UNIVERSITY OF PARMA

Department of Genetics, Biology of Microorganisms, Anthropology, Evolution

Ph.D. in Biotechnologies
XXIV COURSE

SDH assembly in *S. cerevisiae*: focus on the functional role of *SDH6*

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To the Fabulous Four

To A. and V.

"That is the essence of science: ask an impertinent question and you are on the way to a pertinent answer."

- Jacob Bronowski-

"I believe in intuition and inspiration."

- Albert Einstein-

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Chapter I:

Mitochondrial Function and OXPHOS defects

1.1. Mitochondria

1.1.1. Mitochondrial structure and function

Mitochondria are eukaryotic organelles responsible for several essential metabolic processes. They are involved in the cellular signaling, in programmed cell death, in heme and Fe/S clusters biogenesis, in the oxidation of pyruvate, in the Krebs cycle, in metabolism of amino acids, fatty acids, cholesterol, steroids and nucleotides (Scheffler, 2008). However their most important function is to generate most of the cell's chemical energy in the form of adenosine triphosphate (ATP) by the process of aerobic respiration and the oxidative phosphorylation.

Mitochondria are ellipsoidal organelles and have no fixed size, generally with a diameter ranging from 0.2 to 1 μm and a length of 3-4 μm. They contains two membranes that separate four distinct compartments, the outer membrane (OMM), the intermembrane space, inner membrane (IMM) and the matrix (Mcbride *et al.*, 2006).

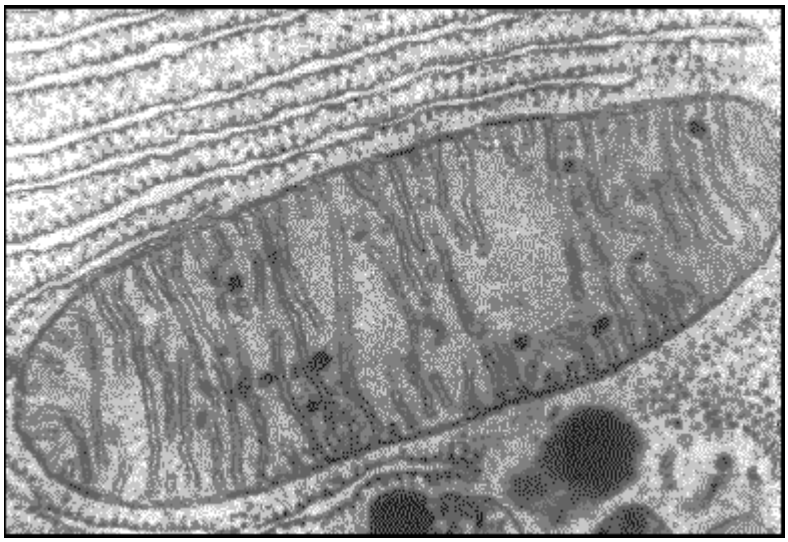


Figure 1.1. A mitochondrion *(http://biology.unm.edu/ccouncil/Biology_124/Summaries/Cell.html)*

There are some differences in phospholipid composition between the outer and inner membrane. The phospholipid: protein ratio is higher in the outer than in the inner membrane (Guérin, 1991) The inner membrane is enriched with cardiolipin whereas the outer membrane is enriched with phosphatidylinositol (Bottema and Parks, 1980; Daum, 1985). The outer membrane has pore-forming proteins called porins which allow the outer membrane to be permeable to molecules of 10kDa or less. The inner membrane is not freely permeable and contains a series of specific carriers that allow connections between the mitochondrial and cytoplasmic metabolism (Guérin, 1991). The inner membrane is

highly folded into special structures called *cristae* in which most of the OXPHOS proteins are located (Griparic and Van der bliek, 2001). The organization in these special structures greatly increases the total surface area of the inner membrane, optimizing the energy production processes.

Mitochondria are very dynamic organelles (Dimmer *et al.*, 2002) and form a functional reticulum whose steady-state morphology is regulated by dynamic fission, fusion and motility events (Mcbride *et al.*, 2006). It is thought that the balance between mitochondrial fission and fusion is essential in mitochondrial function and maintenance (Chen *et al.*, 2003). By definition, mitochondria are able to express an integral genome and generate ATP coupled with electron transport.

In the cell their distribution and morphology vary according to the cell's metabolic demand (Bereiter-Hahn, 1994; Warren and Wickne, 1996; Hermann and Shaw, 1998; Yaffe, 1999). Tissues with high energy demands such as hearth, brain and muscle, have a rich number of mitochondria and these tissues are more vulnerable to mitochondrial defects.

Respect to other organelles, mitochondria have a peculiar characteristic: they posses their own genome, mitochondrial DNA or mtDNA and protein synthesis machinery. More than one copy of mtDNA is present in mitochondria and their number depends on the cellular conditions, environmental factors and in the case of multicellular organisms on the tissues.

1.1.2. mtDNA

Mitochondrial DNA in mammalian cells is circular double stranded DNA of 16.5Kb (fig.1). Mitochondrial DNA is not associated with histones but organized in a very compact structure called nucleoid, anchored to the inner membrane and stabilized by DNA-binding proteins different from the histones organizers on nuclear DNA (Satoh and Kuroiwa, 1991). An average nucleoid in mammalian cells contains 5-7 genomes (Atkinson and Winge, 2009). Furthermore, mtDNA is replicated continuously in a process independent from nuclear genome replication, occurring in both mitotic and post-mitotic cells (Yu-Wai-Man *et al.*, 2011). Mammalian mtDNA encodes for only 13 proteins of total mitochondrial proteins as well as for 22 tRNA and 2 rRNA that are essential for the mitochondrial translation. The 13 mtDNA encoded polypeptides consist of seven subunits of complex I (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), one subunit of complex III (CytB), three subunits of complex IV (COXI, COXII, COXIII) and two subunits of complex V (ATPase 5

and ATPase 6). The other mitochondrial proteins such as all subunits of complex II and the mitochondrial DNA polymerase (γ) are encoded by nuclear DNA and are imported into mitochondria through specialized import machineries (Baker *et al.*, 2007; Neupert and Herrmann, 2007). This is possible thanks to a N-terminal target sequence for mitochondria which is processed when the proteins are traslocated (Hartl, 1989; Glick and Schatz, 1991; Neupert, 1997).

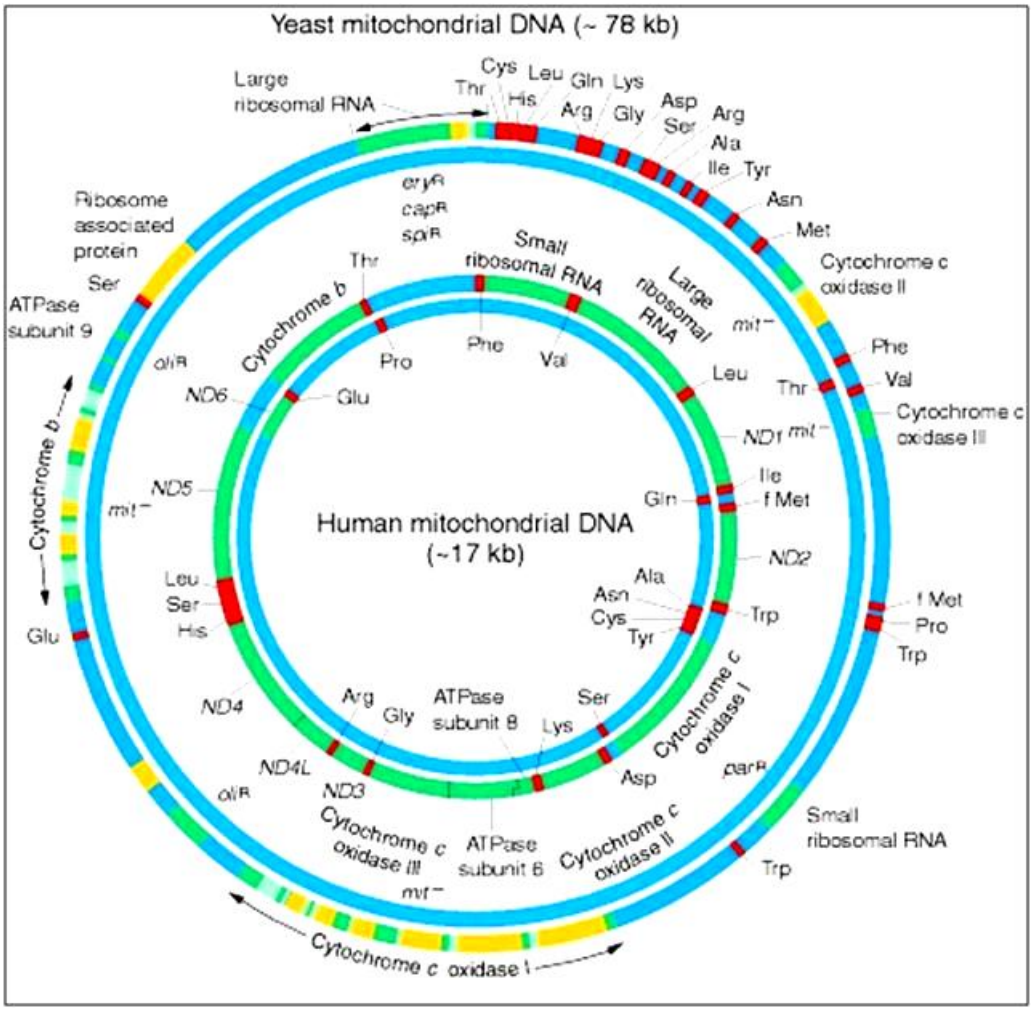


Figure 1.2. Yeast and Human mtDNA maps. Each map is shown as two concentric circles corresponding to the two strands of the DNA helix. Note that the mutants used in yeast mtDNA analysis are shown opposite their corresponding structural genes. Green = exons and uninterrupted genes, red = tRNA genes, and 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***

Yeast mtDNA has a size ranging from 68Kb (short strain) to 86Kb (long strain). Unlike the human mtDNA, the yeast mitochondrial genome is less compact and contains both non coding and intronic regions. The yeast mtDNA encodes for 19 proteins: among these there are the subunits of complex III, *Cob* or *CytB*, and other three subunits of complex IV, *Cox1p, Cox2p, Cox3p* as in human. Moreover, in mtDNA are encoded three subunits of

ATP synthase complex (Atp6, Atp8 and Atp9) so as the Var1p, a ribosomal mitochondrial protein (*VAR1*). The other 11 polypeptides, encoded in introns of 21S rRNA, *COB* and *COX1* genes, consist of maturases, endonucleases and reverse transcriptases and in a protein of unknown function yet. In addition mtDNA whose genetic code is different respect to the universal code, encodes for 24 mitochondrial tRNAs, 2 rRNA and for an RNA belonging to RNAse P. In the short strains all these elements are present except for some non coding and intronic regions.

1.2. Functions

Among mitochondrial functions, the most important one is the conversion of energetic metabolic intermediates into H_2O and CO_2 coupled to ATP production. These substrates come from catabolic pathways that take place in mitochondria such as oxidative degradation of pyruvate, fatty acids and some aminoacids.

1.2.1. Oxidation of pyruvate and fatty acids

Sugars and fatty acids are the main energy sources for the cells. In aerobic organisms, these substrates are oxidized to CO_2 and H_2O with production of ATP through a series of reactions known as cellular respiration. The glycolytic pathway which occurs in cytosol, converts sugars in pyruvate and produces 2 ATP molecules. In anaerobic conditions, pyruvate can be transformed in ethanol or lactate and this pathway is known as alcoholic or lactic fermentation. In presence of O_2, pyruvate can be completely oxidized with a greater ATP production. Therefore it is transported into mitochondria and here it is transformed in acetyl-coA thanks to pyruvate dehydrogenase reaction (Perham, 2000; Ochoa, 2006).

In eukaryotic cells, fatty acids are first activated and converted in acyl-coA. Then they are imported in mitochondrial matrix thanks to acil-carnitine carrier. In mitochondria fatty acids-acyl coA are oxidized through beta oxidation process, a cycle of 4 reactions which releases acetyl-coA and acyl-coA. In yeast, however, beta oxidation occurs in peroxisomes. Here fatty acids are degraded in acetyl-coA units and are subsequently transported in mitochondria through acetyl-carnitine carrier.

Acetyl-coA units either coming from fatty acids oxidation or glycolysis or from catabolism of some aminoacids, are then oxidized in CO_2 through Krebs Cycle.

1.2.2. Krebs Cycle

In eukaryotes the Krebs cycle or tricarboxylic acid cycle occurs in mitochondrial matrix and plays an important role in utilizing non fermentable carbon sources (Lee *et al.*, 2011). This cycle is made of eight reactions (fig.3) and has been proposed in 1947 by H. Krebs. When it was first postulated there were still some major areas of uncertainty especially in production of citrate (Scheffler, 2008). With the discovery of coenzyme A by Kaplan and Lipmann, Ochoa and Lynes were able to show in 1951 that acetyl coA was the intermediate in the reaction with oxaloacetate to form citrate (Scheffler, 2008).

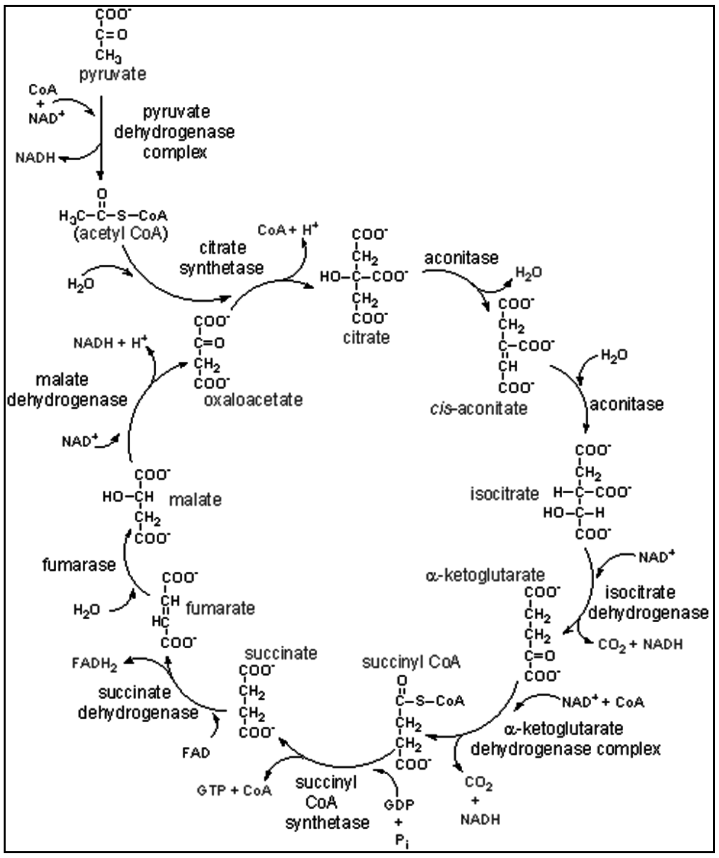


Figure 1.3. The reactions of Krebc Cycle (from chemistry.gsu.edu)

In the cycle two carbons enter as acetyl CoA and leave as CO_2. Four oxidation-reduction reactions take place to yield reduction potential in the form of three molecules of NADH and one molecule of $FADH_2$. A high energy phosphate bond (GTP) is also formed.

TCA cycle reactions are also an important source of biosynthetic building blocks such as α-ketoglutarate and oxalacetate. Indeed these intermediates are used to form aminoacids and numerous precursors for other biosynthetic reactions. For example succinyl-coA is a precursor for heme synthesis (Scheffler, 2008).

The Krebs enzymes have been purified and the catalytic mechanisms have been defined. All enzymes of TCA cycle are soluble in mitochondrial matrix except one: succinate

dehydrogenase. This enzyme is a highly conserved heterotetrameric protein which is anchored to the inner membrane and protrude with the catalytic site into the mitochondrial matrix (Bardella *et al.*, 2011). This enzyme will be object of discussion in the chapter II.

When *Saccharomyces cerevisiae* is grown on acetate, a two carbons compound, the Krebs cycle itself cannot supply adequate amounts of biosynthetic precursors unless alternative reactions are available. Yeast, plants and some microorganisms thus employ a modification of the TCA cycle called glyoxylate cycle (fig.4). This cycle produces four carbon compounds starting from two acetate units bypassing oxidative decarboxylation (Lee *et al.*, 2011).

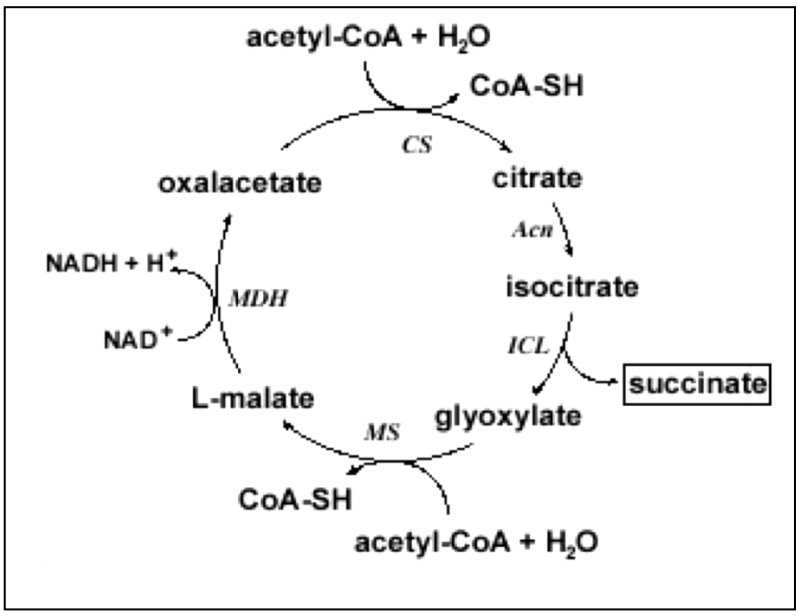


Figure 1.4. The glyoxylate cycle (http://www.scibio.unifi.it/ourlab/icl.html)

1.2.3. Oxidative phosphorylation

A pivotal function of mitochondria is to produce ATP through the process of oxidative phosphorylation (OXPHOS) that takes place in the inner membrane. The OXPHOS is a fundamental energy-conversion process that transfers electrons from high-energy electron donors to oxygen through respiratory enzyme complexes. This process is also known as the electron transport chain (ETC). From Krebs cycle intermediates (NADH and $FADH_2$), electrons feed into complex I or II and are transferred to complex III, then to complex IV and finally to O_2. The redox energy released during the electron transfer process in complexes I, III, and IV is utilized to actively pump out H^+ from the mitochondrial matrix to the intermembrane space (Wallace *et al.*, 1988; DiMauro and Schon, 2003; Balaban *et al.*, 2005) The redox reactions in the respiratory chain, based on

the chemiosmotic theory proposed by Mitchell, produce an electrochemical proton gradient that allows H^+ to diffuse back to the matrix converting ADP and inorganic phosphate (P_i) to ATP through a fifth inner membrane complex (Mitchell and Moyle, 1967). NADH electrons are sufficient to produce three ATP molecules, while from $FADH_2$ electrons two ATP molecules are obtained. The electrons are transported through specific transporters that are cyclically reduced and oxidized. In the ETC the transporters are:

- Fe-S centers: are prosthetic groups that contain iron different from heme. Inorganic sulfur atoms or cysteine residues coordinate the iron atoms. These centers and specifically the iron atoms that pass from $Fe2^+$ to $Fe3^+$ cyclically accept electrons one by one.
- respiratory cytochromes: are mitochondrial proteins bound (covalently or not) to heme groups whose iron atom is able to transfer electrons. There are several different cytochromes, named with alphabetical letters, which absorb UV light with specific adsorption spectra. Typically, c-type cytochromes have a prominent band peak at about 550 nm, b-type cytochromes at around 560 nm, and a type cytochromes at around 602 nm.
- Coenzyme Q or ubiquinone: is the only ETC transporter not bound to a protein complex. It transports either electrons or protons. There are three redox states of coenzyme Q10: fully oxidized (ubiquinone), semiquinone (ubisemiquinone), and fully reduced (ubiquinol).

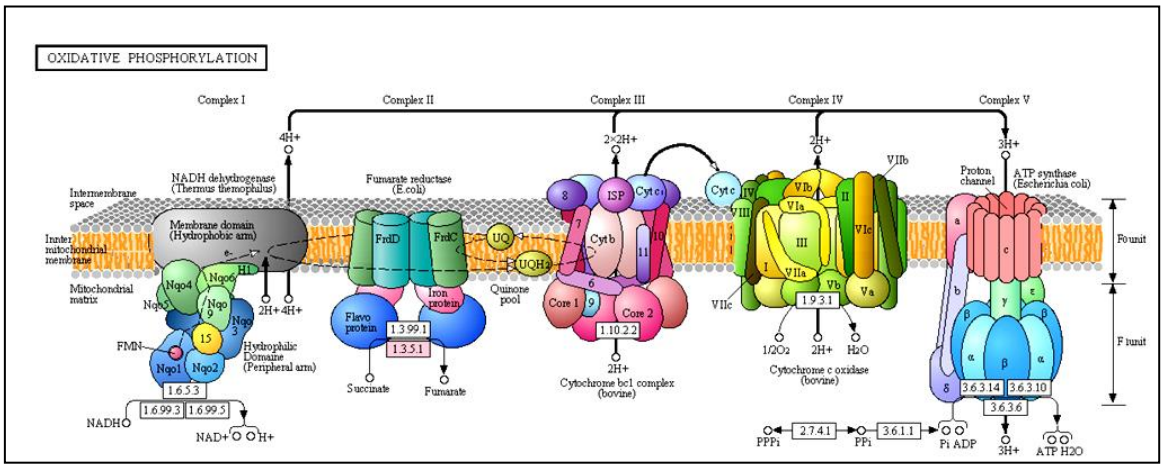


Figure 1.5.Respiratory chain by KEGG pathway

Respiratory complexes are anchored to the inner membrane and are able to transfer electrons across the respiratory chain. These are:

- Complex I or NADH ubiquinone oxidoreductase is the largest complex of the respiratory chain. It has a L shaped structure consisting of a hydrophobic

membrane arm and a peripheral arm that protrudes into the mitochondrial matrix (Mckenzie and Ryan, 2010). This complex comprises 45 subunits (>900 kDa) in mammalian mitochondria, 7 of which (ND1, 2, 3, 4L, 4, 5, 6) are encoded by mtDNA. The remaining 38 subunits are encoded by nuclear genes. It contains 6 iron-sulfur clusters and one flavin mononucleotide cofactor. NADH is oxidized and two electrons are transferred to FMN center. These electrons are then transferred via iron sulfur clusters to the matrix side to reduce ubiquinone to ubiquinol and 4 protons are subsequently transferred to the intermembrane space (Saraste, 1999).

$$\mathbf{NADH + Q + 5H_i^+ => NAD^+ + QH_2 + 4\ H_o^+}$$

Unlike other OXPHOS subunits, however a detailed structure for mammalian complex I remains to be determined. The organization of these redox centers inside the complex, the roles of accessory subunits unique to mammalian complex I and the coupling mechanism between electron transfer and proton pumping are still poorly understood. In addition common yeast such as budding and fission yeasts which are used as powerful genetics tool for studying the OXPHOS system, lacks the complex I but uses a completely different enzyme for electron transfer from NADH to ubiquinone, which does not pump protons out the matrix.

