 600 nm) of 0.6–0.8. The cultures were harvested by centrifugation, and total cellular nucleic acids were isolated using a "smash and grab" protocol essentially as described previously (Hoffman & Winston 1987). The nucleic acid pellet was dissolved in 100 µl of TE buffer containing DNase-free RNase A (10 µg/ml) and incubated at 37°C for 1.5 h. This DNA was quantified by absorbance at 260 nm and various dilutions (≥80-fold) of the template DNA were used in the final PCR reaction to ensure measurements were within the linear range.

Nuclear DNA and mtDNA PCR were done in individual wells for each sample dilution in the following standard 50-µl SYBR Green reaction: 25 µl of Bio-Rad iQ SYBR Green Supermix, 10 µl of diluted template, 3 µl of H_2O, and 1 µl of each nuclear primer (*ACT1* forward and reverse primers) or each mtDNA primer (*COX3* forward and reverse primers).

Each primer was stored separately as a working stock of 6.25 µM, and the final concentration in the above-mentioned reaction for each primer was 125 nM. The PCR reaction was performed in 96-well format using a Bio-Rad iCycler and analyzed using and accompanying iCycler IQ version 3.1 software.

Analysis of the data to generate the relative mtDNA copy number of the strains was

The PCR protocol was empirically designed around a primer annealing temperature of 58°C, a probe annealing temperature of ~68°C, and 45 cycles of real-time PCR amplification and data collection as described in the iCycler manual.

For each strain analyzed, the copy number of the mtDNA and the nuclear DNA is calculated using the threshold cycle number (C_T), making sure the reactions were in a linear range of detection, by running a variety of dilutions. This is done in triplicate to obtain an average value for C_T for *COX1* and *ACT1*, C_T (*COX1*) and C_T (*ACT1*), respectively.

The difference (ΔC_T) between these averages C_T (*COX1*) - C_T (*ACT1*) is determined and used to arrive at a value of the relative mtDNA copy number (RCN) of each strain, which is equal to $2^{\Delta}CT$. The fold change in the RCN of each mutant strain is calculated by dividing its RCN by the wild-type RCN value, which yields the fold change compared with wild type, which now has a value of 1.

At least three experiments of this type were run for each strain analyzed.

5.8. Transformation procedures

5.8.1. E. coli transformation

E. coli transformation was achieved with electroporation. Competent cells were prepared as previously described (Dower *et al.* 1988). Transformation was carried out with 1-3µl of plasmid DNA or ligation product. One mm or 2 mm cuvettes were used, applying respectively a current of 1.75KV, 25 µF 200 Ω or 2KV, 25 µF 200 Ω.

Alternatively, CaCl2 competent cells were prepared and transformed with standard techniques (Maniatis *et al.* 1982).

5.8.2. S. cerevisiae transformation

Yeast transformation was carried out with Lithium Acetate (LiAc) as described by Gietz *et al.* If a greater efficiency was desired, the long protocol was applied (Gietz *et al.* 2002).

For the one-step gene disruption and episomic plasmids integration (Rothstein 1983) the protocol used was modified from Schiestl *et al.* method (Schiestl *et al.* 1989) and performed as follows:

1) Inoculate strain to be transformed in 10ml YPD and grow o/n at 30°C
2) Inoculate from overnight batch into fresh 10ml YPD to OD600 of about 0.1
3) Grow cells at 30°C until OD600 reaches 0.6 to 1

4) Harvest 1ml of culture centrifuging 2000-3000rpm for 1-2 minutes. Resuspend in 1ml of TEL solution and centrifuge 2000-3000rpm for 5 minutes.

TEL solution: 10mM TrisHCl pH 7.5
1mM EDTA pH 8
0.1M LiAc

5) Resuspend pellet in 0.1ml TEL and transfer to a clean sterile eppendorf tube. Add transforming DNA (1-10µg) and 5µl of 10mg/ml salmon sperm DNA solution (previously denatured for 5 minutes at 95°C and cooled 2 minutes on ice). Mix and incubate 30 minutes at room temperature without shacking
6) Add 0.7ml of 40% PEG4000 in TEL buffer and mix by pipetting. Incubate 45-60 minutes at room temperature without shacking
7) Heat shock at 42°C for 10 minutes and incubate on ice 2-5 minutes
8) Pellet for 1 minute and wash once in 0.2ml of TE (10mM TrisHCl pH 7.5, 1mM EDTA pH 8). Pellet again for 1 minute ad resuspend in 100µl of sterile water. Plate on selective medium.