- Complex II or succinate: ubiquinone oxidoreductase or succinate dehydrogenase: catalyses the electrons transfer from succinate to coenzyme Q. It is the only enzyme which is part both of ETC and Krebs cycle. Indeed it catalyses the oxidation of succinate to fumarate and the electrons are channeled to ubiquinone. Thus, complex II links the Krebs cycle directly to the ETC (Davis and Hatefi, 1971; Hanstein *et al.*, 1971; Davis and Hatefi, 1972). Description of this enzyme will be given later in this dissertation.
- Complex III or ubiquinol- cytochrome c oxidoreductase: catalyses the transfer of electrons from ubiquinol to cytochrome c and concomitantly the release of two H^+ in the intermembrane space. It contains a membrane embedded cytochrome b, the Rieske Fe-s protein and cytochrome c1. These are functionally the most important subunits since the only ones participating in electron transfer and in proton translocation (Scheffler, 2008). Transfer direction is from cytochrome b (b562 and b566) through Fe-S centers to cytochrome c. This protein is poorly soluble in water and loosely bound to the inner mitochondrial membrane and, unlike others cytochromes, is not associate to additional proteins.

$$\mathbf{QH_2 + Cyt\ c^{3+} + 2\ H_i^+ => Q + 2\ Cyt\ c^{2+} + 4\ H_o^+}$$

Complex III like complex I has two reaction centers for ubiquinone, Qn and Qp required for the Q cycle. The Qn or quinone reduction center is located in the matrix side (N-side) of the inner membrane, it recycles electrons into the quinone pool and takes H^+ from the matrix. The Qp site is located near to the outer face of the inner membrane where protons are released into the intermembrane space. The electrons are split in two directions, half are used for recycling and the other part is passed through iron-sulfur centers and cyt c1 to cytochrome c.

- Complex IV or cytochrome c oxidase: consists of 13 proteins and catalyzes the electron transfer from cytochrome c to the molecular oxygen, which is the final electron acceptor. Cytochrome c is reoxidized and for each electron transferred four protons are pumped into intermembrane space. There are two processes involved. First four electrons are donated on the IMS side, while four protons are taken up on the matrix side, resulting in a transfer of four positive charges across the membrane. Second an average of one proton is pumped through the enzyme for each electron transferred to oxygen (Brändén *et al.*, 2006). This complex contains cytochromes a, a3 and copper atoms to transport electrons.

$$\mathbf{4\ cyt\ c^{2+} + 4\ H^{+}_{(s)in} + 4H^{+}_{(p)in} + O_2 => 4\ cyt\ c^{3+} + 4H^{+}_{(p)out} + 2\ H_2O}$$

- Complex V or ATP synthase: is not a respiratory complex but produces ATP using the electrochemical energy released during the oxidative phosphorylation. The H^+ pumped from the matrix to the inner membrane space are coupled to the electron transfers and create the gradient used for ATP synthesis starting from ADP and P_i. In addition, the chemical gradient is used for other mitochondrial functions and for metabolites transport across the inner membrane.

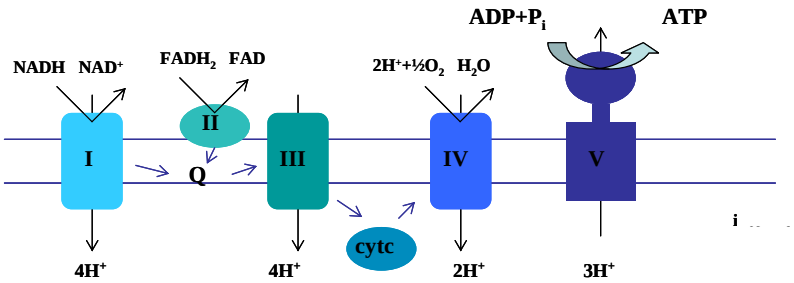


Figure 1.6. Respiratory mitochondrial complexes: I: NADH-ubiquinone oxidoreductase; II: succinate:ubiquinone oxidoreductase; III: ubiquinone -cyitochrome c oxidoreductase; IV: cytochrome c oxidase; V: ATP synthase complex; Q: coenzyme Q; cytc: cytochrome c. The blue arrows indicate the electrons.

1.3. The Assembly of the Electron Transfer Chain Complexes

Despite the compositions, structures and functions of the electron transport complexes are well understood, the biogenesis mechanisms are not completely elucidated. Furthermore, the mechanisms and the pathways leading to the assembly of these multisubunit complexes with integral and peripheral membrane subdomains, to the incorporation of prosthetic groups such as Fe-S centers or heme and other metal centers, are still an intriguing challenge to be understood. In order to gain insights into these mechanisms, several approaches have been developed. One of these approaches takes advantage of the model organism *S.cerevisiae* whose specific knockouts are available and can be made easily. The knockout strains can be used to characterize intermediates that accumulate in the absence of a particular subunit for a complex of interest. In other organisms, the possibility to find suitable mutants can help to identify assembly intermediates. Despite several ETC subunits mutations have been identified, the difficulty is given by the fact that when specific subunits are absent due to mutations, some or all the remaining subunits fail to assemble and are rapidly degraded (Scheffler, 2008). A significant impact on the study of the ETC complexes has been given by Schagger and colleagues who developed the Blue-Native gel electrophoresis. This method allows to achieve in one gel the fractionation of all complexes in their native form (Schägger 1995; Schägger 2001; Schägger 2002; Schägger *et al*., 2004). However, this technique requires the solubilization of mitochondria in detergents and therefore there is the possible risk to destroy *in vivo* the existing unstable intermediates. A brief description of the state of art about the ETC assembly will be now discussed. As regards the assembly of complex I, the human model will be the object of discussion and the motivations are described below. For the other complexes, the studies in yeast will be discussed.

- Assembly of mitochondrial complex I

Complex I is the largest component of the respiratory chain. It consists of 45 different subunits which must be assembled correctly to form the properly functioning mature complex of ~1 MDa (Lazarou *et al*., 2009). Complex I dysfunction is the most common oxidative phosphorylation (OXPHOS) disorder in humans and defects in the complex I assembly process are often observed. This assembly process has been difficult to characterize because of its large size, the lack of a high specific resolution structure and its dual control by nuclear and mitochondrial DNA. However, in recent years, some of the atomic structure of the complex I have been resolved and new insights into complex I

assembly have been generated. Furthermore, a number of proteins have been identified as assembly factors for complex I biogenesis and many patients carrying mutations in genes associated with complex I deficiency and mitochondrial diseases have been discovered (Mimaki *et al.*, 2011). In addition, the yeast *S. cerevisiae* lacks a true complex I (there is a single subunit NADH-Ubiquinone Oxidoreductase, Ndi1p, that catalyzes a similar reaction, see Yang *et al.*, 2011 for details) and this has prevented such detailed analyses into assembly seen for other complexes of the respiratory chain (Lazarou *et al.*, 2009).

The study of cI assembly has employed various eukaryotic model systems such as the green alga *Chlamydomonas reinhardtii*, the fungus *Neurospora crassa*, the nematode *Caenorhabditis elegans* and cultured mammalian cell lines which have been recently reviewed and described by Mimaki *et al.*, 2011. The positions of each subunit within this structure have not yet been fully defined; however, treatments with mild chaotropic agents can dissociate the complex into four subcomplexes (Iα, Iβ, Iλ, Iγ) allowing the identification of subunits within each subcomplex (Sazanov *et al.*, 2000; Carroll *et al.*, 2003; Finel *et al.*, 1992). As regards the nomenclature of these subunits the nuclear encoded subunits contain the prefix "NDU" (NADH-dehydrogenase-ubiquinone) and the mtDNA encoded subunits the prefix "ND" (NADH-dehydrogenase). The assembly factors, furthermore, contain the prefix "NDUFAF": NDU for NADH dehydrogenase, F for alpha subcomplex, AF as assembly factor plus a number indicating when it was first named (Mckenzie and Ryan, 2010).

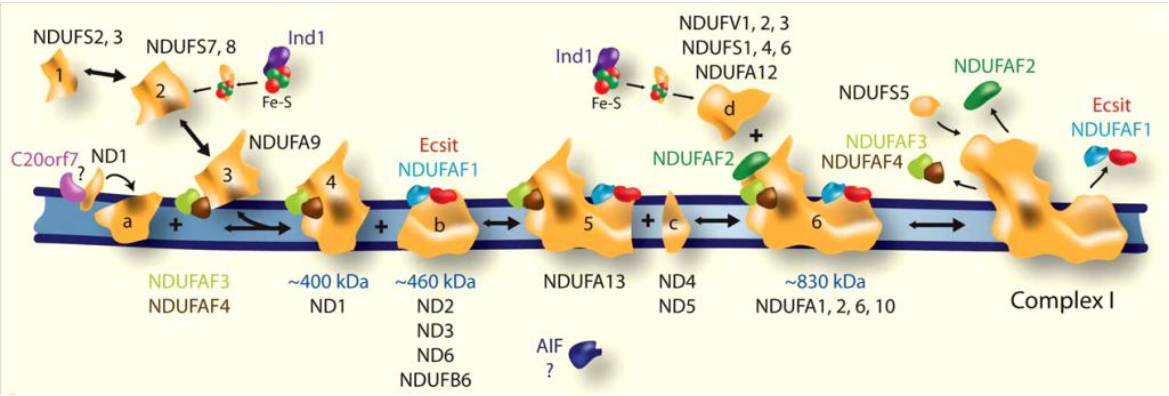


Figure 1.7.Complex I intermediates 1–6 correspond to intermediates described by Vogel and colleagues (Vogel *et al.*, 2007)

Through analyses using tagged intermediates, it was shown that during the early stages of human complex I assembly, intermediate 1 is formed by the addition of subunits NDUFS3 and NDUFS2. The subsequent integration of subunits NDUFS7 and NDUFS8 forms intermediate 2 (fig.7). These four subunits are part of the 14 "core" mammalian

subunits (each with a homolog in complex I from *E.coli*) and form part of the peripheral matrix arm which contains the evolutionarily conserved hydrogenase module (Q module) (Mckenzie and Ryan, 2010). With the addition of NDUFA9 (and possibly other subunits), intermediate 3 is assembled and subsequently anchored to the membrane by the assembly factors Ndufaf3 (C3orf60) and Ndufaf4 (C6orf66). Here, intermediate 3 is assembled with the ND-containing membrane arm intermediate "a" to form the 400 kDa intermediate 4. A second membrane arm intermediate of 460 kDa, intermediate "b", is also formed and is assembled from the membrane arm subunits ND2, ND3, ND6, and NDUFB6 and is associated with the assembly factors Ndufaf1 (CIA30) and Ecsit. Intermediate 4 and "b" assemble together to form intermediate 5 at which stage NDUFA13 is incorporated (Vogel *et al.*, 2007). The subunits ND4 and ND5 are then assembled into the growing complex with other subunits in a small membrane arm intermediate (intermediate c). The resulting ~830 kDa complex is the intermediate 6. The assembly factors Ndufaf1, Ndufaf2, Ecsit, Ndufaf3, and Ndufaf4 remain associated with this intermediate until the last stage of assembly, where intermediate "d" (which contains the N module) is assembled to form mature complex I.

Another specific complex I assembly factors is Ind1p (iron-sulfur protein required for NADH dehydrogenase): it is a mitochondrial protein which binds iron-sulfur clusters *via* a conserved CXXC motif (Bych *et al.*, 2008). It is important for the assembly of the eight Fe-S clusters present in the complex I. Deletion of Ind1p in the yeast *Yarrowia lipolitica* (*S.cerevisiae* lacks this gene) results in a specific defect in the enzymatic activity of complex I but not in other mitochondrial Fe-S containing enzymes such as aconitase, succinate dehydrogenase or cytochrome *bc1* complex (Bych *et al.*, 2008).

The assembly of complex II will be discussed in the chapter II.

- Assembly of complex III

The *bc1* complex or complex III is a homodimeric complex localized in the inner mitochondrial membrane and each monomer is composed of ten (eleven in mammals) different non redundant subunits (Zara *et al.*, 2009). Three of them are the catalytic subunits: cytochrome *b*, cytochrome c_1 and the Rieske protein (ISP), found in all organisms. The remaining subunits (core protein 1, core protein 2, Qcr6p, Qcr7p, Qcr8p, Qcr9p and Qcr10p) do not contain any prosthetic group and their functions are still poorly understood. The yeast crystal structure does not contain Qcr10p even if this protein is an

authentic subunit of the complex likely analogous to mammalian SU11 which has been seen in the bovine structure (Smith *et al.*, 2011). Mammalian complexes contain an additional subunit not present in *S.cerevisiae*, SU9, which is embedded between Cor1 and Cor2 and corresponds to the presequence of the Rieske Fe/S protein (Smith *et al.*, 2011). Only cytochrome *b* subunit is encoded by mtDNA; the other subunits are nuclear encoded and are imported post translationally into yeast mitochondria (Zara *et al.*, 2009).

Name	Mammal (bovine[a])	Fungi (*S. cerevisiae*[b])
Cor 1	SU1 (*UQCRC1*)	Qcr1 (*COR1*)
Cor 2	SU2 (*UQCRC2*)	Qcr2 (*COR2*)
Cyt *b*	SU3 (*MT-CYB*)	Qcr3 (*COB*)
Cyt *c*	SU4 (*CYC1*)	Qcr4 (*CYT1*)
Rieske Fe/S protein	SU5 (*UQCRFS1*)	Qcr5 (*RIP1*)
–	SU6 (*UQCRQ*)	Qcr7 (*QCR7*)
–	SU7 (*UQCRB*)	Qcr8 (*QCR8*)
Acidic hinge	SU8 (*UQCRH*)	Qcr6 (*QCR6*)
–	SU10 (*UQCR10*)	Qcr9 (*QCR9*)
–	SU11 (*UQCR11*)	Qcr10 (*QCR10*)
Rieske presequence	SU9 (*UQCRFS1*)	–

Figure 1.8. Comparison of eukaryotic *bc1* complex subunit nomenclature *(Smith et al., 2011)*.

The assembly status of the cytochrome *bc1* complex has been analyzed in distinct yeast deletion strains in which genes for one or more subunits were deleted. In all the yeast strains tested, a *bc1* sub-complex of approximately 500 kDa was found when the mitochondrial membranes were analyzed by BN-PAGE. The subsequent molecular characterization of this sub-complex, carried out in the 2D-BNGE and immunodecoration, revealed the presence of two catalytic subunits, cytochrome *b* and cytochrome *c1*, associated with the non catalytic subunits core protein 1, core protein 2, Qcr7p and Qcr8p as described below (Zara *et al.*, 2009).

Zara and colleagues have systematically investigated the effects of single or double deletions of these supernumerary proteins or Cyt *b* on the formation of an active complex III. Some of these subunits (QCR6p, QCR10p) were dispensable as single deletions whereas most double deletions had a significant impact on the assembly of a functional complex (Scheffler, 2008). Through these mutants and several studies, an assembly scheme was developed (fig.9) and indicated that the central hydrophobic core of the *bc1* complex was represented by the cytochrome *b*/Qcr7p/Qcr8p sub-complex (Crivellone *et al.*, 1988; Grivell, 1989; Zara *et al.*, 2004). Indeed deletions in one of these three genes lead to the absence of the other two proteins, suggesting that cytochrome *b*, Qcr7p and Qcr8p could functionally interact (Crivellone *et al.*, 1988; Zara *et al.*, 2004;). This subcomplex is referred to as the 'membrane core sub-complex'. Furthermore the authors indicated the presence of a larger core structure of the *bc1* complex that included

cytochrome *b*/Qcr7p/Qcr8p/cytochrome *c1*/core protein 1/core protein 2. A significant difference between the smaller and the larger sub-complexes was the fact that the first one (cytochrome b/Qcr7p/Qcr8p) was very unstable and, consequently, its identification was extremely difficult, whereas the second (cytochrome *b*/Qcr7p/Qcr8p /cytochrome c1/core protein 1/core protein 2) was characterized by a much higher stability (Zara *et al.*, 2009). Therefore they speculated that the larger *bc1* core structure could acquire a higher stability against proteolytic degradation after incorporation of the two core proteins.

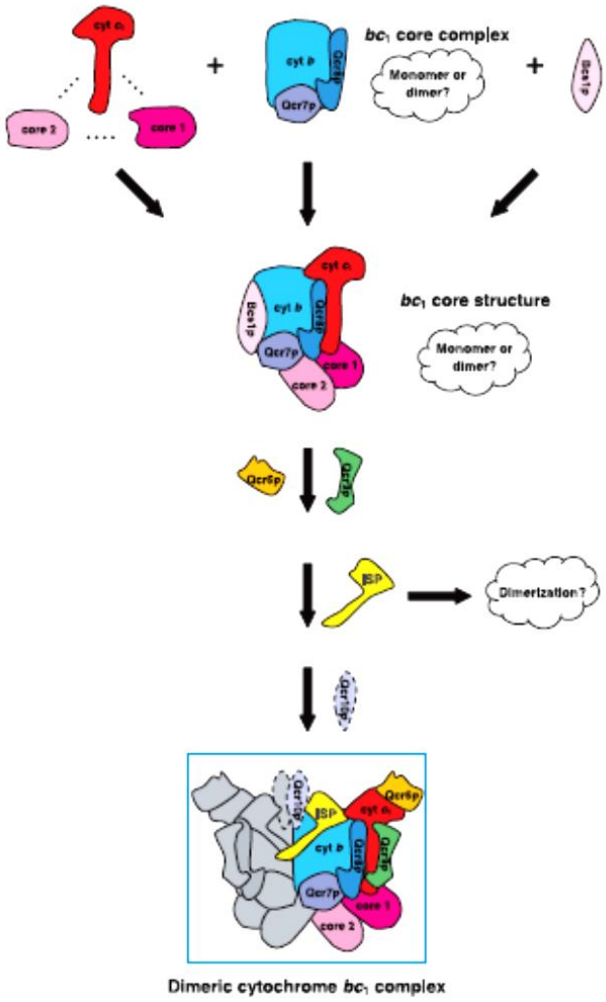


Figure 1.9. Schematic model depicting the putative pathway of assembly of complex of the yeast cytochrome bc1 complex *(Zara et al., 2009)*

So the large *bc1* core structure was capable of binding the chaperone protein Bcs1p. What is known is that Bcs1p is primarily required for the incorporation of Rip1p (ISP), even if further functions cannot be excluded. A possible role of this chaperone in the stabilization of the core structure of the *bc1* complex can be excluded on the basis of the existence of the 500 kDa *bc1* sub-complex also in the *Δbcs1* strain. In addition, the fact

that the molecular mass of the *bc1* sub-complex found in this deletion strain is more or less similar to that of the sub-complex found in all the other deletion strains would suggest that the Bcs1p is present as a monomer in the *bc1* core structure. The role of this chaperone protein has also been investigated in humans, in which molecular defects of *BCS1* are associated with mitochondrial encephalopathy (Fernandez-Vizarra *et al*., 2007). The so formed *bc1* core structure associated with Bcs1p, binds Qcr6p and or Qcr9p and these proteins are not required for the binding of Bcs1p. However both Qcr9p and Bcs1p are required for the subsequent insertion of ISP into the *bc1* sub-complex, but the presence of only one of these two subunits does not substitute for the other. After the addition of the essential ISP, the binding of the last subunit (Qcr10p) finally occurs. These findings are in agreement with the previous studies suggesting that ISP and Qcr10p represent the last subunits incorporated into the *bc1* complex before dimerization (Zara *et al.,* 2009). The dimerization process is still unknown because it is not clear when this process takes place. The 500 kDa complex could represent either a monomeric *bc1* complex in association with various unidentified assembly factors or a dimeric *bc1* complex perhaps associated with monomeric Bcs1p (Smith *et al*., 2011). Recently new complex III assembly factors have been found either in mammals and yeast. *TTC19,* which encodes tetratricopeptide repeat (TPR) domain, has been identified in individuals coming from two families affected by progressive encephalopathy associated with a profound cIII deficiency and accumulation of cIII-specific assembly intermediates (Ghezzi *et al*., 2011). Curious thing is that orthologs to *TTC19* in other eukaryotes (plants or fungi) are not known. On the contrary, the assembly factor Bca1p has been identified only in fungi and controls an early step in complex III assembly. The authors suggested that the supra-molecular organization of Bca1p is dependent upon the assembly level of complex III (Mathieu *et al*., 2011).

- Assembly of complex IV

Mitochondrial cytochrome c oxidase (COX) is a multimeric copper- heme A enzyme of the mitochondrial respiratory chain. It is formed by subunits of dual genetic origin whose assembly, intricate and highly regulated, have been recently reviewed by Barrientos and colleagues. Eukaryotic COX is formed by 11-13 subunits (11 in the yeast *S. cerevisiae* and 13 in mammals) and the catalytic core is formed by three mitochondrial DNA encoded subunits, Cox1, Cox2, Cox3 conserved from prokaryotes to mammals (Soto *et al*., 2011). A significant impact on the study of the assembly of this complex was given by the yeast

S.cerevisiae and by its knockout mutants. However at least one interesting gene (*SURF1/Shy1*) was identified from a molecular genetic analysis of human patients with Leigh Syndrom, a mitochondrial disease discussed further in this dissertation (Scheffler, 2008).

The process of COX assembly has been subject of intense investigations over the last 50 years using different approaches and it is still actively investigated nowadays (Soto *et al.*, 2011). This process seems to be a linear process with the sequential addition of different subunits and cofactors in ordered manner. The concept of a sequential addition was developed in the early 1980s thanks to the data obtained from studies that used rat liver mitochondria and followed the incorporation of radiolabeled subunits into the COX complex (Wielburski and Nelson, 1983). The model was later confirmed by analyses of the human enzyme performed by BN-PAGE (Soto *et al.*, 2011). By analyzing the formation of assembly intermediates, it was concluded that assembly initiates around a seed formed by subunit 1 and proceeds with the formation of several discrete assembly intermediates probably representing rate-limiting steps in the process (Soto *et al.*, 2011). A recently simplified model for the process of COX assembly is represented in fig. 10. After the synthesis of mtDNA subunits forming the core and their insertion in the inner membrane, Cox1p and Cox2p are maturated by the addition of metal cofactors. At some point, substrate-specific chaperones bind Cox1p and Cox2p to maintain them in an assembly-competent state. A specific Cox3p chaperone has not yet been identified. Only after the Cox1p maturation, the nuclear DNA-encoded Cox5p and Cox6p subunits can be added as well as the other core subunits and the rest of the accessory subunits to form the holoenzyme (Soto *et al.*, 2011).

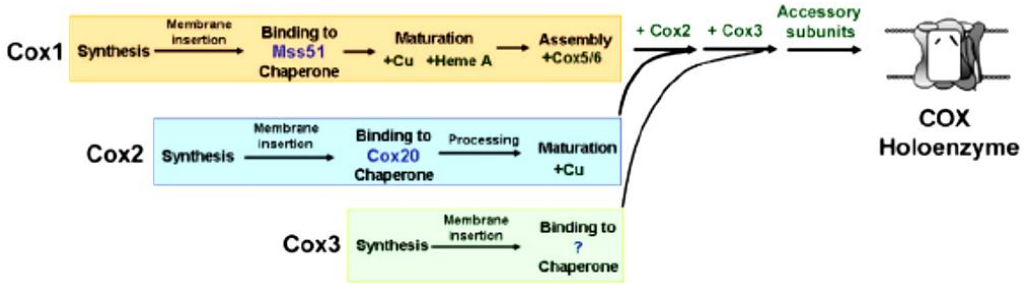


Figure 1.10. Simplified model for the process of COX assembly *(Soto et al. 2011)*.

In yeast, COX assembly requires the assistance of at least 30 nuclear gene products acting at all stages of the assembly process (Fontanesi *et al.*, 2006; Fontanesi *et al.*, 2008) and recent studies suggested that the assembly requires the accumulation of COX

constitutive subunits in a defined stoichiometric ratio (Fontanesi *et al.*, 2011). A list of all genes involved in this biosynthetic process is reported below.

Yeast		Human		Role
Gene	Protein	Gene	Protein	
Catalytic core (mtDNA encoded structural subunits)				
COX1	Cox1	*MTCOX1*	COX1	
COX2	Cox2	*MTCOX2*	COX2	Catalytic core subunits
COX3	Cox3	*MTCOX3*	COX3	
Core protective shield (nDNA encoded structural subunits)				
COX4	Cox4	*COXVb*	COX5b	
COX5a	Cox5a	*COXIV-1*	COX4-1	
COX5b	Cox5b	*COXIV-2*	COX4-2	
COX6	Cox6	*COXVa*	COX5a	Subunits required for COX assembly and function
COX7	Cox7	*COXVIIa*	COX7a	
COX8	Cox8	*COXVIIc*	COX7c	
COX9	Cox7a	*COXVIc*	COX6c	
–	–	*COXVIIb*	COX7b	
–	–	*COXVIII*	COX8	
COX12	Cox9	*COXVIb*	COX6b	Non-essential subunits
COX13	Cox10	*COXVIa*	COX6a	
Expression of catalytic core subunits				
MSS116	Mss116	–	–	Helicase involved in splicing of all *COX1* and *COB* introns Component of the RNA degradosome
SUV3	Suv3	–	–	Helicase involved in *COX1* ai5β intron splicing Stability of intron-containing *COX1* and *COB* transcripts Component of the RNA degradosome
MRS1	Mrs1	–	–	Required for *COX1* ai5β intron splicing Required for excision of the *COB* bI3 intron
MNE1	Mne1	–	–	Required for *COX1* ai5β intron splicing
MSS18	Mss18	–	–	Required for *COX1* ai5β intron splicing Additional unidentified function
COX24	Cox24	–	–	Required for splicing of ai2 and ai3 *COX1* introns Required for *COX1* mRNA translation
NAM2	Nam2	–	–	Required for *COX1* ai4 intron splicing
CCM1	Ccm1	–	–	Required for *COX1* ai4 intron splicing
PET309	Pet309			Yeast: translational activator of *COX1* mRNA
		LRPPRC	LRPPRC	Human: mitochondrial mRNA stability
MSS51	Mss51	–	–	Translational activator of *COX1* mRNA Cox1 chaperone required for its stability/ maturation/assembly
YGR021w	Ygr021w			Yeast: no role on COX biogenesis
		TACO1	TACO1	Human: *COX1* mRNA translational activator
PET111	Pet111			Translational activator of *COX2* mRNA
PET54	Pet54	–	–	Translational activator of *COX3* mRNA Required for *COX1* ai5β intron splicing and translation
PET122	Pet122	–	–	Translational activator of *COX3* mRNA
PET494	Pet494	–	–	Translational activator of *COX3* mRNA
Membrane insertion and processing of catalytic core subunits				
OXA1	Oxa1	*OXA1*	OXA1	Membrane insertion of COX subunits, cytochrome *b* and ATPase proteolipid
COX20	Cox20	*COX20*	COX20	Cox2 chaperone. Preservation of Cox2-precursor to the IMP complex
COX18	Cox18	*COX18*	COX18	Export of the Cox2 C-terminus tail
MSS2	Mss2	–	–	Export of the Cox2 C-terminus tail
PNT1	Pnt1	–	–	Export of the Cox2 C-terminus tail
IMP1	Imp1	–	–	Responsible for the maturation of precursor Cox2
IMP2	Imp2	*IMMP2L*	IMMP2L	Required for the stability and activity of Imp1
SOM1	Som1	–	–	Third component of the yeast IMP complex. It could play a role in substrate recognition
Copper metabolism and insertion into catalytic core subunits				
COX17	Cox17	*COX17*	COX17	Delivery of copper to Scol and Cox11
SCO1	Sco1	*SCO1*	SCO1	Transfer of copper to COX and/or reduction of cysteine residues in subunit 2
		SCO2	SCO2	
COX11	Cox11	*COX11*	COX11	Stable formation of the Cu_B and Mg centers
COX19	Cox19	*COX19*	COX19	
COX23	Cox23	*COX23*	COX23	
PET191	Pet191	*PET191*	PET191	CX_9C proteins. They could play roles in redox control and copper trafficking in the intermembrane space
CMC1	Cmc1	*CMC1*	CMC1	
CMC2	Cmc1	*CMC2*	CMC2	
Heme A biosynthesis				
COX10	Cox10	*COX10*	COX10	Farnesylation of protoheme
COX15	Cox15	*COX15*	COX15	Conversion of heme *o* to heme *a*
YAH1	Yah1	*FDX2*	FDX2	Collaborates with Cox15 in heme *o* hydroxylation
ARH1	Arh1	*ADR*	ADR	Collaborates with Cox15 in heme *o* hydroxylation
Assembly/unknown				
COX16	Cox16	*COX16*	COX16	Unknown function
PET117	Pet117	–	–	Unknown function
PET100	Pet100	–	–	Formation of assembly intermediates containing Cox7, Cox8, and Cox9
SHY1	Shy1	*SURF1*	SURF1	Catalyzes an assembly step involving Cox1
COX14	Cox14	–	–	Binds Cox1 and is required for its stability/maturation/assembly
COA1	Coa1	–	–	Binds Cox1 and is required for its stability/maturation/assembly
COA2	Coa2	–	–	Required for Cox1 stability/maturation/assembly
COA3/	Coa3	–	–	Binds Cox1 and is required for its stability/maturation/assembly
COX25	Cox25			
CMC3/	Cmc3	–	–	CX_9C protein involved in late stages of COX assembly
COA4	Coa4			

Figure 1.11. Homologue COX subunits and COX assembly factors in the yeast *Saccharomyces cerevisiae* and human *(Soto et al., 2011)*.

1.4. Supercomplex organization state of the OXPHOS system

The OXPHOS complexes I-IV and complex V are multisubunit enzymes which are embedded in the lipid bilayer of the inner mitochondrial membrane. The use of Blue Native gel electrophoresis allowed to understand better how these complexes are organized in the membrane. Indeed the OXPHOS complexes besides their expected position as "monomer" in the gel migrate also as bands of higher molecular weight. This indicated that the mitochondrial complexes of the OXPHOS system physically interact with each other to form supramolecular structures in the inner mitochondrial known as supercomplexes or respirasomes (Wittig *et al.*, 2006; Bultema *et al.*, 2009; Strecker *et al.*, 2010). It is thought that since the electron transport chain is one of the major contributors to free radicals in the cell, respirasomes could minimize the generation of reactive oxygen species (ROS) by allowing a more efficient electron transfer and substrate channeling among the complexes avoiding thus the diffusion of reactive intermediates (Genova *et al.*, 2008; Lenaz and Genova, 2010). The assembly of supercomplexes has been observed in a wide variety of organisms, including bacteria, plants, fungi, and mammals, although their composition might vary from organism to organism (Diaz *et al.*, 2012). Mammalian supercomplexes are composed mainly by CI, CIII, and CIV in different stoichiometries (I/III, I/III_2, I_2III_2, I/III/IV, I/III_2/IV, I/III/IV_2, III/IV, and III_2/IV_{1-2}) (Schägger and Pfeiffer, 2001; Wittig *et al.*, 2006). In yeast *S.cerevisiae* where complex I is absent, complex III and complex IV have been found in organized supramolecular structures (Stuart, 2008). In particular *bc1* is capable of forming three complexes: its dimeric form and supercomplexes with either one or two monomers of complex IV (III_2,III_2-IV, III_2-IV_2) (Smith *et al.*, 2011). It is thought that the aggregation state of these complexes is influenced by the lipid composition rather than phospholipids amount of the inner mitochondrial membrane. Indeed it was shown that cardiolipin (CL) is specifically required for super-complex association (Lenaz *et al.*, 2010). Studies in a CL-lacking yeast mutant demonstrated that the III_2-IV_2 super-complex was significantly less stable than supercomplexes in the parental strain (Pfeiffer *et al.*, 2003; Zhang *et al.*, 2003). It is suggested that the putative protein–protein interaction of COX and Complex III involves CL and phosphatidyl ethanolamine which is tightly embedded in the cIII (Lange *et al.*, 2001). In addition, a subpopulation of the cIII-COX complex has been found to interact with TIM23 machinery and also to associate with the Shy1 and Cox14 proteins (van der

Laan *et al.,* 2006; Mick *et al.*, 2007; Saddar *et al.*, 2008; Stuart, 2009). In yeast another supercomplex described is the dimeric ATP synthase complex.

1.5. OXPHOS Defects

In the late 1980s, human pedigrees with mtDNA mutations and a phenotype were discovered and the term "mitochondrial disease" was introduced (Scheffler 2008). Today the term "mitochondrial diseases" indicates a vast group of clinical phenotypes characterized by biochemical and genetic abnormalities of oxidative phosphorylation (OXPHOS) (Zeviani and Carelli, 2007). Actually all the diseases with impaired mitochondrial activities are considered mitochondrial diseases.

There are a number of genetically determined abnormalities of mitochondria that cause human diseases. The mitochondrial genome accumulates mutations at a significantly faster rate compared to the nuclear genome and several factors contribute to this higher mutational rate: the absence of protective histones, the lack of effective repair mechanisms, the high mtDNA replication rate increasing the likelihood of errors and the close proximity of mtDNA molecules to the respiratory chain complexes where they are exposed to high levels of reactive oxygen species (ROS) (Howell *et al.*, 1996; Jazin *et al.*, 1998; Raha and Robinson, 2000; Yu-Wai-Man *et al.*, 2011).

Since there are a large numbers of copies of mtDNA within the cells (from 1000 to 100000 depending on the cell type), these can be identical in a healthy individual at birth, a condition known as homoplasmy (Chinnery, 2003). By contrast, two populations of mtDNA molecules may also be present, one being normal (wild type) and the other being mutated (Scheffler, 2008) and in this case the definition is heteroplasmy (Holt *et al.*, 1988). Single cell studies have shown that the proportion of mutant mtDNA must exceed a critical threshold level before the cell expresses a biochemical defect of the mitochondrial respiratory chain (Schon *et al.,* 1997). This threshold level varies from tissue to tissue and partly explains the tissue-selectivity seen in mitochondrial disorders (Wallace, 1994). The percentage level of mutant mtDNA can also vary between and within individuals harbouring a pathogenic mtDNA defect and this partly explains the clinical variability that is a hallmark of mtDNA disorders (Macmillan *et al.*, 1993).

Mitochondrial disorders usually involve organs that are heavily dependent upon the energy produced by mitochondria such as brain, peripheral nerves, limb, muscles, heart and hormone-producing glands. These disorders can cause muscle weakness on its own

and are usually associated with neurological, heart and hormone problems including diabetes (Chinnery *et al.*, 2009). They are usually progressive and often cause significant disability and premature death (Leonard and Schapira, 2000; DiMauro and Schon, 2001). Based upon recent epidemiological studies, mitochondrial disorders affect at least 1 in 5000 of the general population (Smeitink *et al.*, 2006).

Mitochondrial diseases are classified in two distinct groups depending on they are caused by mtDNA mutations or nuclear DNA mutations of genes involved in respiratory chain. As regards the first group, mtDNA mutations are inherited down the maternal line, thus the specific mtDNA disorders either are passed from mother to child or they affect sporadic cases (Chinnery *et al.*, 1998). These disorders may be caused by:

- mtDNA point mutations: are maternally inherited. They include aminoacids substitutions and protein synthesis mutations (tRNA, rRNA, mRNA that are then translated in proteins). Individuals with point mutations in their mtDNA are generally heteroplasmic and the mutant DNA is inherited in a variable proportion from the maternal germline. Even if more than one hundred point mutations have been described in association with an extremely heterogeneous spectrum of clinical presentations, only a few of them are frequent and associated with well-defined clinical syndromes. The A3243G mutation in the $tRNA^{Leu}$ gene (UUR) is for example responsible for the mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) syndrome (Goto *et al.*, 1990). It is characterized by stroke like episodes due to focal brain lesions, lactic acidosis and red ragged fibers. Other associated manifestations are headache, vomiting, ataxia, diabetes or cardiomyopathy. Other point mutations associated with MELAS have been reported, although they are much rarer than the A3243G (www.mitopedia.org).

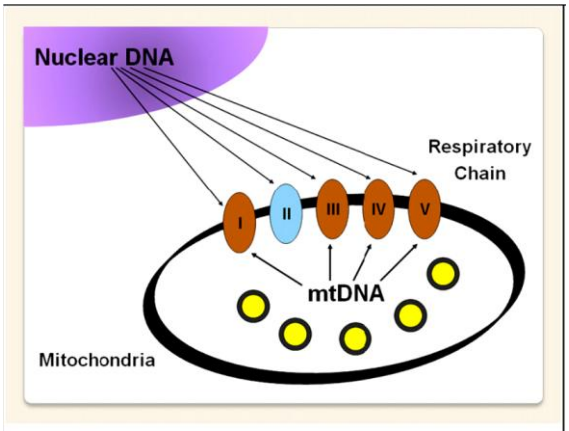
  As regards mitochondrial genes encoding structural proteins, mutations have been reported in Leber's hereditary optic neuropathy (LHON) and in Neurogenic, Ataxia and Retinitis Pigmentosa (NARP)/Leigh syndrome (Ruiz-Pesini *et al.*, 2007). LHON is characterized by adult onset blindness due to retinal ganglia cell death followed by optic nerve atrophy (Wallace *et al.*, 1988). The majority of patients with LHON (90-95%) harbors one of three mtDNA point mutations affecting in subunits of complex I: m.3460G>A in ND1 subunit (Howell *et al.*, 1991; Huoponen *et al.*, 1991), m.11778G>A in ND4 (Wallace *et al.*, 1988) and m.14484T>C in ND6 (Johns *et al.*, 1992; Mackey and Howell, 1992). The m.11778G>A mutation was identified in 1988 by Wallace *et al.*, and it was the first identified mtDNA

substitution confirmed to cause human disease. Other mutations, all present in complex I mtDNA genes, have recently been identified.

NARP or Neurogenic muscle weakness, ataxia, retinitis pigmentosa is a disease caused by T8993G mutation in the gene encoding subunit 6 of mitochondrial ATPase (complex V of the respiratory chain) (Holt *et al.*, 1990). In these patients there is a significant defect in the rate of ATP synthesis. The same mutation when present in >90% of total mitochondrial genomes, leads to the a more severe disease called maternally inherited Leigh syndrome (MILS).

- mtDNA rearrangements: these mutations can be mtDNA deletions or duplications and are associated with severe progressive mitochondrial diseases which are often lethal. They are usually sporadic, heteroplasmic and unique and frequently occur between directly repeated sequences, suggesting that they are caused by *de novo* rearrangements arising during oogenesis or early development (Rötig, 2010). mtDNA deletion are usually associated with Kearns-Sayre syndrome, Progressive external ophthalmoplegia and Pearson's syndrome.
- mtDNA copy number mutations (depletions): these are quantitative reductions of mtDNA amount without mutations or rearrangements of mtDNA. These disorders are usually infantile with a progressive course and are caused by mutations in the nuclear genes such as Thymidine kinase 2 (*TK2*) in the myopathic form, Deoxyguanosine kinase (*DGUOK*) or *POLG* in the hepatic form (Mandel *et al.*, 2001; Saada *et al.*, 2001; Naviaux and Nguyen, 2004). The most involved organs are skeletal muscle and heart, liver and brain (www.mitopedia.org).

The second group is represented by mutations in the nuclear genome. Mitochondria have limited autonomy since they heavily rely on the nuclear genome for their biogenesis. Mitochondrial disorders can therefore arise secondary to both primary mtDNA mutations and nuclear genetic defects which disrupt mitochondrial-related proteins (Yu-Wai-Man *et al.*, 2011).



Respiratory Chain Complex	Gene Location	
	MtDNA	Nuclear DNA
Complex I	7	~ 39
Complex II	0	4
Complex III	1	10
Complex IV	3	10
Complex V	2	14

Figure 1.12. Mitochondrial and nuclear-encoded subunits of the mitochondrial respiratory chain complexes *(Yu-Wai-Man et al. 2011).*

Because of the extreme complexity of OXPHOS and its peculiar genetic organization, the number of genes potentially involved in disease is enormous and tends to coincide with the size of the mitochondrial proteome itself (Calvo *et al.*, 2006). Over 90% of mitochondrial proteins are expressions of nuclear genes and these proteins can be grouped in three categories:

1. structural components of the respiratory chain
2. proteins that control OXPHOS or mtDNA metabolism
3. proteins indirectly correlated to OXPHOS

Nuclear mitochondrial disorders.

Mutations involving structural subunits of the mitochondrial respiratory chain
Leigh syndrome: with complex I deficiency – mutations in *NDUFS1, NDUFS4, NDUFS7, NDUFS8, NDUFV1*; with complex II deficiency – mutations in *SDHA*
Cardiomyopathy and encephalopathy with complex I deficiency – mutations in *NDFUS2*
Optic atrophy and ataxia with complex II deficiency–mutations in *SDHA*
Hypokalaemia and lactic acidosis with complex III deficiency – mutations in *UQCRB*

Mutations involving assembly factors of the mitochondrial respiratory chain
Leigh syndrome–mutations in *SURF1* and *LRPPRC*
Hepatopathy and ketoacidosis – mutations in *SCO1*
Cardiomyopathy and encephalopathy – mutations in *SCO2*
Leukodystrophy and renal tubulopathy – mutations in *COX10*
Hypertrophic cardiomyopathy – mutations in *COX15*
Encephalopathy, liver failure, and renal tubulopathy with complex III deficiency – mutations in *BCS1L*
Encephalopathy with complex V deficiency – mutations in *ATP12*

Nuclear genetic disorders of intra-mitochondrial protein synthesis
Leigh syndrome, liver failure, and lactic acidosis – mutations in *EFG1*
Lactic acidosis, developmental failure, and dysmorphism – mutations in *MRPS16*
Myopathy and sideroblastic anaemia – mutations in *PUS1*
Leukodystrophy and polymicrogyria – mutations in *EFTu*
Encephalomyopathy and hypertrophic cardiomyopathy – mutations in *EFTs*
Oedema, hypotonia, cardiomyopathy, and tubulopathy–mutations in *MRPS22*
Hypotonia, renal tubulopathy, and lactic acidosis – mutations in *RRM2B*

Nuclear genetic disorders of mitochondrial protein import
Mohr–Tranebjaerg syndrome or deafness-dystonia-optic neuronopathy (DDON) syndrome – mutations in *TIMM8A* (*DDP*)
Early-onset dilated cardiomyopathy with ataxia (DCMA) or 3-methylglutaconic aciduria, type V–mutations in *DNAJC19*

Nuclear genetic disorders of mitochondrial DNA maintenance
Chronic progressive external ophthalmoplegia – mutations in *POLG1, POLG2, PEO1, SLC25A4, RRM2B*, and *OPA1*)
Mitochondrial neurogastrointestinal encephalomyopathy – mutations in *TYMP*
Alpers syndrome–mutations in *POLG1* and *MPV17*
Infantile myopathy and spinal muscular atrophy – mutations in *TK2*
Encephalomyopathy and liver failure – mutations in *DGUOK*
Hypotonia, movement disorder and/or Leigh syndrome with methylmalonic aciduria – mutations in *SUCLA2* and *SUCLG1*
Optic atrophy, deafness, chronic progressive external ophthalmoplegia, myopathy, ataxia, and peripheral neuropathy – mutations in *OPA1*

Miscellaneous
Co-enzyme Q10 deficiency – mutations in *PDSS2, APTX, COQ2*, and *ETFDH*
Barth syndrome –mutations in *TAZ*
Cardiomyopathy and lactic acidosis associated with mitochondrial phosphate carrier deficiency – mutations in *SLC25A3*

Alpers syndrome: epilepsy, cortical blindness, micronodular hepatic cirrhosis, episodic psychomotor regression; Barth syndrome: cardiomyopathy, hypotonia, weakness, and neutropenia.