5.9. Whole cell analyses

5.9.1. Spot Assay

Spot assay is a classical phenotypic analysis used to test growth of single strains in different conditions. The principle is a 10-fold serial dilution of a starting culture at 1*10e7 cells/ml, performed four/five times to a final 1*10e4 cells/ml or 1*10e3 cells/ml.

After a o/n culture at 28 °C in YPD or YNBD the OD600 was measured for each strain. A first dilution was made to dilute each strain to a concentration of 1 OD (1*10e7). Then three or four 10-fold serial dilutions were made. From these suspensions 5µl or 10µl were spotted in ordered rows on agar plates and then incubated for several days.

5.9.2. Filter paper disk-method

This assay allows to evaluate the strain sensitivity to specific substances. 20 ml of or YNBD + DO - Ura + 2% agar were plated and once solidified covered with a 10 ml of cells/top agar in order to have a total concentration of 1*10e6 cells.

These cells were pre inoculated in YNBD +DO - Ura and incubated o/n at 28 °C. After measuring OD_{600}, cells were inoculated in 10 ml of Top agar. Once the second lay was solidified, a Whatman disk was placed in the plates centre.

10 µl of the testing substance at desired concentrations were added on the disk and after an incubation at 28 °C for 3-4 days, the halos were observed.

5.9.3. Cytochromes spectra absorption

(Ferrero et al., 1981)

Mitochondrial respiratory chain intactness can be assessed easily recording the cytochromes absorption profile on whole cells. All the experiments were carried out with a Cary 300Scan UV-vis spectrophotometer (Varian Inc.), recording continuously from 630 to 540nm. The following general protocol was used:

1. Pre inoculate the strains in 10ml YPD or YNBD+DO- Ura and incubate o/n at 28°C.
2. Inoculate 2.5ml in 100ml of YPD 0.6% or YNBD+DO-Ura and incubate 24hrs at 28°C.
3. After checking that glucose is finished (to be sure that any repression is affecting the cells) harvest cells centrifuging 5000rpm 10 minutes, 4°C.
4. Resuspend the cells in water proportionally to their humid wet (to normalize concentration among different strains).
5. Register the spectra oxidized versus reduced.

Raw data were processed using Excel functions (Microsoft® Office). WT strain was included in each analysis.

5.9.4. Oxygen consumption

(Ferrero et al., 1981)

Oxygen consumption was recorded using Oxygraph system from Hansatech, composed of a S1 Clark electrode core controlled with Oxygraph Plus software (Hansatech Instruments Ltd.).

Cells were obtained as described for cytochromes spectra recording. 100µl were added to 900µl of K-ftalate 0.1M pH5 in the chamber. Oxygen decrease was recorded for at least 1 minute. 100µl of cell suspension were exsiccated at 37°C for 48hrs and dry wet estimated.

Oxygen consumption rate was expressed as nmol O_2 for minute for mg of cells (nmol/min mg).

5.9.5. In Vivo Labeling of Mitochondrial Products with 35S-met

1. Strains were grown on YPD plates and inoculate at very low concentration in 10ml of synthetic minimum media with galactose and auxotrophic requirements. This step can also be performed in YPD media. Grow until OD600 is 0.6 – 2 maximum.
2. Harvest equivalent of 0.6 OD in eppendorf tube. Centrifuge 1 minute 6000-8000rpm.
3. Wash the pellet with 500µl of 40mM phosphate pH 6, galactose 2% (Important: repeat twice this step if cells were grown in complete media to eliminate any residual methionine). Resuspend cells in 500µl of the same buffer.
4. Add 10µl of freshly prepared cycloheximide (10mg/ml in water), mix and incubate 2.5 minutes at 30°C (or the temperature at which the cells were grown).
5. Add 2.5-4µl of 35S-methionine (10Ci/ml). Mix and incubate for a definite time depending on the experiment (usually 5 to 15 minutes).
6. Chase of mitochondrial translation products was performed by stopping the reaction with the addiction of 40 mM of puromycin and 25 Mm cold methionine. After different time periods (from 30 to 120 minutes) aliquots were removed and protein extracted.
7. Centrifuge 1 minute at maximum speed and resuspend cells in 75µl of Rodel Mix (protect stock solution from light). Immediately add 500µl of bidistilled H2O. Add 575µl of 50% tri-chloroacetic acid (TCA). Incubate at least 15 minutes on ice. Centrifuge 15000rpm, 10 minutes.