Figure 1.13. Nuclear mitochondrial disorders *(Yu-Wai-Man et al. 2011)*

The most common and well known group of diseases associated with abnormalities in the nuclear genes related to OXPHOS system is the Leigh Syndrome. In about 30% of the cases the biochemical defect is a profound decrease in the activity of Complex IV activity (cytochrome c oxidase, COX) and the genetic alteration is due, in most cases, to mutations in an assembly gene of complex IV, called *SURF-1* (Dahl 1998; Tiranti *et al.*, 1998; Zhu *et al.*, 1998; Tiranti *et al.*, 1999; Borisov 2002). In other cases, the biochemical defect is found in Complex I or Complex II of the respiratory chain. In particular, mutations in the subunits *NDUFS1, NDUFS4, NDUFS7, NDUFS8, NDUFV1* of Complex I and mutations in *SDHA* of complex II have been identified in some patients.

A few mutations of complex III are known. These mutations have been associated with two genes encoding CIII subunits, *UQCRB* and *UQCRQ* (Haut *et al.*, 2003; Barel *et al.*, 2008) and with *BCS1L* gene which is involved in complex III assembly (Tuppen *et al.*, 2010).

Nuclear gene mutations are also associated with multiple mtDNA deletions. Indeed in 2001, the first nuclear genes, *POLG1* and *PEO1*, were identified among families with autosomal - dominant chronic progressive external ophthalmoplegia (CPEO) associated with multiple mtDNA deletions (Spelbrink *et al.*, 2001; Van Goethem *et al.*, 2001). Also the nucleotide transporter *ANT1* has been associated with this disease (Van Goethem *et al.*, 2002). Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an

autosomal recessive disease associated with both deletions and mtDNA depletion due to a mutation in thymidine phosphorylase causing an imbalance in mitochondrial nucleotide pool (Bardosi *et al.*, 1987).

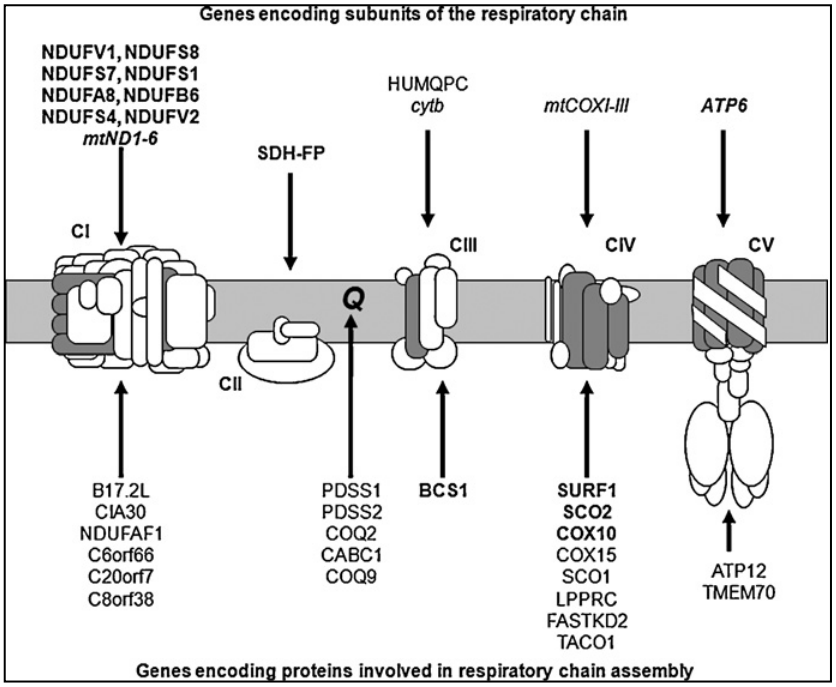


Figure 1.14. Gene mutations resulting in specific respiratory chain complexes *(Rötig, 2010)*

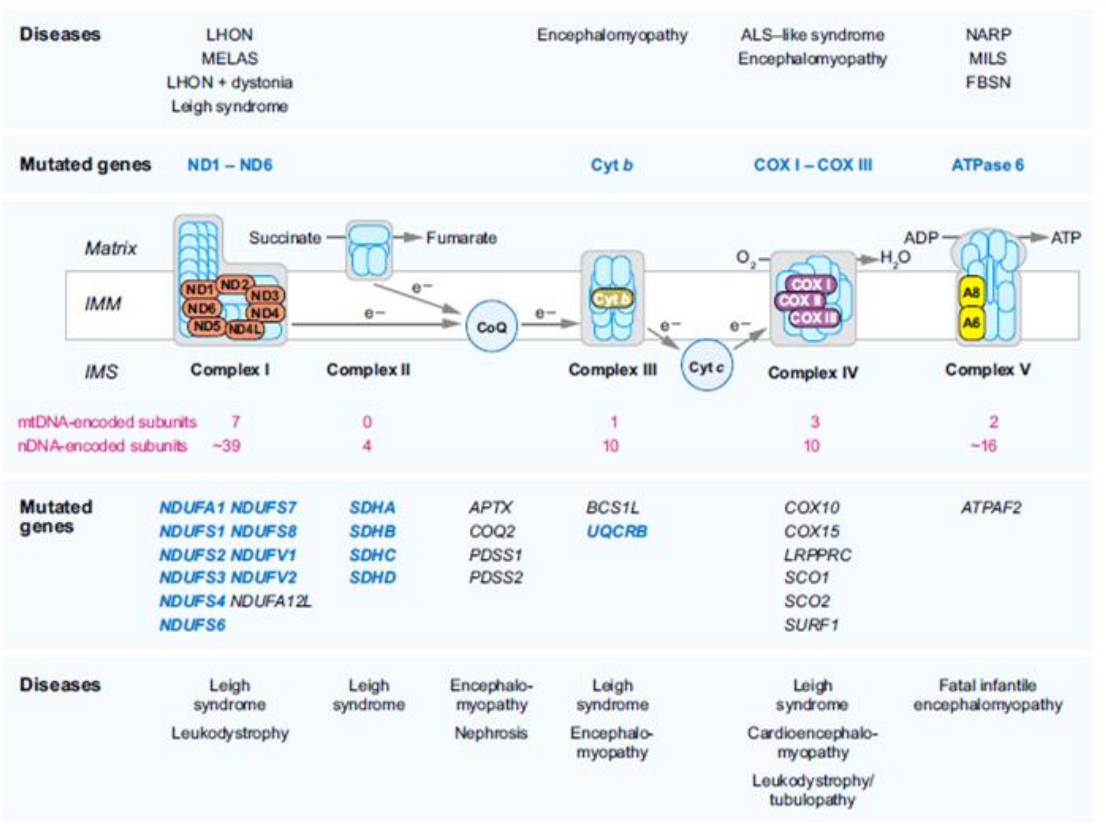


Figure 1.15. The mitochondrial respiratory chain (RC). The diseases caused by mutations in mtDNA (above the RC) and in nDNA (below the RC) are listed according to the correspondingly affected RC complex *(DiMauro and Schon, 2008).*

1.6. Diseases associated with impaired SDH activity

The various nuclear genes encoding RC subunits have all been identified and mapped. The RC complexes contain from four (CII) to 45 subunits (CI) each. Therefore, a functional complex requires structural integrity and tight regulation of each of its subunits (Rötig, 2010). Mutations in one of these genes lead to neurodegenerative diseases. However, mutations in one of subunits of complex II are associated with different diseases not necessarily responsible for neurodegenerative disorders. Indeed, germinal mutations in *SDHA* mutations cause Leigh Syndrome while mutations in the three other genes encoding subunits B, C and D of CII have been reported in hereditary paraganglioma and pheochromocytoma (HPGL/PCC), suggesting that such 'housekeeping' genes may be involved in carcinogenesis (Baysal, 2008). It has been hypothesized that SDH mutations cause an accumulation of succinate and reactive oxygen species (ROS), which could act as downstream signaling molecules to activate hypoxia-inducing pathways (Baysal, 2008). Interestingly, also mutations in genes encoding for the assembly factors, *SDHAF1* and *SDHAF2*, cause both typical mitochondrial diseases and cancer (Ghezzi *et al.*, 2009; Hao *et al.*, 2009).

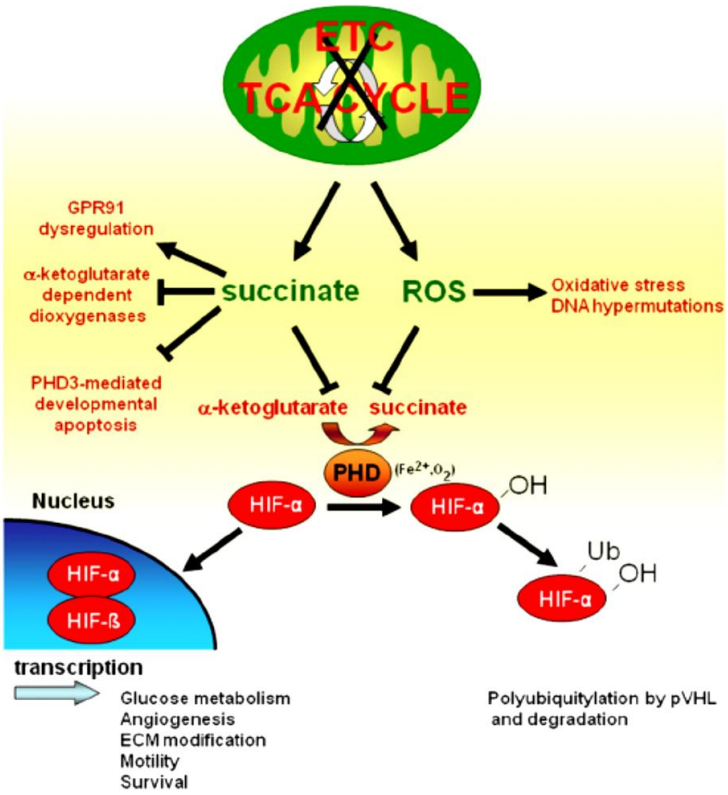


Figure 1.16. Succinate dehydrogenase complex (SDH) in the electron transport chain and Krebs cycle *(Bardella et al. 2011)*

SDHA The first mutation in a nuclear gene encoding a RC subunit was reported in 1995 in two sisters with Leigh syndrome and CII deficiency (Rötig, 2010). The pathogenic mutation was in the *SDHA* gene encoding the flavoprotein of CII (Bourgeron *et al.*, 1995). Mutations in the same gene were subsequently reported in other patients affected by Leigh Syndrome (Parfait *et al.*, 2000; Van Coster *et al.*, 2003; Horvath *et al.*, 2006). Leigh Syndrome is a genetically heterogeneous disease with multiple causes for alteration in mitochondrial function including defect or deficiencies in: electron transport chain Complex I-V, the pyruvate dehydrogenase complex (PDHC), mitochondrial DNA (mtDNA) and mutations in the *SURF1* gene (Rutter *et al.*, 2010). A single case of a pathogenic *SDHA* mutation (c.1664 G>A, p.Gly555Glu) not associated to Leigh Syndrome has been described in a patient with a lethal infantile presentation, which has led to death due to respiratory infection and acute hypoglycemia, before any sign of the syndrome could develop (Van Coster *et al.*, 2003). The same *SDHA* missense mutation, which was reported to cause a multisystemic failure leading to neonatal death (Van Coster et al. 2003) and a relatively mild Leigh syndrome (Pagnamenta *et al.*, 2006), was also described in a familial neonatal isolated cardiomyopathy (Levitas *et al.*, 2010). Recently germline

mutations in *SDHA* have been identified in patients affected by apparently sporadic (para)sympathetic paragangliomas and pheochromocytomas (Korpershoek et al. 2011).
Even if *SDHA* is part of the same complex which comprise *SDHB*, *SDHC*, *SDHD* there are no evidences that mutations in these other SDH genes could lead to neurological disorders. The molecular mechanism causing these diseases is not well understood. Bourgeron *et al.*, reported an increased sensitivity of *SDHA* to the inhibitory effect of oxalacetate and speculated that the mutation could alter SdhAp conformation or redox state which might alter enzyme catalysis and response to oxalacetate (Bourgeron *et al.*, 1995; Rutter *et al.*, 2010).

SDHB (PGL4) The PGL4 syndrome, which was identified for the first time by Astuti in 2001, is due to mutations in the *SDHB* gene which encodes an iron-sulfur protein that together with *SDHA* constitutes the catalytic domain of SDH complex. *SDHB* mutations mainly predispose to extra-adrenal paraganglioma (PGLs) with high malignant potential and to a lesser extent to adrenal pheochromocytomas (PCCs) and head and neck PGLs (Young *et al.*, 2002; Neumann *et al.*, 2004; Benn *et al.*, 2006; Brouwers *et al.*, 2006; Timmers *et al.*, 2007). *SDHB* mutations are some of the most common germline mutations in Familial Paraganglioma Syndrome (FPS) and 98 different alterations have been identified in 216 index cases (Ricketts *et al.*, 2010). The majority of these *SDHB* mutations were missense mutations (46%), followed by frameshift mutations (23%) and splicing mutations (13%). The mean age of PGL diagnosis has been reported from 27.4 (PCCs) to 42.3 (HNPGLs) years old by one study (Ricketts *et al.*, 2010) and ~ 30 years old by another study (Timmers *et al.*, 2007) but there are cases in which the index cases have been diagnosed before 10 years of age (Benn *et al.*, 2006; Mora *et al.*, 2006; Armstrong *et al.*, 2009; Ricketts *et al.*, 2010). This suggests that tumor screening of asymptomatic *SDHB* carriers should start as early as 10 years of age (Bardella *et al.*, 2011).

SDHC (PGL3) The PGL3 syndrome is caused by mutations in the *SDHC* gene. *SDHC* constitutes the membrane domain of complex II together with *SDHD*. The association of *SDHC* (1q21) mutations with PGL3 wad described for the first time by Niemann and Muller in 2000 (Niemann and Müller 2000). *SDHC* mutations were originally believed to be associated only with HNPGLs but recently rare cases of adrenal PCCs and extraadrenal PGLs were reported (Burnichon *et al.*, 2009; Mannelli *et al.*, 2007; Mannelli 2009; Peczkowska *et al.*, 2008; Pasini *et al.*, 2008). Germline *SDHC* mutations appear to be less frequent than *SDHB* and *SDHD* mutations and a limited number of *SDHC* mutation carriers

have been identified worldwide (Niemann and Müller, 2000; Bauters *et al.*, 2003; Niemann *et al.*, 2003; Baysal *et al.*, 2004; Schiavi *et al.*, 2005; Bayley *et al.*, 2006; Mannelli *et al.*, 2007; Peczkowska *et al.*, 2008; Boedeker *et al.*, 2009; Burnichon *et al.*, 2009; Cascón *et al.*, 2009; Neumann *et al.*, 2009). In general, the clinical features of SDHC-associated cases are similar to those found in patients with sporadic HNPGLs. Mutation carriers typically present with solitary head and neck tumors with incomplete penetrance and a very low tendency to malignant transformation (Schiavi *et al.*, 2005). Only a single case of malignant catecholamine-producing carotid body tumor has been reported in a patient with IVS5 +1 G>T *SDHC* mutation (Niemann *et al.*, 2003).

SDHD (PGL1) The hereditary syndrome PGL1 was identified in 2001 by Baysal *et al.*, and is caused by mutations in the *SDHD* gene (11q23) which encodes the other membrane – anchoring subunit of complex II. *SDHD* mutations are typically associated with multifocal HNPGLs and less frequently with adrenal PCCs and extra-adrenal PGLs, which are usually benign (Neumann *et al.*, 2004; Benn *et al.*, 2006; Burnichon *et al.*, 2009). Rare cases of metastatic HNPGLs have been described within *SDHD* mutation carriers and their estimated prevalence is 0-10% (Astrom *et al.*, 2003; Neumann *et al.*, 2004; Amar *et al.*, 2005; Benn *et al.*, 2006; Ogawa *et al.*, 2006; Havekes *et al.*, 2007; Papaspyrou *et al.*, 2008; Burnichon *et al.*, 2009; Ricketts *et al.*, 2010).

Although PCCs and extra-adrenal PGLs are relatively rare in patients with *SDHD* germline mutations, recently Ricketts described that *SDHD* mutations predicted to result in an absent or unstable SdhD protein were associated with an increased risk of PCCs and extra-adrenal PGLs, compared to missense mutations or in-frame deletions, which were not predicted to impair protein stability (Ricketts *et al.*, 2010). The mean age of PGL diagnosis in PGL1 patients ranges from 20.7 (PCCs) to 40.1 (HNPGLs) years old (Neumann *et al.*, 2004; Ricketts *et al.*, 2010). *SDHD*-related diseases have been characterized by a parent-of-origin effect since it is transmitted only when the mutated allele is inherited from the father (Baysal *et al.*, 2002; Neumann *et al.*, 2004; Benn *et al.*, 2006). This pattern of inheritance suggested maternal genomic imprinting (suppression) of this gene. The influence of genomic imprinting in disease susceptibility is well recognized in developmental disorders, such as Prader-Willi/Angelman syndrome (Nicholls and Knepper, 2001) but the mechanisms underlying the imprinted penetrance of *SDHD* mutations (and *SDHAF2* mutations, both genes present on the long arm of chromosome 11), are poorly understood, because this PGL gene does not reside in known imprinted domains and molecular evidence of imprinting has been lacking (Baysal *et al.*, 2011).

However, recently, Baysal and Shadel indicated a mechanism explaining this maternal inheritance and the parent-of-origin-dependent tumor susceptibility through a maternal downregulation of *SDHD*. Indeed, they identified imprinted methylation within an alternative promoter for a large intergenic non coding RNA located at the boundary between the *SDHD* locus and a flanking gene desert. Partial inactivation of *SDHD* by imprinting might provide an advantage for early detection of hypoxia, because PGL tumors, where SDH is completely inactive, show constitutive activation of hypoxia-inducible pathways (Gottlieb and Tomlinson, 2005; Baysal, 2008; Raimundo *et al.*, 2011).

SDHAF2 (SDH5; PGL2) The *SDHAF2* gene has been identified recently by Hao *et al.*, and is known as *SDH5*. It is a susceptibility gene for the PGL2 syndrome and was first described in a previously identified large Dutch kindred with multiple HNPGLs (Baars *et al.*, 1992; Mariman *et al.*, 1995). The position of the involved gene in these affected families was localized by linkage analysis to 11q11.3, but for almost two decades the specific gene remained unknown. This gene encodes a highly conserved protein, necessary for the incorporation of FAD cofactor in the subunit A of the SDH complex. Correct flavination of the *SDHA* subunit is essential for the SDH enzyme activity (Hao *et al.*, 2009). To date *SDHAF2* mutations have been associated to benign, often multifocal head and neck paragangliomas, with young age of onset (Hao *et al.*, 2009; Bayley *et al.*, 2010; Kunst *et al.*, 2011).

SDHAF1 (SDH6) The identification and the diseases associated to this gene, will be discussed in chapter 2 of this dissertation.

	PGL1	PGL2	PGL3	PGL4
SDH Gene	*SDHD*	*SDH5 (SDHAF2)*	*SDHC*	*SDHB*
Chromosomal Location	11q23	1lqll.3	lq21	lp35-36.1
First Described	Baysal et al. (2000)	Hao et al. (2009)	Niemann and Muller (2000)	Astuti et al. (200l)
Most common mutation	frameshift	point	nonsense	missense
HNPGL	++	++	++	+
PCC (any abdominal)	+	–	+/–	++
Catecholamine secreting	+/–	–	+/–	++
Malignant	–	–	Unknown	++
Biochemistry	Structural SDH subunit	SDH assembly factor-FAD insertion	Structural SDH subunit	Structural SDH subunit

"–" = Rare

"+" = Occasionally

"++" = Frequently

HNPGL = Head & Neck Paraganglioma

PCC = Pheochromocytoma

Figure 1.17. Summary of clinical features of the four described familial paraganglioma syndromes *(Rutter et al. 2010)*

Chapter II:

SDH6 a new nuclear gene required for SDH assembly

2.1. The complex II or succinate dehydrogenase (SDH)

2.1.1. The SDH structure

Succinate dehydrogenase (SDH) is a ubiquitous mitochondrial enzyme that catalyzes the oxidation of succinate to fumarate and the generated reducing equivalents are transferred to ubiquinone (coenzyme Q) in the electron transport chain (ETC):

$$\text{Succinate} \leftrightarrow \text{fumarate} + 2\ H^+ + 2\ e^-$$

Also known as complex II its position is unique in energy metabolism since it provides a direct link between the tricarboxylic acid cycle and the respiratory chain and it is the only citric acid cycle enzyme that is membrane-bound. A highly conserved and similar complex is also found as part of the electron transport system in bacterial membranes; *Bacillus subtilis* and *Escherichia coli* have been very useful model systems for the study of the structure, function and assembly of this complex (Scheffler, 2008).

The yeast SDH, like its mammalian counterpart, consists of four subunits (Sdh1p-Sdh4p in yeast, SdhA-SdhD in mammals) all encoded by the nuclear genome.

The fundamental role of succinate-coenzyme Q reductase in the electron transfer chain of mitochondria makes it vital in most multicellular organisms. Removal of this enzyme from the genome has also been shown to be lethal at the embryonic stage in mice. As previously described, human mutations in genes encoding SDH subunits result in different diseases depending on which gene is mutated. In yeast SDH complex is essential for growth on respiratory carbon sources and disruption of any one of the four genes leads to the loss of SDH activity.

The SDH structure (fig.1) consists of a hydrophilic head that protrudes into the matrix compartment and a hydrophobic tail that is embedded within the inner membrane. The hydrophilic head is composed of two subunits forming the catalytic core: *SDH1* and *SDH2* in yeast and *SDHA* and *SDHB* in humans (for simplicity the yeast nomenclature will be used in this dissertation).

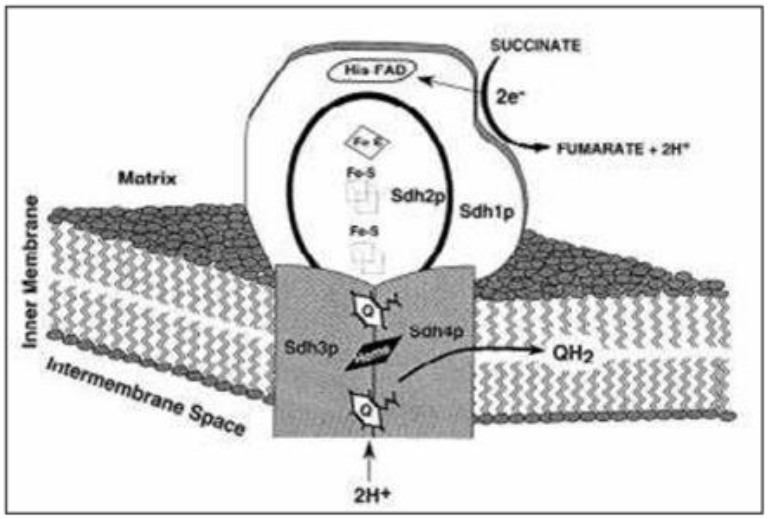


Figure 2.1. Model of the yeast SDH *(Lemire and Oyedotun, 2002)*

The *SDH1* gene encodes the flavoprotein containing subunit (Fp) and is located on chromosome XI. The FAD cofactor is covalently linked to Sdh1p through the HIS90 residue. By mutating His-90 it was shown that covalent flavinylation was not required for SDH assembly to occur but that it was required for activity in succinate oxidation (Robinson and Lemire 1996a; Robinson and Lemire 1996b). Sdh1p with its 70kDa size is the largest polypeptide of complex II and is made as a precursor of 640 amino acids with a cleavable 28 amino acid N- terminal mitochondrial targeting sequence (Robinson and Lemire, 1992; Schülke *et al*., 1992; Bullis and Lemire, 1994). Loss of the *SDH1* gene results in loss of SDH activity. Sdh1p contains also the binding site for succinate.

Sdh2p, together with Sdh1p, is the other catalytic subunit also known as the iron-sulfur subunit (Ip). It contains three non heme Fe-S centers that mediate electron transfer to ubiquinone (Hägerhäll, 1997; Sun *et al*., 2005). These Fe/S centers consist of a 2Fe-2S center proximal to the FAD site, an adjacent 4Fe-4S center followed by a 3Fe-4S center (Yankovskaya, 2003; Sun *et al*., 2005). *SDH2* encodes a polypeptide of 266 aminoacids (30kDa) with a mitochondrial targeting sequence of 20 aminoacids in length. The seven amino acids at the extreme C-terminus of the Sdh2p subunit containing a Lys-Lys sequence, are essential for activity and assembly (Schmidt *et al*., 1992). Sdh2p forms the interface between the catalytic domain and the membrane anchor domain of the complex (Rutter *et al*., 2010).

In the catalytic dimer, as reviewed by Lemire and Oyedotun (2002), the absence of one subunit leads inevitably to the absence of the other suggesting that dimer formation is required for subunit stability (Lombardo and Scheffler, 1989; Robinson *et al*., 1991; Schmidt *et al*., 1992). However, subsequently studies which are discussed in the next

section opened up new perspectives about the stability of catalytic dimer. It is not clear whether the catalytic dimer could be stably formed and could exist in the absence of one or both of the membrane anchors. What is known in literature is that a yeast lacking one of the membrane anchor subunits shows a marked decrease in abundance of both of the hydrophilic subunits, Sdh1p and Sdh2p (Rutter *et al.*, 2010) and this is in agreement with experiments performed in our laboratory (data not shown).

From succinate the electrons flow to the FAD and sequentially through the 2Fe/2S, the 4Fe/4S and the 3Fe/4S clusters and then pass into the membrane dimer (Lemire and Oyedotun, 2002).

The membrane heterodimer is composed of two hydrophobic subunits, Sdh3p and Sdh4p, small peptides of 15kDa and 12-13kDa in mammals and 22kDa and 20kDa in yeast, respectively. Sequence analysis suggested that each subunit has three transmembrane segments along with a N-terminal domain extending into matrix where are localized the peptidase that cleaves this segment (Scheffler, 2008) (fig.2).

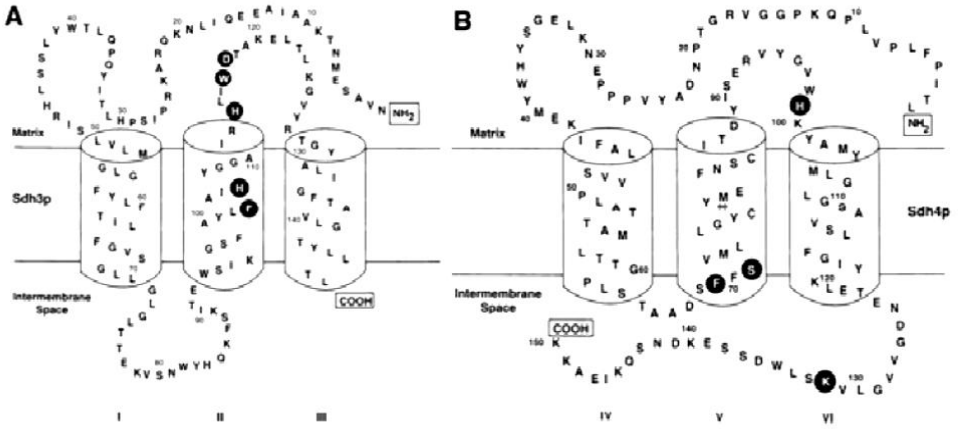


Figure 2.2. Topological models of the membrane subunits.(A) Sdh3p model is indicated and the three transmembrane segments are referred to as I-III. (B) Sdh4p model with the three transmembrane segments IV-VI. *(Lemire and Oyedotun, 2002)*

The membrane domain contains a bound heme *b* moiety and the binding sites for ubiquinone. Unlike the catalytic dimer, whose subunits exhibit remarkable evolutionary conservation in aminoacid sequences and in cofactor composition across species, the membrane anchor subunits vary considerably in subunit composition, subunit primary structure and in heme content (Hägerhäll and Hederstedt, 1996; Hägerhäll, 1997). The currently accepted model for the membrane dimer in all SDH enzymes has a core of four anti parallel helices and is based and supported by two crystal structures (Iverson *et al.*, 1999; Lancaster and Kröger, 2000) (Fig. 3).

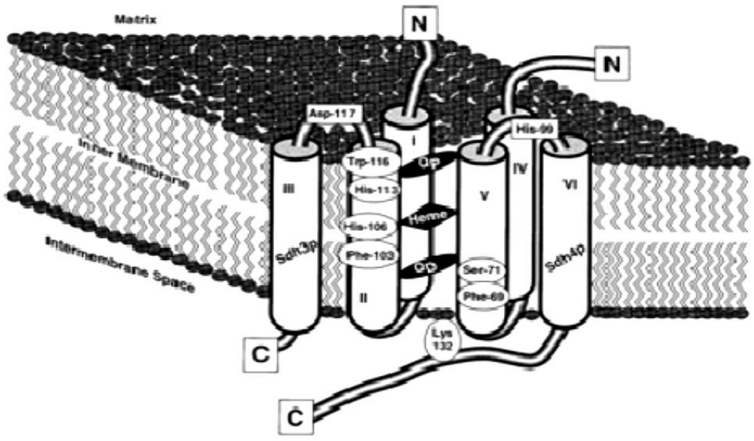


Figure 2.3. Structural model of the membrane dimer *(Lemire and Oyedotun, 2002)*

According to this model, Sdh3p contains three transmembrane helices numbered I-III while Sdh4 helices are numbered IV-VI. Transmembrane helices I,II, IV and V form an antiparallel helix bundle with helices III and IV dangling by the sides (Lemire and Oyedotun, 2002). Several observations with the yeast SDH suggested that transmembrane helices III or VI are not dispensable and that they may be necessary for providing rigidity or stability to the helix bundle. It has been proposed that helices III and VI may serve as hydrophobic zippers to stabilize the helix bundle (Hägerhäll and Hederstedt, 1996). Mutations that truncate Sdh3p or Sdh4p and remove helices III or VI severely impair respiratory growth and enzyme activity but do not lead to a complete loss of function (Oyedotun and Lemire, 1999; Oyedotun and Lemire, 2001). This suggests that helices III and VI are not required for catalysis but rather play roles in maintaining the structural integrity of the membrane dimer (Lemire and Oyedotun, 2002).

Furthermore, Sdh4p contains a hydrophilic carboxylterminus of about 30 amino acids that extends into the intermembrane space which is absent in any other known SDH (Lemire and Oyedotun, 2002). The complete deletion of this region does not impair the respiratory growth and therefore it is not implied in the reduction of quinone. It rather stabilizes the helix bundle of the membrane dimer and affects the enzyme conformation.

Two ubiquinone binding sites have been identified in SDH complex in mammals and in *E. coli* (Yankovskaya 2003; Sun *et al.*, 2005). The high affinity ubiquinone site (Qp-proximal) lies on the matrix side of the IM and is formed by residues in Sdh2p, Sdh3p, Sdh4p. The Qp site lies within 7Å to the 3Fe-4S redox center and is the dominant ubiquinone site in yeast SDH (Lemire and Oyedotun, 2002; Silkin, 2007). The second low affinity ubiquinone site (Qd-distal) resides closer to the IMS side of the IM. Ubiquinone

reduction occurs in two stepwise single electron reactions, in contrast to the two electron reduction of FAD. The Qp site is effective in stabilizing the partially reduced semiquinone thereby permitting its full reduction to the ubiquinol and reducing the ROS generation (Yankovskaya, 2003). Protonation of ubiquinol is likely attained by a conserved Tyr residue in the Qp pocket (Silkin *et al.*, 2007).

The heme moiety, associated with Sdh3p and Sdh4p, is present in mammalian, yeast and *E. coli* SDHs. However the number of heme moieties and their redox properties vary in different species. This is consistent with the observation that membrane domain subunits show greater variability between SDHs than the highly conserved catalytic core domains (Hägerhäll, 1997). All SQRs characterized use two His residues for heme coordination (Hägerhäll, 1997; Lancaster and Kröger, 2000; Yankovskaya, 2003; Huang *et al.*, 2005; Sun *et al.*, 2005). The *S. cerevisiae* SDH is unusual because it uses a Cys residue as one of the heme axial ligands (Oyedotun *et al.*, 2004). The two *S.cerevisiae* SDH heme axial ligands are: Sdh3p His-106 and Sdh4p Cys-78. Mutations in either of these ligands produce an enzyme that retains a significant content of heme but with altered spectral properties. In addition a heme free SDH of a *S.cerevisiae* mutant, without both axial ligands, is capable of an efficient assembly, of supporting growth on a non-fermentable carbon source and of succinate-dependent quinone reduction. Heme b therefore confers some structural stability to the enzyme but it is not an obligatory requirement for electron transfer from succinate to ubiquinone (Oyedotun *et al.*, 2007). The significance and the role of the conserved heme moiety in eukaryotic SDHs is still unclear. The heme *b* is not necessary for the reduction of ubiquinone at the Qp site but it may mediate electron transfer to the distal Qd site (Rutter *et al.*, 2010) and protect against oxidative damage by serving as electron sink (Yankovskaya, 2003). Baysal and colleagues identified mutations in the *SDHD* gene in individuals affected by paraganglioma and one of these mutations was the His102 residue substituted with a leucine (Baysal *et al.*, 2000). They speculated that heme in SDH could play a role in oxygen sensing and in hypoxia response in paraganglioma tissues (Baysal *et al.*, 2000). This histidine residue is located in an analogous position to His-71, a heme ligand in the *E.coli SDHD* (Vibat *et al.*, 1998).

Furthermore, in the Saccharomyces Genome Database (SGD) are present three intriguing entries. One is *SDH1b*, a gene located on chromosome IX and able to rescue only in multicopy the respiratory growth defect of *Δsdh1* (Colby *et al.*, 1998). This result suggested that *SDH1b* is unlikely to play an important role in mitochondrial respiration. The other two entries are represented by Sdh3p and Sdh4p paralogs. *SDH3* paralog is

YMR118c and is located on chromosome XIII and its primary sequence is 57% identical to the *SDH3* but its function is still unknown. *SDH4* has two paralogs: *YLR164w* and *YOR297c* which are respectively 52% and 36% identical to Sdh4p. Noteworthy *YLR164w* contains the His -102 residue which is a potential axial ligand for heme (Lemire and Oyedotun, 2002).

2.1.2. The SDH assembly

Assembly of respiratory complexes is mediated by a large number of accessory proteins; despite complex II is the simplest respiratory complex and its structural and catalytic properties are well elucidated, little is known about the assembly mechanism. Intermediates, factors involved, timing as well as the mechanism of the addition of the various prosthetic groups are still poorly understood.

Prior to 2009, only a couple of factors, Tcm62p and Flx1p, were known to be required for SDH assembly. But these factors are either not evolutionarily conserved or only act on SDH assembly indirectly (Rutter *et al.*, 2010). In 2009 two new factors, Sdhaf1p (Sdh6p) and Sdh5p, have been described with dedicated and evolutionarily conserved roles in SDH assembly (Ghezzi *et al.*, 2009; Hao *et al.*, 2009).

***TCM62*:** the gene encoding Tcm62p was originally identified in a screen for mutants specifically lacking SDH activity (Dibrov *et al.*, 1998). Tcm62p is a 572 aminoacids (64kDa) polypeptide and has 16-17.3% sequence identities, distributed along the entire protein sequence, to *E.coli* GROEL and yeast Hsp60 respectively (Dibrov *et al.*, 1998). The Tcm62p amino terminus is in the mitochondrial matrix, whereas the carboxyl terminus is accessible from the intermembrane space. The *Δtcm62* mutant had normal levels of components of complexes III, IV and V but undetectable Sdh2p. Overexpression of *TCM62* results in the production of intramitochondrial inclusion bodies that also trap Sdh1p and Sdh2p subunits (Dibrov *et al.*, 1998). According to this result, the authors proposed that Tcm62p functions as a chaperone in the assembly of yeast SDH. However, its role as well as its biochemical function are still unknown. Dibrov and colleagues speculated that Tcm62p might be involved in the formation or insertion of the clusters into apo- Sdh2p or, alternatively, might stabilize the holo-Sdh2p subunit before it forms a stable complex with the Sdh1p subunit (Dibrov *et al.*, 1998). Afterwards Langer and colleagues showed that Tcm62p is required for thermostability of mitochondrial respiratory function and ensures mitochondrial gene expression at elevated temperatures preventing heat-aggregation of the ribosomal subunit Var1p (Langer *et al.*, 2001). Var1p

is the only soluble protein encoded by the mitochondrial genome. At 24°C it was predominantly in the soluble fraction in both wild type and *tcm62* strains. At 37°C however Var1p became insoluble in the *tcm62* mutant but not in the wild type strain (Langer *et al.*, 2001). Therefore, the authors suggested that Tcm62p could act more generally in the SDH assembly by supporting mitochondrial protein stability under stress (Rutter *et al.*, 2010).

FLX1: this gene was originally described by Tzagaloff and colleagues and is required for maintenance of a normal FAD/FMN ratio in mitochondria. However Flx1p role is still unclear and literature of the last six years did not clarify its function. It is clear that Flx1p is a mitochondrial transporter probably dedicated to flavin and it has been shown that in *flx1* mutant the activities of *SDH1* and *LPD1*, which contain FAD, were impaired (Rutter *et al.*, 2010). Hao and colleagues in 2009 observed a very modest decrease of Sdh1 protein levels in the *flx1* mutant but a complete loss of covalent FAD incorporation. Overexpression of *SDH5* which is required for FAD incorporation, was able to partially restore the *SDH1* - FAD covalent interaction that was lost in the *flx1* mutant but not to rescue the growth defect on non fermentable carbon sources (Hao *et al.*, 2009). Therefore the authors speculated that Flx1p is required for FAD incorporation into Sdh1p in a wild-type strain but it is also necessary for additional functions required for respiratory growth thus it being not specific for SDH complex (Hao *et al.*, 2009).

SDH5: it was found recently by Hao and colleagues in a screen for mutants incompetent to grow on glycerol, a non fermentable carbon source. They identified the *yol071* mutant which had an OXPHOS growth defect and an undetectable SDH activity, while the activity of other TCA cycle enzymes and electron transport chain complexes were normal. The SDH complex seemed to be partially assembled in the absence of Yol071p but was unstable. Based on its requirement for SDH function, the authors renamed *YOL071* as *SDH5* (Hao *et al.*, 2009). The key observation that pointed them toward the SDH complex came from purifying the Yol071 protein and discovering that it specifically copurified with Sdh1p (Rutter *et al.*, 2010). In addition, they observed that Sdh5p was completely degraded in the *sdh1* mutant supporting the idea of a Sdh1p-Sdh5p interaction. Moreover overexpression of *SDH5* partially reduced the FAD incorporation defect of *flx1* mutant. Finally and most directly, co-expression of *SDH5* but not *SDH2* with *SDH1* in *E. coli* increased FAD incorporation (Rutter *et al.*, 2010). The authors, therefore, proposed that Sdh5p is a dedicated SDH assembly factor required for the covalent insertion of FAD into the catalytic Sdh1p subunit. In addition, the discovery of *SDH5* led to a further knowledge

about SDH assembly and in particular about the stability of Sdh1/Sdh2 dimer. Indeed if Lemire suggested that the absence of one catalytic subunit determined the absence of the other one, in 2009 Hao *et al*. observed a complete loss of Sdh2p in *Δsdh1* yeast mutant but the presence of Sdh1p in *Δsdh2* mutant (Hao *et al*., 2009). This result suggested that Sdh1p is required for the stability of Sdh2p but no *viceversa*. This can be explained with two hypothetical reasons: Sdh1p might be either in a pre assembled complex or be partially stabilized by other assembly factors or functional partners. For instance Sdh5p was absent in a *Δsdh1* strain but was more abundant in a *Δsdh2* mutant presumably due to enhanced Sdh1/Sdh5 complex formation in the absence of the major Sdh1p partner (Hao *et al*., 2009).

SDHAF1/SDH6: it is the other assembly factor that together with Sdh5p is conserved and has a dedicated role in SDH assembly. In humans it's known as *SDHAF1* and in yeast as *SDH6* (Ghezzi et *al*., 2009). The role of this protein in SDH assembly whose characterization is the subject of this dissertation is not clear. SDHAF1p presents a LYR tripeptide motif, a signature for proteins involved in the biogenesis of Fe/S clusters and for this reason it has been speculated that this protein could be important for the insertion or retention of the Fe-S centers within cII protein backbones.

2.2. Iron-sulfur clusters biogenesis

Iron sulfur clusters are important cofactors utilized by numerous proteins involved in several biological processes such as nucleotide biosynthesis and stability, protein translation, enzyme catalysis and mitochondrial metabolism (Rawat and Stemmler, 2011). Chemically the Fe-S clusters are made of ferrous (Fe^{2+}) and/or ferric (Fe^{3+}) iron and inorganic sulfide ions (S^{2-}) linked together in specific structures and are usually integrated into proteins through coordination of the iron ions by cysteine or histidine residues or by alternative ligands such as Asp, Arg, Ser, CO, CN^- and so on (Lill, 2009). The several structures in which they are organized ranging from the simplest rhombic [Fe2–S2] and cubane [Fe4–S4] forms up to [Fe8–S8] clusters, more complex form found in bacteria and archea (Brzóska *et al*., 2006). Fe-S clusters are therefore ubiquitous cofactors of numerous bacterial and eukaryotic proteins; they were discovered in the early 1960s and the first Fe-S proteins were found indeed in plant and bacterial ferredoxins so as in respiratory complexes I, II and III of bacteria and eukaryotic mitochondria (Lill, 2009). Since 1960 and for several decades chemists and biochemists thought that Fe-S clusters

assembled spontaneously on proteins. Only in the 1990s genetic and biochemical studies revealed that the biogenesis process was very complex and underlined the primary role of mitochondria in it (Scheffler, 2008). Thanks to the researches made in bacteria and in yeast, ample evidences of how this process was tightly regulated and coordinated were provided abolishing the idea of a spontaneous formation *in vivo*. What's more, surprisingly in contrast to the chemical simplicity of Fe–S clusters, their biosynthesis *in vivo* appeared to be a complex but coordinated reaction (Lill, 2009). To date, the most of biogenesis components have been identified and the first insights into the mechanism of biogenesis have been obtained. In this section, the mechanism proposed for the assembly of yeast Fe-S will be discussed. Actually the studies in yeast allowed to obtain a large amount of details regarding eukaryotic mitochondrial Fe-S cluster assembly because of the high correlation and conservation between human and yeast systems and the facility of yeast biology (Rawat and Stemmler, 2011).

As reviewed by Lill, the Fe/S clusters in proteins can serve for several functions that can be grouped into three categories namely electron transfer, enzyme catalysis and regulation (Lill, 2009). The most common function is in the redox processes: indeed this function is based on iron capability to formally switch between oxidative states +2 and +3. Thus Fe-S clusters can serve as excellent donors and acceptors of electrons and the redox potential depends mainly on the protein environment of the clusters. Considering that Fe-S proteins are present in mitochondria, cytosol and nucleus, this discussion will focus mainly on the mitochondrial Iron Sulfur Cluster (ISC) assembly since complexes I, II and III of the electron transfer chain are typical proteins involved in the redox reactions and contain Fe-S clusters.

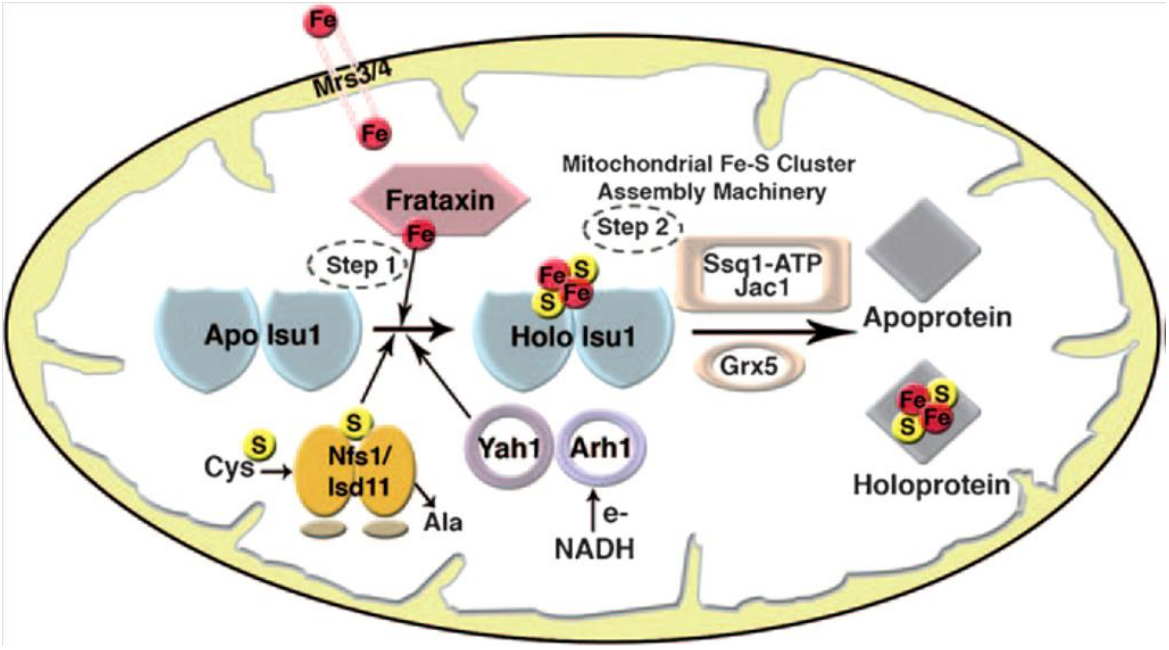


Figure2.4. Model of yeast mitochondrial ISC assembly (step 1) and release(step 2) prior to the cluster being exported from the mitochondria *(Rawat & Stemmler 2011).*

A schematic model of yeast ISC assembly is indicated in the fig.4: the mechanism which is very similar to that of bacteria, is made of two steps, the first based on the cluster synthesis on a scaffold protein and the second based on the transfer of the formed cluster to target apo proteins and its subsequent assembly into the polypeptide chain (Rawat and Stemmler, 2011). All these steps are tightly controlled and regulated since the free iron and sulfur ions are toxic for the yeast cells.