Rodel Mix: 5.56ml 5M NaOH
1.11ml ß-mercaptoethanol
6.84ml H2O
1.5ml 0.1M PMSF

8. Wash pellet once without resuspending with 0.5M Tris-Base (no pH adjusted) to neutralize TCA. Wash once without resuspending with bi-distilled H2O.

9. Resuspend in 25µl of 1X Loading Buffer without heating. Load on 17.5% SDS-PAGE (10X15cm separating gel at least).

Loading Buffer 1X: 2% SDS
10% Glycerol
60mM Tris-HCl, pH 6.8
2.5% ß-mercaptoethanol
bromophenol blue

10. Run until blue reaches the bottom, without exiting the gel. Maximum 40mA.
11. Semidry transfer to nitrocellulose membrane for 1hr 30minutes at 200mA. Let the membrane dry for few minutes at 37˚C and perform a first exposition of about 24 hrs.
Buffers and gel composition

Solution	Separating Gel 17.5%	Stacking Gel 5%
30% acrylamide 0.2 bis-acrylamide	12.61ml	1.079ml
1.825M TrisHCl pH 8.8	4.55ml	-
0.6M TrisHCl pH 6.8	-	0.650ml
water	4.55ml	4.620ml
SDS 10%	217µl	65µl
APS 10%	109µl	32.5µl
TEMED	7.8µl	6.5µl
final volume	22.1ml	6.5ml

Running Buffer: 50mM Tris-HCl, pH 8.3
384mM glycine
0.1% SDS

Prepare a 5X stock solution (30gr Tris-Base, 144gr glycine, 5gr SDS for 1 liter). Dilute to 1X and adjust the pH precisely to pH 8.3. The pH is crucial for optimal proteins resolution. Check frequently the pH of 1X running buffer.

5.9.6. Crude Total Protein Extraction

1. Grow cells overnight to 0.7-1.2 OD
2. Harvest the equivalent of 1.2OD of cells by centrifugation 1min 6000-8000rpm
3. Wash once with water and resuspend the pellet in 75µl of Rodel mix (see above)
4. Add 500µl of water

5. Add 575µl of TCA50%
6. Incubate on ice at least 15 minutes. Centrifuge 15000rpm 10min
7. Wash pellet once with 0.5M Tris-base without resuspending (about 1ml)
8. Wash pellet once with water without resuspending (about 1ml)
9. Resuspend in 25µl of 1X LB and add few µl of Tris-base if the color is yellowish

5.9.7. Chronological lifespan assay

Yeast strains were pre-inoculated in 10 ml of YPD or SD O.N and after were inoculated to an OD600 of 0.05 in 100 ml total volume in 25o ml flasks and incubated at 28° C in an orbital shaker moving at 200 rpm.

Viability was assayed either by staining a 100 ml sample of the culture with 100 ml of trypan blue (0.4 mg/ml), incubating at 28° C for 5 min, and counting clear versus blue cells or by plating serial dilutions onto YPD plates, as indicated.

5.9.8. Treatment with ethidium bromide

1. Cells were pre-grown in YPD medium.
2. Cells were inoculated at $1*10^6$ cells/ml in 2 ml of YPD supplemented with ethidium bromide 30 µg/ml and incubated at 28° C for one day.
3. Repeat point two twice.
4. 300 colonies were plated on YPD and after 24-48 hours some of the grown colonies are streaked on YPD and YPE plates.
5. After 3 days ρ^0 cells will be grown on YPD plates but not on YPE.