Briefly, in the first step of Fe/S clusters assembly, the cysteine desulfurase Nfs1p functions as sulfur donor releasing the sulphur from cysteine to produce alanine and forms a stable complex with Isd11p. A persulfide intermediate is formed on the conserved cysteine residue of Nfs1p that can be transferred to Fe/S scaffold proteins. The source of iron is still unclear: it has been suggested that iron is imported into mitochondria in a membrane potential proton motive force driven fashion by Mrs3 and Mrs4 (mitoferrin in humans) which are inner membrane proteins (Mühlenhoff *et al.*, 2003). Since it cannot be free in solution, iron is bound by specific proteins which functions as iron donors and mediates the iron delivery to the scaffold proteins. In particular Yfh1p seems to play a role in this mechanism since it functions as iron chaperone protein and binds iron, Nfs1-Isd11 complex and the scaffold proteins (Zhang *et al.*, 2006). In this step the ferredoxin reductase Arh1 and ferredoxin Yah1 are involved since they transfer electrons from NADH or NADPH, needed for the reduction of sulfane (S^0 in cysteine) to sulfide (S^{2-} Fe/S clusters). In this way the cluster is formed on the scaffold protein Isu1 or Isu2 which

contain conserved cysteine residues and bind Fe/S cluster in a labile manner suggesting that this cluster can be transferred to target proteins in the second step of biogenesis process (Lill, 2009; Rawat and Stemmler, 2011).

In the step 2 the labile Fe/S clusters bound to the scaffold are transferred to the apoproteins which are converted from the apo form to the holo form (Lill, 2009). Several proteins are involved in this process and although the mechanism is not yet well defined, four proteins are thought to participate in this transfer: the chaperon protein of Hsp70 ATPase family Sssq1, the DnaJ like co chaperone protein Jac1, the nucleotide exchange factor Mge1 and the monothiol glutaredoxin Grx5 (Rawat and Stemmler, 2011).

All the aforementioned proteins plus other ancillary proteins are known as the ISC proteins and are required for the biogenesis of all mitochondrial Fe/S proteins. Most of these proteins are essential for viability of yeast and human cells. In yeast the depletion of these proteins results in growth defects or death. Several diseases in human are known to be associated with defects in Fe-S protein biogenesis components or Fe-S proteins. An example is given by the depletion of frataxin *FXN* gene (*YFH1* in yeast), the putative iron donor for Fe-S cluster formation, which causes the Friedreich's ataxia, a neurodegenerative inherited disease. Therefore it's evident how the research about these proteins is pivotal since it can provide indications for development of therapeutic strategies in the treatment of Fe-S diseases (Lill, 2009).

2.2.1. Fe-S clusters and SDH

Within the respiratory chain complex I, II and III contain Fe-S clusters as cofactors. In particular, as previously described, the subunit II of SDH complex contains three Fe-S centers (2Fe/2S, 4Fe/4S and 3Fe/4S) and a heme b group. Therefore the correct biogenesis of these centers is necessary for a functional SDH enzyme. How the Fe/S are assembled and integrated in the complex II and which proteins are involved is not known. What is known is that in *S.cerevisiae* frataxin interacts physically with complex II providing electrons to ubiquinone (González-Cabo *et al.*, 2005). Frataxin is a controversial protein conserved throughout eukaryotes which is thought to be an iron chaperone since it is able to bind iron and to promote the assembly of clusters on Nfs1/Isd11/Isu1 scaffold. In *S. cerevisiae* the mutant lacking for frataxin shows a pleiotropic phenotype: up-regulation of the cellular iron uptake system and severe accumulation of iron in mitochondria (Stemmler *et al.*, 2010). In addition this mutant exhibits an impairment of succinate dehydrogenase and aconitase activities, both enzymes which contains Fe-S

clusters. In 2005, Gonzales *et al*., showed the functional relation between SDH complex and *YFH1* through genetic synthetic analysis. They therefore suggested a direct role of frataxin in regulating the entry of electrons toward the ETC. Moreover the authors identified a direct physic interaction between subunit 1 and 2 of SDH with Yfh1p speculating a direct participation of respiratory chain in the pathogenesis linked to the *YFH1* absence (González-Cabo *et al*., 2005).

ISD11 (Lyrm4 in humans) is another important gene linked to the Fe-S biogenesis, conserved from fungi to humans and is essential for cell viability. It was discovered by Wiedemann *et al*. in 2006. Isd11p participates to the first step of Fe-S biogenesis mechanism and binds the cysteine desulfurase Nfs1p and the scaffold protein. Its role is not well established yet. Yeast *ISD11* conditional mutants identified by Wiedemann are characterized by a decrease of aconitase, succinate dehydrogenase (complex II) and cytochrome c reductase (complex III) activities (Wiedemann *et al*., 2006). Isd11p is characterized by the presence of LYR motif (LYK motif) which is a conserved tripeptide that seems to be important for the correct folding and function of the cysteine desulfurase Nfs1p (Adam *et al*., 2006; Shan *et al*., 2007; Wiedemann *et al*., 2006). The presence of Isd11p is therefore required for *in vivo* activity and stability of Nfs1p.

2.3.Background: "*SDHAF1,* encoding a LYR complex-II specific assembly factor, is mutated in SDH-defective infantile leukoencephalopathy" Ghezzi *et al*., Nature Genetics, 2009

As previously mentioned the first *bona fide* SDH assembly factor which has been found is *SDH6* (in yeast) or *SDHAF1* (in humans) (Ghezzi *et al*., 2009). The authors have identified two family sets, one consisting of a large multiconsaguineous kindred of turkish origin with several affected children, the other composed of three affected children originating from a small village in an alpine valley of Lombardy in Italy. Two of the Italian affected children were second-degree cousins, one being born from first-degree cousin parents. Although they were not able to formally ascertain the consanguinity of the other parents and to connect the family of the third child with the other two, they assumed that all affected individuals had inherited by descent the same, presumably homozygous, mutation on the basis of virtually identical clinical presentations and common geographic origin.

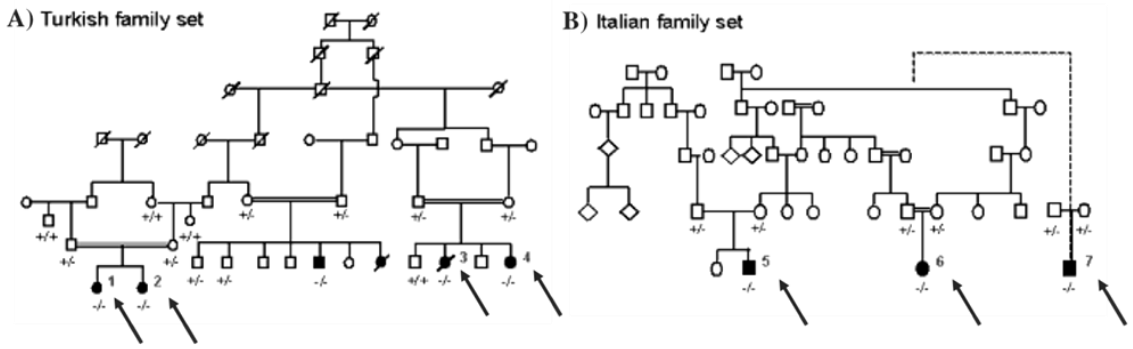


Figure 2.5. Pedigrees of the Turkish and Italian family sets. Red numbers indicate the affected individuals who were studied. Allele genotyping symbols are +/+, for homozygous wild-type individuals, +/- for heterozygous individuals, -/- for homozygous mutant individuals. Black symbols indicate affected subjects. The dotted line indicates that the pedigree relation is suspected but not established (Ghezzi, 2009).

The clinical features of the Turkish and Italian subjects were very similar and accompanied by a significant decrease in SDH activity and by a marked reduction of cII holoenzyme in muscle and fibroblasts.

Symptoms consisted essentially of rapidly progressive psychomotor regression after a 6- to 11-month disease free interval with lack of speech development, followed by spastic quadriparesis and partial loss of postural control with dystonia. Brain magnetic resonance imaging showed severe leukodystrophic changes with sparing of the peripheral U-fibers and basal ganglia. Proton magnetic resonance spectroscopy revealed a decreased N-acetylaspartate signal and abnormal peaks corresponding to accumulation of lactate and succinate in the white matter (Brockmann *et al.*, 2002; Bugiani *et al.*, 2006). Lactate and pyruvate were variably elevated in blood. The subjects underwent relative stabilization of their clinical conditions, with survival beyond the first decade of life in several cases, although their growth was consistently and severely impaired. Biochemical analysis of mitochondrial respiratory chain (MRC) complexes in muscle and fibroblasts showed a ~20–30% residual activity of SDH and SCoQR, whereas the other MRC activities were normal. Protein blot analysis on one- and two-dimensional blue-native gel electrophoresis showed marked reduction of cII holoenzyme in muscle and fibroblasts.

The gene encoding Sdhaf1p was identified through a genome wide linkage analysis using SNP array genotyping performed in the Turkish and Italian family. The authors identified a 1.2 Mb region on chromosome XIX which contained 42 annotations. A single anonymous entry in the region termed *LOC644096*, consisting in a single exon that predicted the translation of a 115 a.a putative protein of unknown function (Fig. 6).

>gi|111038124|ref|NP_001036096.1| hypothetical protein LOC644096 [Homo sapiens]

```
MSRHSRLQRQVLSLYRDLLRAGRGKPGAEARVRAEFRQHAGLPRSDVLRIEYLYR   55
RGRRQLQLLRSGHATAMGAFVRPRAPTGEPGGVGSQPDDGDSPRNPHDSTGAPET  110
RPDGR                                                      115
```

Figure 2.6. Protein sequence of LOC644096. The LYR motifs are indicated in the box and the residues involved in pathological mutations, R55P and G57R, are in grey.

This region contained also two homozygous missense mutations segregating with diseases: G57R in the Italian individuals and R55P in the Turkish ones and in addition the residues involved in these mutations are highly conserved across species (Fig.7).

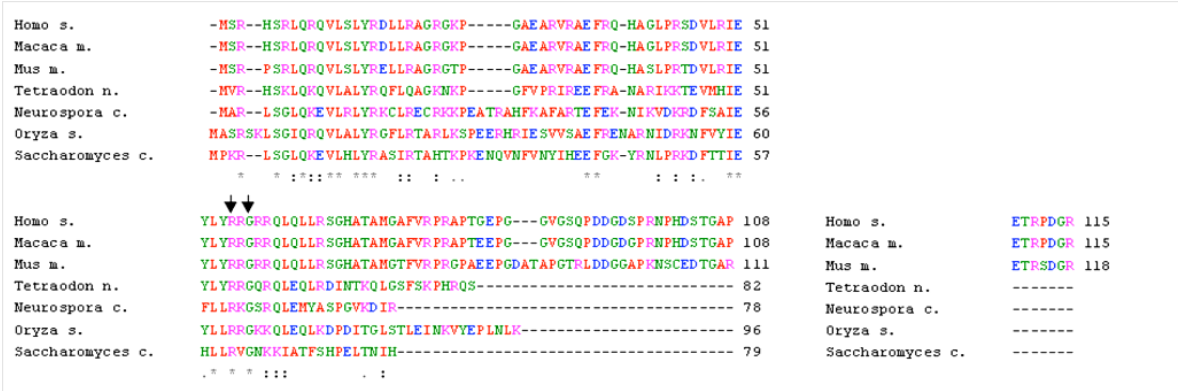


Figure 2.7. Multiple alignment of human SDHAF1 aminoacid sequence with orthologs form monkey (Macaca mulata), mouse (Mus musculus), fish (Tetraodon nigroviridis), fungi (Neurospora crassa), plant (Oryza sativa) and yeast (Saccharomyces cerevisiae), obtained with ClustalW .

Within this aminoacid sequence, there are two LYR tripeptide motifs (Leu- Tyr- Arg) which are conserved and are present in the N-terminal region of several proteins. There are at least eight LYR-motif proteins (LYRM) in humans, including Sdhaf1p. Lyrm-4p is the human ortholog of yeast Isd11p, a protein that, as previously reported has an essential role in the mitochondrial biosynthesis of Fe-S centers. Indeed it forms a complex with the cysteine desulfurase Nfs1p and is required for formation of an Fe/S cluster on the Isu scaffold proteins (Wiedemann *et al.*, 2006). Another example is represented by the two complex I subunits *NDUFA6* (LYRM-6, 14kDa) and *NDUFB9* (22kDa). Both contain the LYR motif but they have different functions (Cardol *et al.*, 2004; Ye and Connor 2000). These data suggest that the LYR motif is a signature for protein involved in Fe-S metabolism. In particular, *NDUFA6*, *NDUFB9* and probably *SDHAF1* itself could be involved in the insertion or retention of the Fe-S centers within protein backbones of cI and cII. In *S. cerevisiae* the proteins that contain LYR motifs are Acn9p, Isd11p, Fmc1p, Sdhaf1p/Sdh6p and Mzm1p, recently identified (Atkinson *et al.*, 2011).

Because the trasfection of fibroblasts with the gene *LOC644096* was not suitable to examine whether the disease-segregating missense mutations of *SDHAF1* were indeed causing cII deficiency, the yeast *S. cerevisiae* was used as a model and a functional

analysis was performed. Yeast possesses a putative *SDHAF1* ortholog*, YDR379c-a* an uncharacterized ORF of 239 bp localized on chromosome IV which encodes a 79 amino acid protein predicted to be localized in mitochondria.

The alignment between *LOC644096* and *YDR379c-a* (Fig.8) shows how the missense mutations are conserved, as well as the LYR motifs, although the identity between the two sequences is 21%.

```
LOC644096       -MSRHSRLQRQVLSLYRDLLRAGRGKPGAEAR-----VRAEFRQHAGLPRSDVLRIEYLY 54
YDR379C-A       MPKRLSGLQKEVLFLYRASIRTAHTKPKENQVNFVNYIHEEFGKYRNLPRKDFTTIEHLL 60
                  .* * **::** ***  :*:.: **  :       :: ** :: .***.*.  **:*

LOC644096       SRGRRQLQLLRSGHATAMGAFVRPRAPTGEPGGVGSQPDDGDSPRNPHDSTGAPETRPDG 114
YDR379C-A       PVGNKKIATFSHPELTNIH---------------------------------------- 79
                * *.:::  :   . * :

LOC644096       R 115
YDR379C-A       -
```

Figure 2.8. CLUSTAL-W Alignment between LOC644096 and YDR379c-a. In grey are pointed out the LYR motifs. The pathological residues in black.

The *YDR379C-A* gene was disrupted in yeast by homologous recombination in BY4742 background. The *Δydr379c-a* yeast strain was OXPHOS incompetent because of a profound and specific reduction of SDH activity ~ 65%, whereas complex IV activity was normal. The more severe phenotype was observed on acetate and this carbon source was chosen for the next experiments. Respiration in standard YNB medium containing 0.6% glucose was only slightly reduced and cytochrome spectra were normal (Fig.9). These results, taken together, suggested that *YDR379c-a* encoded a protein which was specific for complex II.

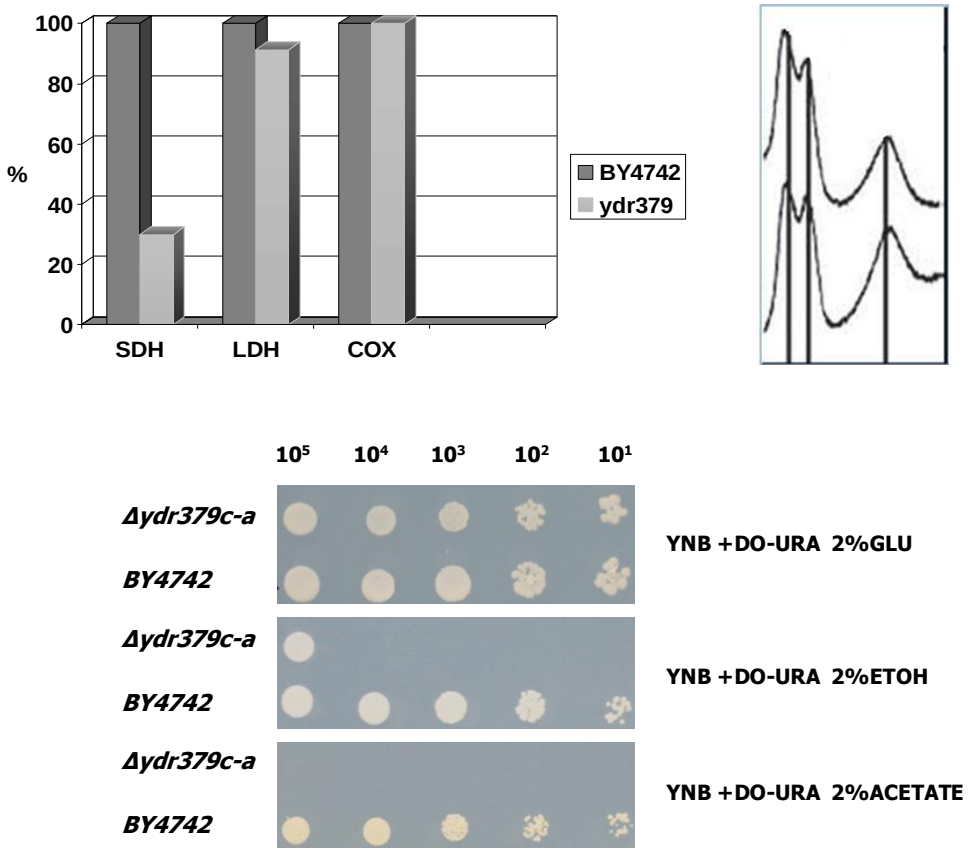


Figure 2.9. Respiratory complex activity and cytochromes spectra. Spot assay at 28°C on glucose, ethanol and acetate-based medium of *Δydr379c-a* and *BY4742* wt.

Transformation with the wild-type *YDR379C-A* restored OXPHOS growth of the *Δydr379c-a* strain. Expression of wild-type human *SDHAF1* failed to complement the yeast mutant strain, possibly because of the low similarity between yeast and human protein species (Fig.10).

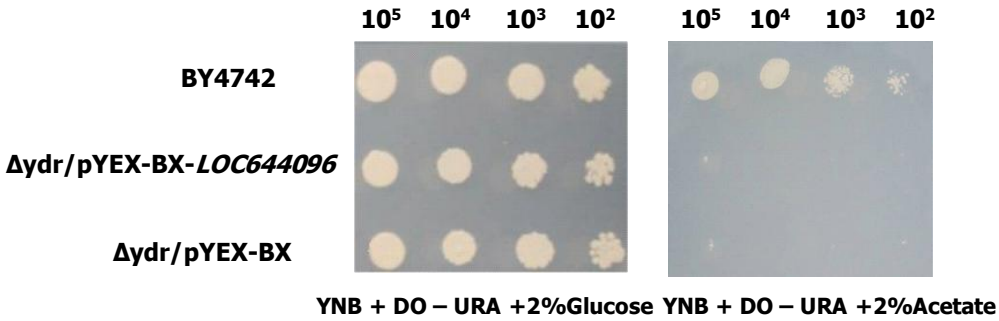


Figure 2.10. Functional complementation with *LOC644096* in *Δydr379c-a.*

Then yeast mutant alleles carrying the equivalent human mutations were created and introduced into the *Δydr379c-a* mutant. The mutant strains behaved like the null mutant and therefore the mutations had a pathological effect in yeast since they did not restore the growth on acetate (Fig.11).

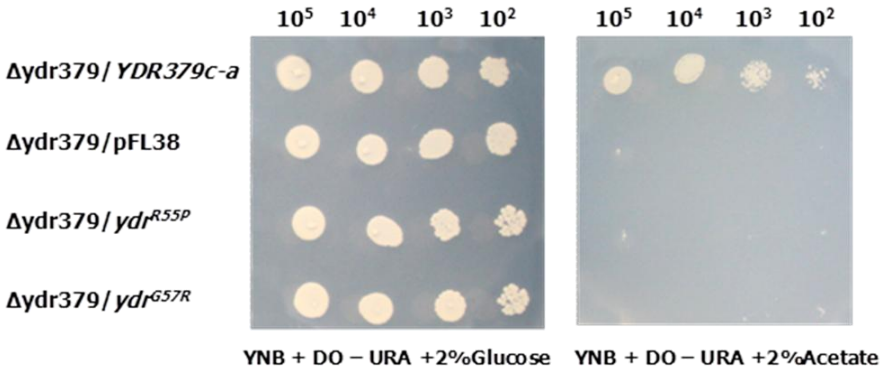


Figure 2.11. Mutant alleles phenotype on acetate and glucose containing plates.

Accordingly, SDH assay on mutant strains carrying the pathological mutations, indicated a specific reduction of the complex II activity. The other components of MRC were intact (Fig.12).

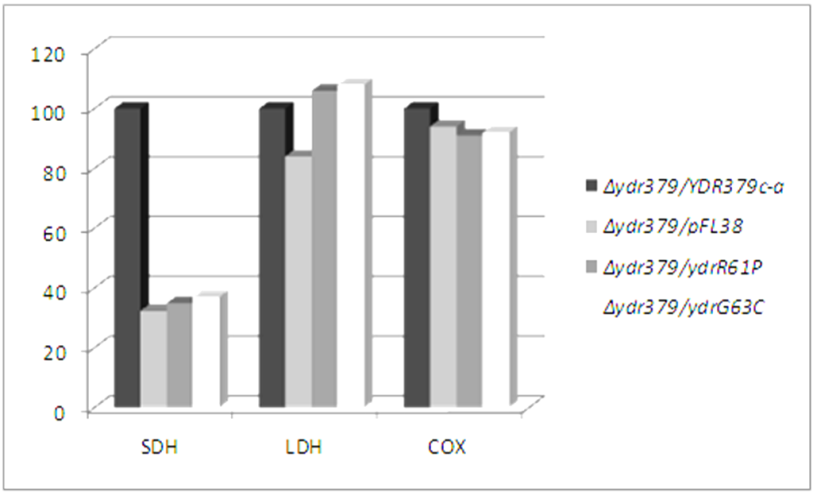


Figure 2.12. SDH assay on mutant alleles *R55P* and *G57R*.

Then the authors measured the apparent Km value for succinate and it was 1 mM in wild-type and 1 mM in the null mutant, suggesting that defective SDH activity was caused by reduced number of enzyme units rather than by qualitative alterations of cII.

Taken together, these results demonstrated that mutations in *SDHAF1* caused an isolated cII defect associated with a specific leukoencephalopathic syndrome and the *SDHAF1* product was the first *bona fide* assembly factor specific to cII, as its loss determined severe reduction in the amount of the enzyme in both yeast and humans. In yeast this gene was renamed *SDH6*.

Even though the experiments carried out in human and in yeast, have indicated that Sdh6p is required for assembly and full function of SDH complex the biochemical function of this protein remains to be elucidated. It could be speculated that *SDH6* gene product interacts with the entire SDH complex or with a specific SDH subunit. The experimental

work described in this thesis has been focused on the biochemical and genetic characterization of *SDH6*.

2.4. Results

2.4.1. Characterization of *Δsdh6* mutant

SDH6 mutations do not affect mtDNA stability

One of the classical analysis carried out on respiratory mutant yeast strains is the evaluation of mtDNA stability. It is known, indeed, that 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, which are referred to as *petites* or rho$^-$ mutants. These mutants do not grow on respiratory carbon sources but they can grow on glucose thanks to the energy produced by fermentation.

In order to evaluate whether the null mutation as well as the two pathological mutations of *SDH6* were linked to an increased mtDNA instability a *petite* analysis was undertaken as described in the material and methods section. The *petite* mutants were counted after 5 days of growth at 28°C. The values indicated below are the mean of five independent experiments.

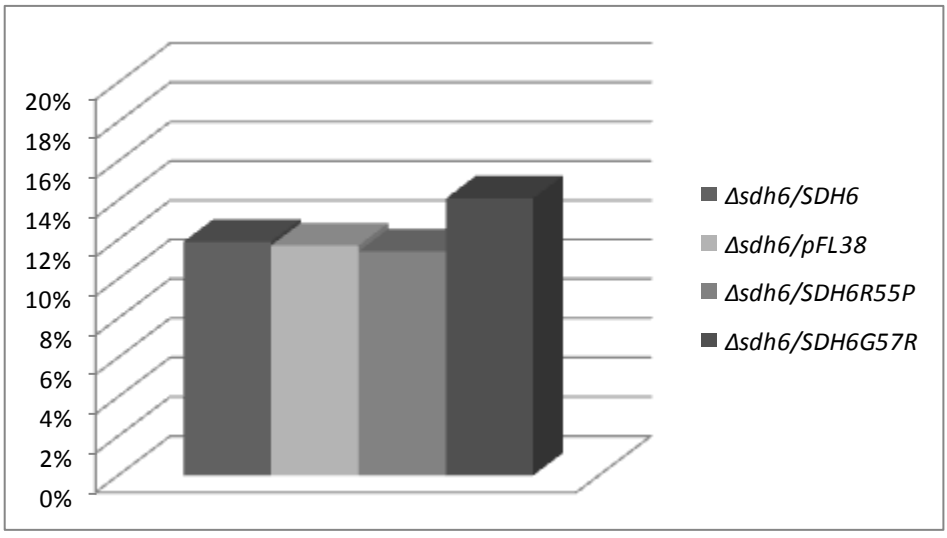


Figure 2.13. Analysis of mtDNA stability. It is indicated the percentage of petite mutants in the several mutants.

As shown in the graphic, the percentage of *petites* was similar in the strains analyzed indicating that neither the absence of Sdh6p nor its mutated forms affect the mtDNA stability.

Induction of SDH6 expression by oxidative carbon sources

The *SDH6* expression was analyzed in several carbon sources and determined by Reverse Transcription PCR (RT-PCR) (Weis et.al, 1992). This method allows to analyze gene expression in different conditions from different samples. It is based on a two steps process: the first step in which mRNAs are transformed in cDNA by reverse transcriptase and the second one in which the obtained cDNAs are specifically amplified with a common PCR reaction.

Total RNA were extracted as described in the material and methods section from the wt strain BY4742 in exponential phase grown in YP medium added with:

- 2%Glucose
- 2%Ethanol
- 2%Acetate
- 2%Glycerol

Then total RNA were quantified and prepared. 2µl of each cDNA samples were used for PCR reaction. The method was first set up with *SDH4* gene. Indeed it is known that SDH genes are induced by ethanol and repressed by glucose. A first reaction of 30 cycles was performed in order to evaluate the effectiveness of the method. This reaction was performed using *SDH4* primers which matched within the ORF region. This analysis was able to point out the induction by ethanol and repression by glucose (data not shown). We then analyzed *SDH6* expression carrying out 25 cycles- PCR amplification by using YDRF and YDRR primers. As loading control *TAF10* was used which encodes a subunit of *TFIID* involved in RNA polymerase transcription initiation. This gene was chosen as suitable internal control (reference gene) as suggested by literature (Teste *et al.*, 2009).

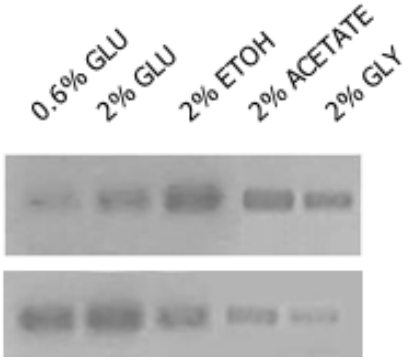


Figure 2.14. Expression of *SDH6* in glucose and respiratory carbon sources. Transcripts of *SHD6* were detected by RT-PCR of total RNA from BY4742 grown in YP added with the different carbon sources. RT-PCR control for all samples is represented by the amplification of *TAF10*. The cells were stocked at 1 OD.

The *SDH6* expression pattern depicted in fig.14 showed a significant increase of *SDH6* expression in non fermentable sources especially in acetate. Furthermore, it was not repressed by glucose where its transcription was basal.

Overexpression of ISD11 does not rescue the OXPHOS defect of Δsdh6 mutant

As previously argued, the SDHAF1 human protein sequence contains two LYR motifs (Leu-Tyr-Arg) and one of these motifs is fully shared with Sdh6p. The other one is still shared but the Tyr-residue is substituted with a Leu-residue. Ghezzi and co-authors speculated that Sdhaf1p could be involved in the insertion of Fe/S clusters within complex II due to the presence of this iron signature but no experiments were made in order to elucidate this putative role. In addition a BLASTP-based search of SDHFA1p in the *Homo sapiens* database showed a significant similarity with Lyrm-4, a protein involved in the iron-sulfur biogenesis whose ortholog in yeast is Isd11p. However, the BLASTP search with Sdh6p as query did not identify any significant protein.

These two yeast proteins, Sdh6p and Isd11p, were aligned (fig.15). The similarity and identity values were respectively 59% and 16%.

```
SDH6       MPKRLSGLQKEVLHLYRASIRTAHTKPKENQVNFVNYIHEEFG-KYRN-LPRKDFTTIEH 58
ISD11      MPGFTAPTRRQVLSLYKEFIKNAN---QFNNYNFREYFLSKTRTTFRKNMNQQDPKVLMN 57
           **  : :::** **: *:.*:  : *: ** :*: .:  .:*: : ::* ..: :

SDH6       LLRVGNKKIATFS-HPELTNIH--------------- 79
ISD11      LFKEAKNDLGVLKRQSVISQMYTFDRLVVEPLQGRKH 94
           *:: .::.:..:. :. :::::
```

Figure 2.15. Alignment between Sdh6p and Isd11p with CLUSTAL-W.

It is worth mentioning that also Isd11p contains a LYK motif within its sequence, a signature similar to LYR motif where the Arg-residue is substituted with a Lys-residue. In addition *ISD11* conditional mutants possess aconitase, succinate dehydrogenase (complex II) and cytochrome c reductase (complex III) activities diminished by 75-95% (Wiedemann *et al.*, 2006).

Since the presence of LYR/LYK motifs and the high protein sequence similarity we wondered whether *ISD11* in multicopy could suppress the OXPHOS defect of *Δsdh6* mutant and *viceversa* whether *SDH6* could rescue the mutant phenotype of *Δisd11*.

The whole *ISD11* ORF was amplified with PCR by using FISD11B and RISD11H primers. The amplified and purified fragment (~900 bp) was digested with *BamHI* and *HindIII* and then was ligated in YEplac195 vector. The recombinant construct was then sequenced.

Δsdh6 mutant was transformed with *ISD11*/YEplac195 and with the empty YEplac195 with the protocol described in material and methods section. Three independent clones were used for spot assays on glucose, ethanol and acetate containing plates. *Δsdh6/SDH6* was also spotted as growth control. The mutant phenotypes were observed after 72h at 28°C.

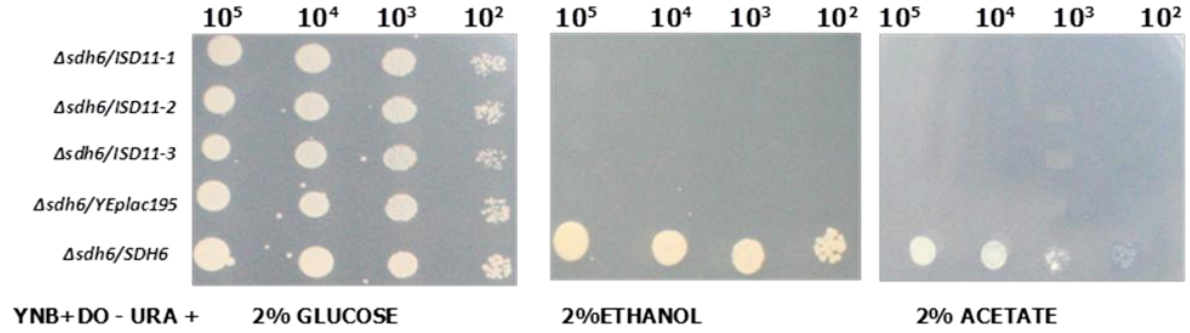


Figure 2.16. Spot assay of three independent clones of *Δsdh6/ISD11* performed on glucose, ethanol and acetate medium at 28°C.

As depicted in fig.16 *ISD11* in multicopy was not able to rescue the growth defect of *Δsdh6* mutant. Therefore the two sequences which shared a sequence similarity did not share a similar function. Since *ISD11* deletion is lethal a diploid *ISD11*/*Δisd11* strain was transformed with wild-type *SDH6* cloned in a multicopy vector (YEplac195/*SDH6*). The transformants, selected on minimal medium without uracile, were sporulated. The tetrad dissection was performed on glucose synthetic medium without uracile to ensure the presence of the plasmid. The viability of all the 4 spores was expected if *SDH6* would have rescued the lethality of *ISD11* deletion. The observation that only two of the four spores were able to germinate led us to conclude that *SDH6* was not able to complement in multicopy the *ISD11* deletion. An analogous experiment was done in order to evaluate the possible interaction between *SDH6* and three other essential genes involved in the mitochondrial ISC biogenesis namely *NFS1*, *JAC1* and *YAH1*. Also in this case we did not observe a complementation by *SDH6*.

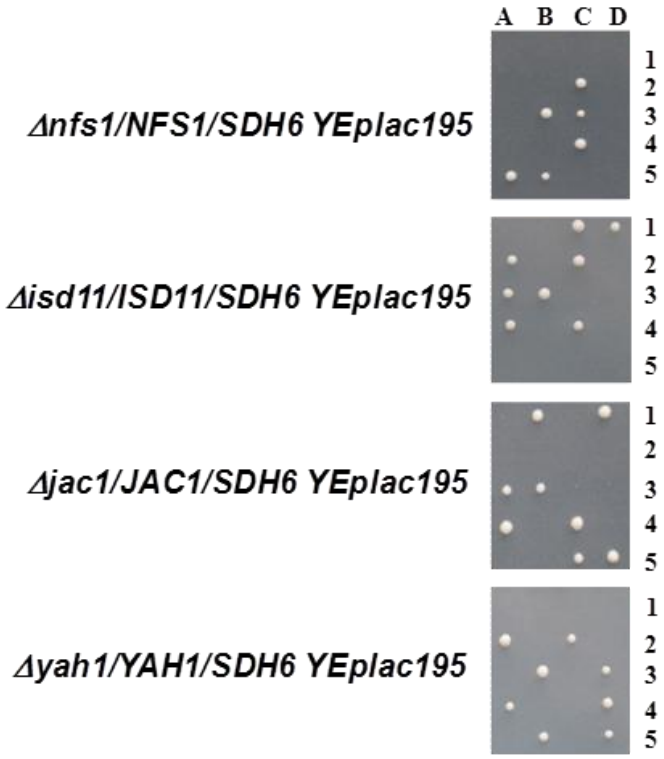


Figure. 2.17. Tetrad analysis performed on *NFS1*, *ISD11, JAC1* and *YAH1* diploids (BY4743) transformed with *SDH6/YEplac195.*

Overexpression of YFH1 does not rescue the OXPHOS defect of Δsdh6 mutant

Another important gene of ISC complex is *YFH1*, encoding for the yeast frataxin homolog. Several physiological functions for frataxin in mitochondria have been proposed. Among these, the most relevant roles are related with the homeostasis of mitochondrial iron: in particular several works speculated that frataxin acts as an iron storage protein maintaining iron in a non toxic and bioavailable form (Adamec *et al.*, 2000; Park *et al.*, 2003; Nichol *et al.*, 2003). What's more, literature data, as described in the previous section, suggested a closely relation between *YFH1* and complex II. In particular Gonzales Cabo *et al.*, through coimmunoprecipitation studies supported by genetic analysis identified a functional relationship between *YFH1* and SDH subunit 1 and 2. Considering the primary role on Fe-S biogenesis and the putative role on the SDH complex, we wondered similarly to *ISD11* whether *YFH1* in multicopy could suppress the OXPHOS defect of *Δsdh6* mutant.

The whole *YFH1* ORF was amplified by using YFH1FBh1 e YFH1RPs primers. The purified fragment (1200 bp) was digested with *BamHI* and *PstI* and then was cloned in YEplac195

vector. The sequenced construct and the empty vector were used to transform *Δsdh6* mutant. Spot assay were performed.

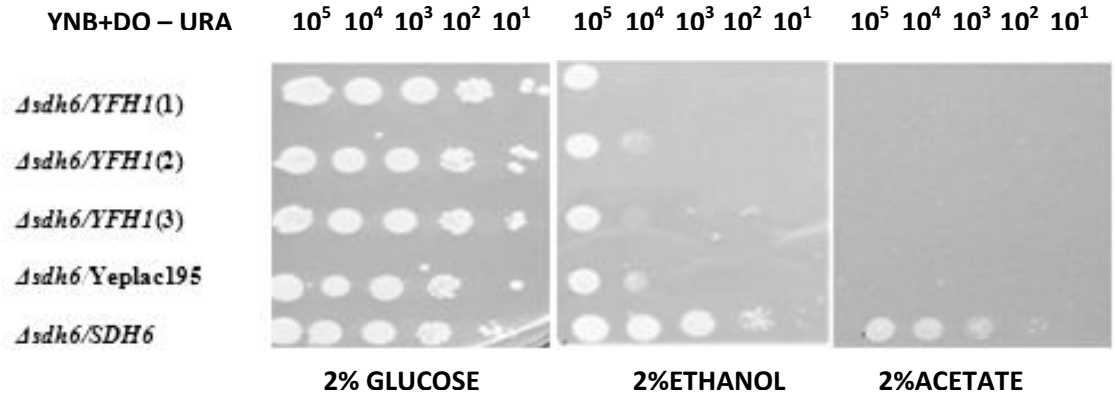


Figure 2.18 . Spot assay at 28°C of three independent clones of *Δsdh6/ISD11.* 10^5, 10^4, 10^3, 10^2, 10^1 cells were plated on glucose and acetate containing plates after serial dilutions.

As indicated in the fig.18, no growth rescue was observed. Further analysis are however necessary to exclude a putative role of Sdh6p with frataxin pathway.

Overexpression of SDH5 and TCM62 does not rescue the OXPHOS growth defect of Δsdh6 mutant

To gain insight into the function of *SDH6*, we analyzed if the other SDH assembly factors, *SDH5* and *TCM62*, could in multicopy rescue the growth defective phenotype of *sdh6* mutant and *viceversa*. Therefore both genes were cloned in YEplac195. *SDH5* was amplified with PCR by using FSDH5B and RSDH5H primers and the purified PCR product was digested with *BamHI* and *HindIII*; subsequently the 1000 bp fragment was cloned in YEplac195. As regards *TCM62*, the ORF region was amplified by using FTCME and RTCMP primers and the amplified fragment (~2700 bp) was digested with *EcoRI* and *PstI* and cloned in YEplac195.

Both the constructs were used to transform the *Δsdh6* mutant. Then a spot assay analysis with the transformant clones was carried out and the *Δsdh6/SDH6* was plated as growth control.

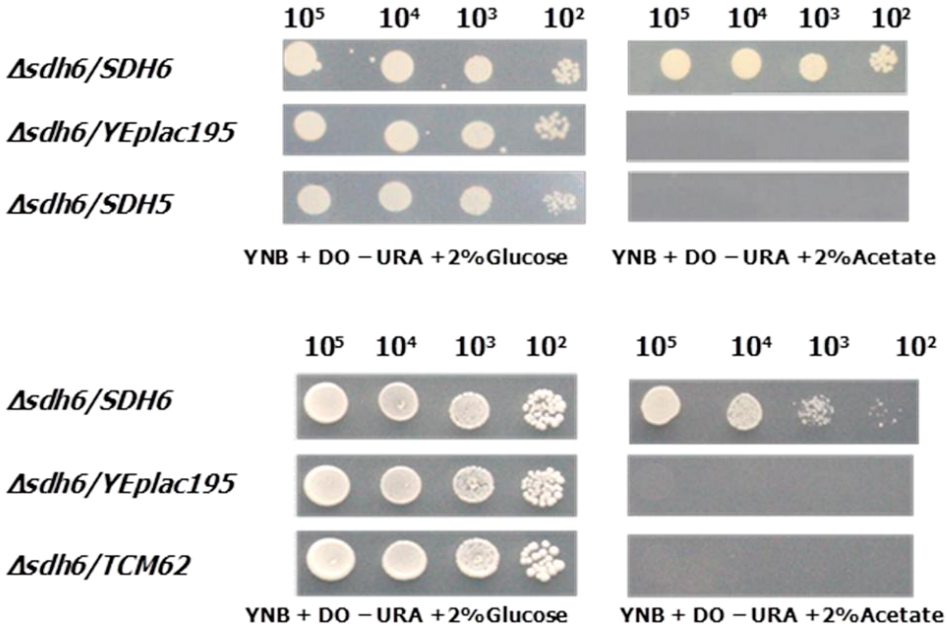


Figure 2.19. Spot assays at 28°C of *Δsdh6/SDH5* and *Δsdh6/TCM62.* 10^5, 10^4, 10^3, 10^2, cells were plated on glucose and acetate containing plates after serial dilutions. The growth was observed after 72h at 28°C.

On acetate where the *Δsdh6* phenotype is more severe, neither *SDH5* nor *TCM62* were able to rescue the respiratory growth defect of the mutant. These results suggested that Sdh6p had a different function in the SDH assembly. The same results were obtained overexpressing *SDH6* in *Δsdh5* and *Δtcm62* mutants (data not shown). The *SDH6* ORF was amplified by PCR using FY379 and RY379 primers, digested with *EcoRI* and *PstI* and cloned both in pFL38 and YEplac195 plasmids. This result further indicated that the three SDH assembly factors were not interchangeable.

Overexpression of the four SDH subunits does not enhance enzyme activity in the absence of Sdh6 protein

In order to investigate the role of Sdh6p, we wondered if overexpression of the four SDH subunits could increase the SDH activity in *Δsdh6* mutant. Actually the aim was to evaluate the possibility to increase SDH activity to 100% and to analyze in this case the *sdh6* phenotype and eventually the state of complex. Therefore *SDH1* and *SDH2* were cloned in YEplac181 and *SDH3* and *SDH4* in YEplac195. The *Δsdh6* strain was transformed with both the plasmids and mitochondria were isolated as described in material and methods section. The SDH assay was performed on the *Δsdh6* transformed both with the four SDH subunits and with the two empty vectors.

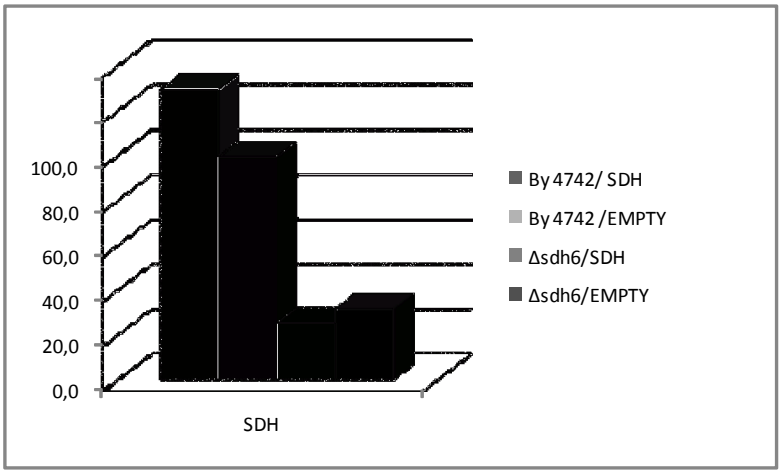


Figure 2.20. SDH assay of BY4742/SDH, BY4742/EMPTY, *Δsdh6*/EMPTY and *Δsdh6*/SDH: the results are the average of three independent experiments.