5.9.9. Aconitase activity assay

1. Harvest 50 ml of cells at OD between 0.5 and 1. All samples must have the same OD, because aconitase activity is susceptible to OD changes.
2. Pellet and get rid of the supernatant.
3. Resuspend pellet in 30 ml of ice cold lysis buffer, pellet and get rid of the supernatant.
4. Resuspend pellet in 1 ml of ice cold lysis buffer.
5. Transfer cells to ice-cold eppendorf tubes.
6. Spin at max speed for 5 minutes in cold room.
7. Get rid of supernatant and resuspend pellet in 200 µl of ice-cold lysis buffer.
8. Store at -80 °C for 1 hour or even days.

9. Thaw cells on ice. Resuspend cells.
10. Add cold glass beads to cells, vortex cells for 30" and put them on ice. Repeat this step 7 times.
11. Spin at maximum speed in cold room for 5 minutes.
12. Transfer the top layer to a new tube and perform the assay within 1 hour.

Cell lysis buffer:

50 mM Tris pH 8.0, 50 mM KCl, 2 mM sodium citrate, 10% glycerol.

Reaction mixture (for 25 reactions):

1 M Tris HCl pH 8.0, 10 mM MgCl2, 10 mM NADPH+, 8 units of ICDH.

Aconitase assay is carried out by mixing 240 µl of reaction mix, 16 µl of citrate and 48 µl of cell extract in a final volume of 800 µl. A Cary 300Scan UV-vis spectrophotometer (Varian Inc.)is used to perfor the assay, recording continuously at 340 nm.

5.10. Mutational rate analysis

5.10.1. mtDNA stability analysis

mtDNA stability was evaluated by the frequency of spontaneous cytoplasmic *petite*. They grow as small colonies on medium supplemented with respiratory carbon sources such as ethanol and glycerol. The protocol is the following:

1. The pre-existing *petite* are counter-selected on YPE 2% medium.
2. Replicate the YPE 2% plates on selectable medium supplemented with 2% glucose. The strains are grown for 24h at 28°C.
3. Replicate on a new plate of the same medium and incubate again for 24h.
4. Resuspend a part of cells in H_2O and evaluate the cellular concentration.
5. Plate 250 cells on YPD or YNB medium supplemented with 0.6% glucose and 2% ethanol. Incubate at 28°C for 5-6 days and count the cells.

0.6% glucose is added in order to allow the growth of *petite* mutants which are respiratory deficient and are unable to use ethanol like the "wt" cells. When glucose is exhausted the wild-type cells uses ethanol to grow, while the *petite* mutants arrest their growth. Indeed the *petite* mutants will form small colonies unlike the big colonies

formed by wt cells. For further controls, the single colonies can be patched on a medium containing a respiratory carbon source.

5.10.2. Determination of Ery^R mutant frequency

A clone from each mutant and wt strains is streaked on SCD or YPD plates. After 1 day at 28° C, cells are picked up with a toothpick, suspended in water, counted, diluted and plated on SCD or YPD to a final concentration of 100 cells/plate.

After 2 days at 28° C, nine colonies from each clone are picked up and suspended separately in 2 ml of SCE or YPGLY. After 2 days at 28° C under vigorous shaking, 1.5 ml of each culture is transferred into an Eppendorf tube. The cell pellet is collected by centrifugation at 5000g for 15 s, suspended in 200 µl of water and spread in a YPAEG-Ery plate.

To determine the number of cells spread on YPAEG-Ery plates aliquots of the cell cultures are diluted to 1:500,000, plated on YPGLY plates and counted after 3 days of growth. After 8 days of incubation, the EryR colonies grown on YPAEG-Ery plates are counted. For each of the nine clones, the ratio of the number of EryR colonies to the total number of plated cells is calculated. The EryR mutant frequency is defined as the median of the nine ratios. For each strain the experiment is performed at least three times, and the EryR mutant frequency is calculated as the mean value of the three median values.

5.10.3. Nuclear DNA point mutability

Canavanine is a toxic equivalent of the arginine, transferred in to the cytosol by the same carrier used by the aminoacid. Point mutations on the gene that encodes for the carrier lead to the generation of strains resistant to canavanine (CAN^R). the number of CAN^R strains that spontaneously arise in a population is used as an indicator of nuclear genome mutability rate. The measure of the CAN^R mutant frequency was performed as indicated below:

1. Cells are pre-grown in YP or YNB supplemented with 2% glucose.
2. For each strain $2*10^5$ cells/ml are inoculated in nine tubes containing YPD or YNB+D and incubated at 28° C under vigorous shaking until they reach the final concentration of $1*10^8$.
3. Cells are collected by centrifugation at 5000 rpm for 5', resuspended in 1 ml of sterile water and washed.

4. Cells are finally resuspended in 100 µl of sterile water and plated on YNB+ Glu 2%+ the aminoacids required and canavanine (6 mg/100 ml).
5. Plates are incubated at 28° C for 6 days and the number of resistant colonies is counted.

5.11. Mitochondrial analysis

5.11.1. Preparation of yeast mitochondria with intact outer membrane

(Glick et al., 1995)

1. Harvest cells at 2000-3500 rpm for 7 minutes and wash once with sorbitol 1.2M.
2. Resuspend washed cells in digestion buffer at concentration of 1gr of cells in 3ml

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

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

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

Buffer A: 1.2M deionized sorbitol (BioRad® AG501-X8 resin)
20mM KPO4 pH 7.5

4. Suspend washed spheroplasts in 0.6M sorbitol, 20mM KMES pH 6, 0.5mM PMSF at concentration of 1gr/3ml. Homogenize with and glass/Teflon pestle and overhead stirrer (Wheaton Science Products).
5. Centrifuge 3000rpm for 5 minutes to pellet debries. Collect the supernatant and centrifuge 12000rpm for 10 minutes to sediment mitochondria. The supernatant is the post mitochondrial fraction (PMS) and represent the cytosolic component.

6. Suspend mitochondria briefly with plastic sticks in 0.6M sorbitol, 20mM KMES pH 6 (1ml/g of starting cells wet weight) in eppendorf tubes. Centrifuge at 3000rpm for 5 minutes to pellet broken mitochondria.
7. Collect supernatant and centrifuge at 12000rpm for 10 minutes. Suspend mitochondrial pellet in 0.6M sorbitol, 20mM KMES (100 µl/g of starting cells wet weight) and dilute with 20ml of 0.6M sorbitol, 20mM HEPES pH 7.4 (1ml/g of starting cells wet weight). Centrifuge 12000rpm for 10 minutes.
8. Suspend mitochondrial pellet in 0.6M sorbitol, 20mM HEPES accordingly to the pellet dimension. Mitochondria can be stored at -80°C.

5.11.2. Quantification of proteins with Bradford method

Protein concentration was determined with Bradford method (Bradford, 1976). The "BioRad Protein Assay" commercial kit was utilized according to the manufacturer's instructions.

5.11.3. Succinate Dehydrogenase (SQDR) enzymatic activity

This assay measures the rate of reduction of an artificial electron acceptor as dichlorophenolindophenol (DCPIP) by complex II as modified by Kim and Beattie, 1973.
Solutions needed:

100 mM K-phosphate pH 7.8 (30°C)
10 mM succinate pH 7.4
2.5 mM phenazyne methosulfate (PMS)
0,15 mM DCPIP
100 mM NaN3

The SDH activity was recorded at 600nm.
Specific Activity: SQDR activity was calculated with ε of 21 and the following formula (ΔOD*ε/min mg).

5.12. Protein separation with SDS-page

Protein separation with SDS-page was performed with classical Laemmli system (Laemmli 1970). Separating gels were prepared at 12% polyacrylamide (30:0.8 acrylamide-bis), stacking gel at 6%. Running was performed for 1hr 30' at 100-120volts.

5.13. Western Blotting and Ig-detection

Separated proteins were transferred to nitrocellulose membrane by electroblot for 1hr 15' at 200mA. Semi-dry blotting system was used. Quality of the blot was assessed with Ponceau S staining.

Transfer Buffer: 200mM glycine
25 mM Tris
20% methanol

Membranes were blocked 1hr with 5% non-fat dry milk prepared in washing buffer and incubated o/n with appropriate primary antibody (mono or polyclonal).

Washing Buffer: TBS 1%
tween 0.1%

Blocked membranes were washed 4 times 15 min with washing buffer prior incubation with suitable secondary antibodies (Anti-Rabbit, Anti-Mouse), conjugated with horseradish peroxidase (1:5000 in 5% milk if anti rabbit Ig, 1.5% milk if anti mouse). After 1hr incubation membranes were washed as above and developed with ECL based immune revelation. If greater sensitivity was necessary SuperSignal West Femto or Pico commercial kits were implemented (© Thermo Scientific).
Primary antibodies used:

- Anti-Cox2p (1:1000) (G. Shadel laboratory)
- Anti-Cox3p (1:1000) (G. Shadel laboratory)
- Anti-Cox4p (1:1000) (G. Shadel laboratory)
- Anti-Sod2p (1:1000) (G. Shadel laboratory)
- Anti-actin (1:1000) (G. Shadel laboratory)
- Anti-porin (1:1000) (G. Shadel laboratory)
- Anti-8-oxodeoxyguanine (1:1000) (G. Shadel laboratory)
- Anti-cytochrome c (1:1000) (a kind gift from A. Barrientos)
- Anti-Atp4 (1:1000) (a kind gift from Prof. J. Velours)

All the antibodies listed below were incubated with Anti-Mouse secondary antibodies with the only exception of anti-Sod2p (anti-rabbit) and anti-8-oxodeoxyguanine (anti-goat).

5.14. Dot Blot assay on nuclear and mtDNA

Total cellular DNA, from cells grown in YPD until OD600 1, was extracted by "smash and grab" protocol (previously described). DNA was resuspend in 100 µl of TE+RNase, incubate 1 hour 65° C and then incubated with 20 µl of proteinase K for an additional hour at 65° C.

DNA was extracted again with phenol:chloroform, and resuspended in TE. DNA was quantified using the nanodrop and aliquots of 5 and 10 µg diluted in 250 µl of TE are prepared per each sample.

The nylon membrane was cut and pre-soak in TE, dot blotter was assembled, samples were placed in wells, and vacuum was turned on to adhere samples to the membrane. Wells were washed with 250 µl TE in case any DNA remains stuck to the sides of the wells. When all liquid has passed through the membrane, disassemble the dot blotter. Membrane is dried for at least 1 hour at 55° C or overnight at 37° C. From this point on, the dot blot was treated as a normal western blot.

5.15. Total RNA extraction from yeast and Northern Blot analysis

Yeast cells were grown O.N in the appropriate medium until they reach an OD600 between 0.8-1.2 and total RNA was prepared by phenol extraction as previously described (Ausubel et al. 1994).

30 µg of RNA for each sample are separated on agarose gel containing formaldehyde (as denaturing agent to limit the formation of secondary RNA structures), running at least 3 hours at 100 V.

RNA is then transferred to a nylon membrane Hybond-N^+ (Amersham) by capillarity (Sherman et al. 1986). Labeling of the oligonucleotide probes is performed by using the "Rediprime II DNA labeling System" kit (GE Healthcare) plus 5 µl of ^{32}P dCTP.

Hybridization is carried out O.N and thereafter the filter is washed many times and revealed using Kodak films. As control a probe direct against the ACT1 mRNA is used.The signals obtained are then quantified using Phosphorimager (Molecular Dynamics).

5.16. Flow cytometry

Cell from a growing culture, once reached OD600, were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). For ROS amount measure cells were resuspended with 500 µl of PBS supplemented with DHE 5mM and incubated at 28° C for 15 minutes.

For measurement of mitochondrial potential, cells from a growing culture were pelleted DiOC6 (Molecular Probes) was diluted to a final concentration of 200 nM in PBS and used to resuspend the cells. The cell suspension was then incubated for 30 minutes at 28 ° C.

Once incubation was finished cells were washed twice with PBS, and analyzed by flow cytometry using the FL1 (for DHE staining) or FL3 (for DiOC6) channel without compensation. All analysis was performed on a Beckton-Dickenson FACSCalibur.

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