As indicated in the histogram (Fig.20), only in BY4742 there was an increase of SDH activity from 100% (*BY4742/EMPTY*) to 150% due to the four overexpressed subunits (*BY4742/SDH*). No differences between *Δsdh6/EMPTY* and *Δsdh6/SDH* were observed so the presence of all SDH subunits was not sufficient to increase SDH activity. This result was very intriguing and not obvious. It underlined that Sdh6p was the limiting factor of complex II assembly. Indeed, even if the four SDH subunits were present and abundant in the cell, only in presence of a functional Sdh6p assembly factor the complex could be assembled showing an extra-enzymatic activity as occurred in wt strain. In addition, spot assays of *Δsdh6/SDH* and *Δsdh6/empty* were performed on 2%ethanol.

Parallel to the activity, neither the *Δsdh6* phenotype was rescued on respiratory carbon source (Fig.21).

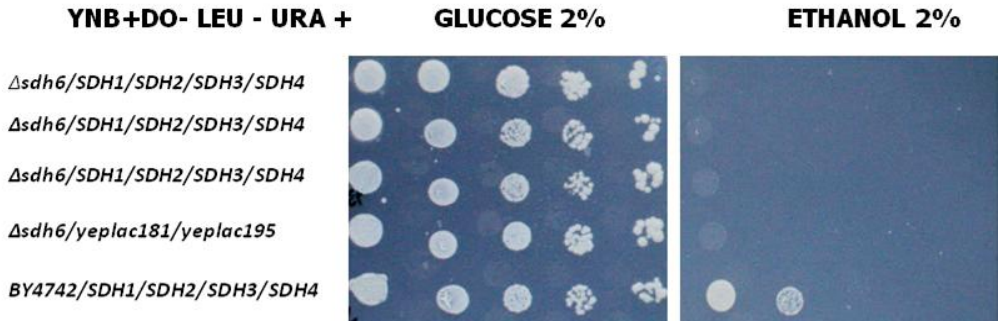


Figure 2.21. Spot assays of *Δsdh6*/SDH, *Δsdh6*/EMPTY, BY4742/SDH at 28°C. 10^5, 10^4, 10^3, 10^2, 10^1 cells were plated on glucose and ethanol containing plates after serial dilutions. The growth was observed after 1 week at 28°C.

Even the wt strain showed a slowly growth on non fermentable carbon sources suggesting that all SDH subunits overexpressed at the same time affected negatively the cell, probably causing an energetic cell imbalance.

In addition we overexpressed the four SDH subunits in the *Δsdh5* mutant, the other specific SDH assembly factor. However, it is worth mentioning that unlike *sdh6* mutant, *Δsdh5* is characterized by a complete loss of SDH activity. Again we did not observe an increase of SDH activity or any phenotype rescue on respiratory carbon sources (Fig.22 and fig.23)

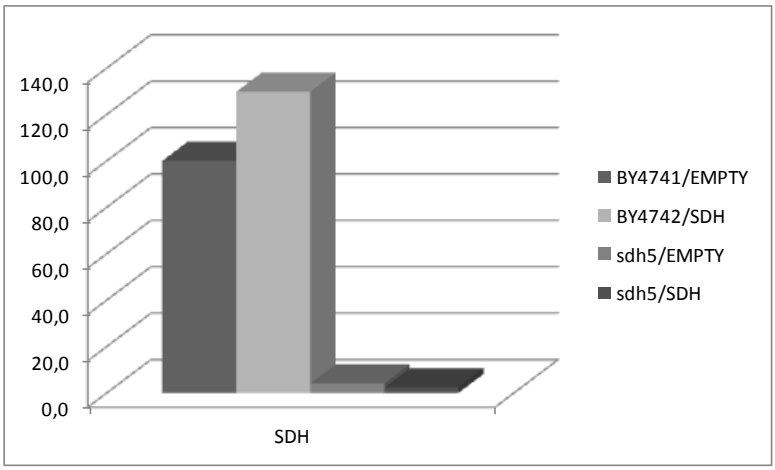


Figure 2.22. SDH assay of BY4741/SDH, BY4741/EMPTY, *Δsdh5*/EMPTY and *Δsdh5*/SDH: the results are the average of three independent experiments.

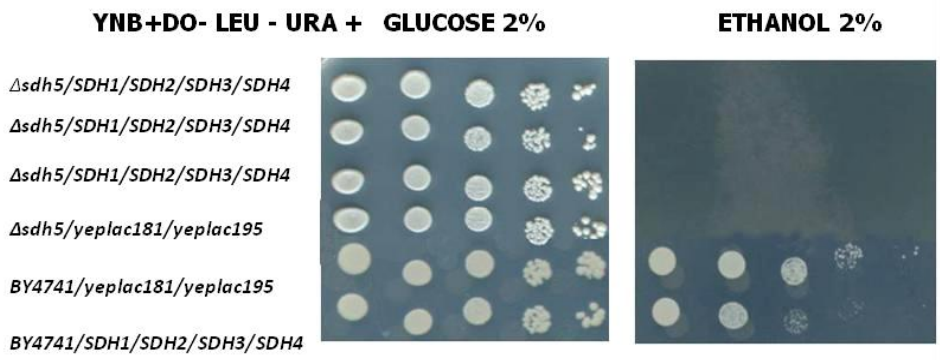


Figure 2.23. Spot assays of *Δsdh5*/SDH, *Δsdh5*/EMPTY, BY4742/SDH at 28°C. 10^5, 10^4, 10^3, 10^2, 10^1 cells were plated on glucose and ethanol containing plates after serial dilutions. The growth was observed after 1 week at 28°C.

Once more this result highlights the involvement of Sdh6p in SDH assembly process: this protein is indeed necessary to ensure a fully assembled and functional complex II and its role could not be bypassed by increasing the number of SDH subunits in the cell.

Steady state levels of SDH subunits are decreased in Δsdh6 mutant

As previously demonstrated *Δsdh6* showed a 70% decrease in SDH activity and a Km value for succinate similar in wild type and in the null mutant. This result indicates that the reduced SDH activity could be due to a reduced complex II amount rather than a

qualitative alteration of it. Furthermore, the overexpression of four SDH subunits in the *Δsdh6* mutant did not restore the SDH activity supporting the idea of a quantitative problem of complex II in this strain. Therefore, SDS-PAGE and 2D-BNGE were performed to evaluate the SDH assembly state in the null mutant. Mitochondria were extracted as described in materials and methods section and quantified with Bradford. 30µg and 60 µg of mitochondria were separated on a 15% polyacrylamide gel and transferred to nitrocellulose membranes.

Western Blots with Anti-Sdh1p and Anti-Sdh2p antibodies were performed and the immunoblot for Atp4 subunit (complex V) was used to show equivalent protein loads per lane (Fig.24)

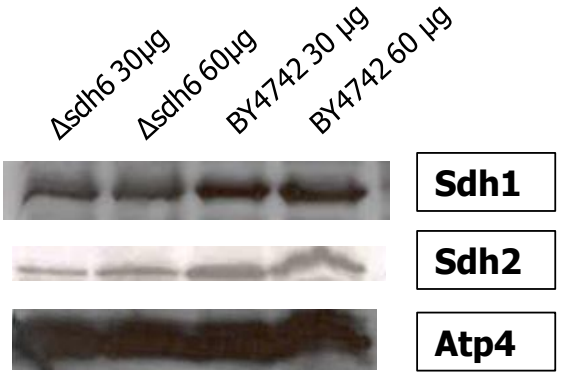


Figure 2.24. SDS-PAGE Western Blot of *Δsdh6* and BY4742 mitochondria. Two different concentrations of each sample were loaded with Laemmli sample buffer 1X.

In agreement with the reduction of SDH activity, the Sdh1p and Sdh2p levels were significantly decreased in the *Δsdh6* mutant. To evaluate the complex II state of assembly, a 2D-BNGE was then performed. The anti-Sdh2p and anti-Atp4p antibodies were used for the immunoblot (Fig.25).

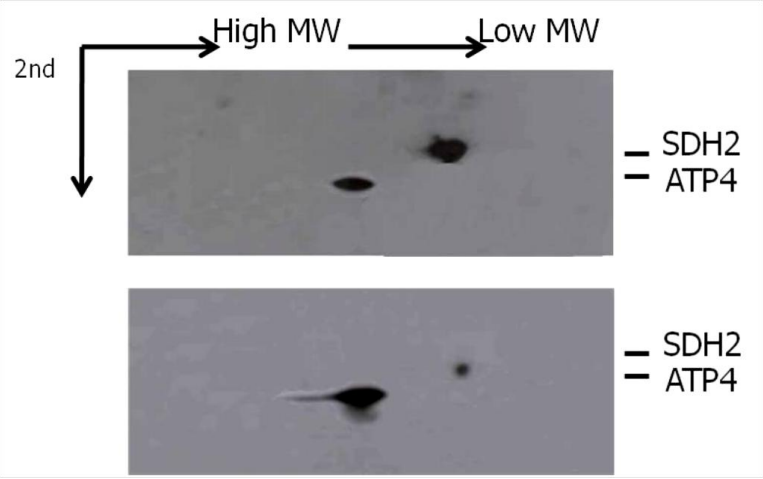


Figure 2.25. 2D-BNGE Western Blot of *Δsdh6* and BY4742 mitochondria. 150 µg of mitochondria were solubilized with 1% lauryl maltoside and loaded on a gradient gel. Then each lane was excised and treated for 1 h at room temperature with 1% SDS and 1% b-mercaptoethanol and then run through a 16.5% tricine-SDS–polyacrylamide gel.

1% Lauryl maltoside extracts of mitochondria isolated from *Δsdh6* cells and separated on 2D-BNGE showed:

- ✓ a fully assembled complex II (~30kDa)
- ✓ a decreased amount of complex II.

In the first dimension, the hybridization Sdh2p signal was not detectable probably because of the Sdh2p folding which did not allow the antibody binding.

These results definitely demonstrated that the defective SDH activity is caused by a reduced number of enzyme units rather than by qualitative alterations of complex II and underlined the Sdh6p specific role in SDH assembly. This specificity is due to the fact that the other respiratory complexes are intact both in human and in yeast.

2.4.2. Characterization of Sdh6p

The results previously obtained analyzing HeLa cell fractions expressing *SDHAF1*^{HA} indicated that Sdhaf1p is localized in the mitochondrial matrix and that is not physically associated with cII *in vivo* (Ghezzi *et al.*, 2009). In order to assess these results a biochemical characterization of Sdh6p protein was performed in yeast. First *SDH6* was tagged with HA tag or HIS tag and then its localization was determined. The proteinase K assay was carried out to establish whether Sdh6p was inaccessible to proteinase K action being inside the mitochondria and then a 2D-BNGE electrophoresis was performed to evaluate where Sdh6p-HA localized.

Construction of *SDH6* TAG

The *SDH6* ORF was amplified with primers FY379 and PRYDRHA to obtain the *SDH6*-HA construct which contained the *SDH6* promoter region and the tag tail a 3' terminus. The primers FY379 and RYDRHIS were used to obtain the same construct with the His tag tail at 3' terminus.

The amplified regions were digested with *EcoRI* and *PstI* and cloned in pFL38 and YEplac195.

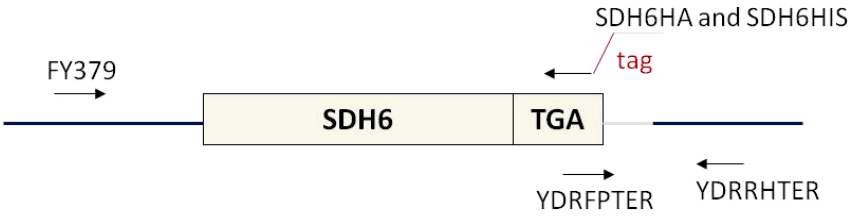


Figure 2.26. Strategy scheme of SDH6-tag construction

Subsequently, the endogenous *SDH6* terminator was amplified in order to increase the efficiency of the recombinant protein translation. The terminator region of Sdh6 was amplified by PCR using YDRFPTER and YDRRHTER primers. The amplified terminator fragment (300 bp) was digested with *PstI* and *HindIII*. So it was ligated to the *SDH6*-TAG and then the whole constructs were cloned in the plasmids pFL38 and YEplac195.

The recombinant constructs obtained were used to transform the *Δsdh6* strain. Neither HA tag nor HIS tag affected the Sdh6p expression (data not shown). However, it was shown with a SDS PAGE that the terminator region absence affected negatively the *SDH6* expression, maybe because mRNA was less stable and therefore the protein translation was less efficient. 3 different mitochondria concentrations of Sdh6p-HA-TER and Sdh6p-HA were loaded and immunodecorated with α-HA antibody. The Western Blot indicated that the terminator region was necessary for the *SDH6* efficient expression and therefore all the experiments were carried out with the strains expressing the plasmids containing it.

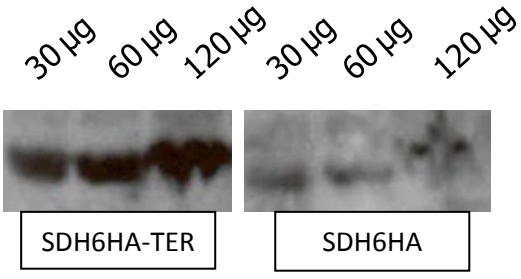


Figure 2.27. Western Blot SDS PAGE of Sdh6p-HA mitochondria with or without the terminator region. 3 different concentrations of mitochondria were loaded and solubilized with Laemmli buffer 1X.

Proteinase K assay treatment

The proteinase K assay is a treatment that may indicate in which mitochondrial subcompartment a protein resides. Indeed it degrades the mitochondrial outer membrane and what is contained inside mitochondria is protected by its action. Therefore isolated mitochondria were treated as described in material and methods section and resolved with SDS-PAGE. Also the Post Mitochondrial Fraction (PMS) was loaded as control resembling the cytosol.

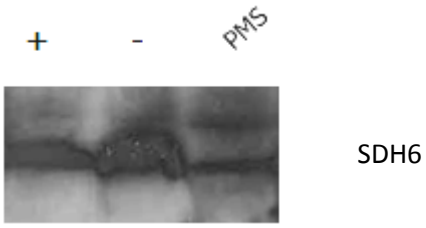


Figure 2.28. Proteinase K assay after1h of treatment(+/-). Also the post mitochondrial fraction (PMS) was loaded.

The western blot with anti-HA indicated that proteinase K did not affect Sdh6p-Ha signal (fig.28). Therefore Sdh6p is localized inside mitochondria, confirming what seen in HeLA cells by Ghezzi *et al.*,. Indeed there are no significant differences between the fraction treated or not with Proteinase K underling how this protein resides inside mitochondria. However a lower signal intensity of Sdh6p-HA was present in the PMS fraction but this was due to some contamination of mitochondria in the cytosol fraction as indicated by Western Blot anti COXIII (data not shown).

Sdh6p^{HA} is not associated to SDH complex nor to other molecular complexes

In order to determine whether Sdh6p associated with any higher molecular weight complexes such as fully assembled complex II or cII assembly intermediates and to exclude the possibility that Sdh6p is actually an unidentified subunit of the mature complex, mitochondria isolated from the strain expressing Sdh6p-HA were analysed in 2D-BNGE. For Western blot analysis antibodies directed against the HA epitope and Sdh2p subunit were utilized. A single signal for both Sdh6p-HA and Sdh2p was detected in *Δsdh6* transformed with pFL38-*SDH6-HA* as depicted in the fig. 29. Sdh6p showed a mobility of 12 kDa in the second dimension. This corresponded to the expected molecular weight of a monomeric molecule.

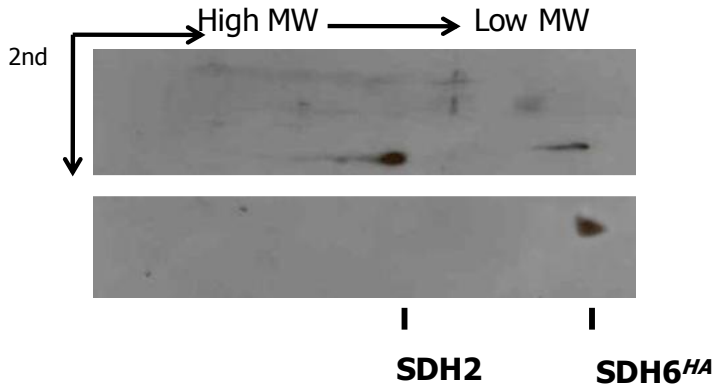


Figure 2.29. 2D-BNGE Western Blot of Sdh6p-HA mitochondria. 150 µg of mitochondria were solubilized with 1% lauryl maltoside and loaded on a gradient gel. Then the lane was excised and treated for 1 h at room temperature with 1% SDS and 1% b-mercaptoethanol and then run through a 16.5% tricine-SDS–polyacrylamide gel.

These results indicated that Sdh6p was not physically associated with CII *in vivo* nor took part, at least stably, in other complexes in these analysis conditions accordingly with what seen in HeLa-cells by Ghezzi.

Sdh1p and Sdh2p are not Sdh6p interactors

Despite the 2D-BNGE showed no Sdh6p-Sdh2p interaction, these results were not sufficient to exclude a possible interaction of Sdh6p with the SDH subunits. Therefore an affinity purification was carried out exploiting the interaction between His6x residues and Ni-Nta agarose beads. The whole *SDH6* ORF was expressed with a tail of six histidine residues before the stop codon, as previously described, and was able to complement the *Δsdh6* null mutant (data not shown). Once obtained the strain, mitochondria were isolated and an affinity purification was performed as described in the material and methods section. However, in the Western Blot of final eluate no Sdh6p-His signal was detected. This result maybe was due to the extraction conditions suggested by the protocol, which indicated a solubilisation buffer containing 3% digitonin. Therefore the mildest extraction conditions for Sdh6p-His6x were identified: three digitonin concentrations were assayed included the 3% digitonin. As indicated in the fig.30 (A) the best condition for the Sdh6p extraction was 0.75% digitonin which was enough to solubilise at least the 50% of protein (the pellet is the fraction not solubilised). Strikingly with 3% digitonin the Sdh6p-His signal was almost completely loss in the supernatant and this could explain the absence of the signal observed in the first purification. In addition, to improve the solubility efficiency, in the extraction buffer was added NaCl either 50 mM and 150 mM (fig.30 B). This latter concentration was the best to extract completely the

protein in the supernatant although it was not recommended since it could abolish probably ionic interactions in a dose dependent manner.

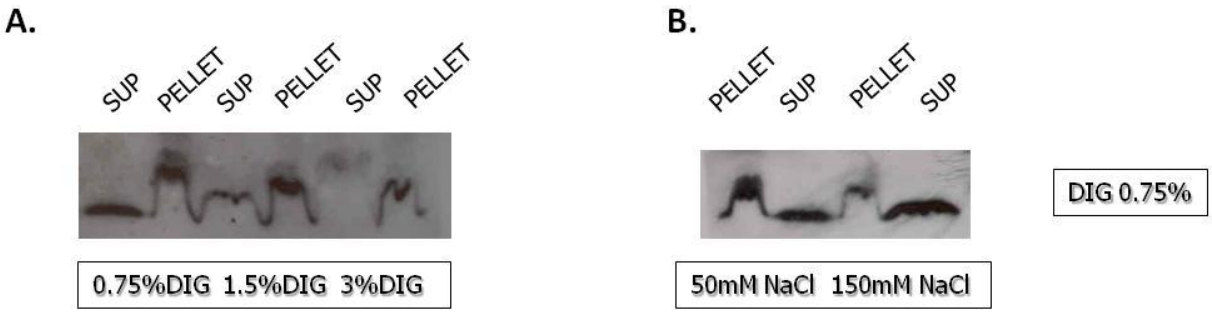


Figure 2.30. Sdh6p-HIS extracting conditions: SDS PAGE 12% Western Blot.

Therefore a first purification was carried using the mildest condition (digitonin 0.75%) on *Δsdh6* transformed with *pFL38-SDH6-HIS6X*. The final eluate was resolved with SDS-PAGE 12% and visualized by immunoblot with antibodies against Sdh2 and His. The supernatant and pellet fractions were also loaded as extraction controls (fig.31).

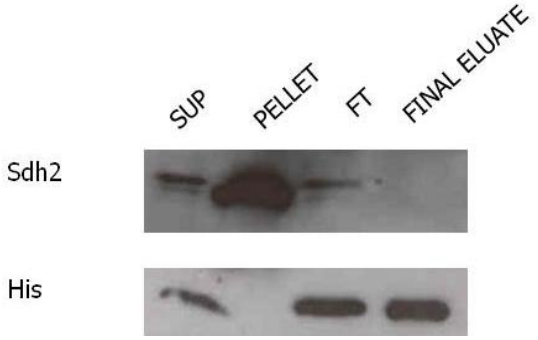


Figure 2.31. SDS-PAGE 12% Western Blot on final eluate of *Δsdh6* transformed with *pFL38-SDH6-HIS6X* and immunodecorated with α-Sdh2 and α-His. Supernatant, pellet and flow through fractions were loaded as controls.

The Western Blot indicated that α-His hybridization signal was present both in the final eluate and in the supernatant suggesting that the extraction was efficient. However a Sdh6p-His signal was detected also in the flow through fraction indicating that the binding Sdh6-His/beads was not optimal. Furthermore α-Sdh2 signal was not detected in the final eluate. The strong Sdh2p signal in pellet might indicate that the optimal Sdh2p extraction condition was not the same of Sdh6p.

To identify the Sdh1p signal a SDS-PAGE 15% was performed since Sdh1p was 70kDa. The western blot is reported below (fig.32).

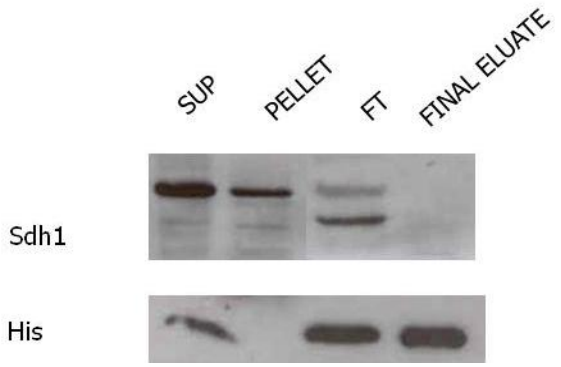


Figure 2.32. Western Blot SDS-PAGE 15% on final eluate of *Δsdh6* transformed with *pFL38-SDH6-HIS6X* and immunodecorated with α-Sdh1. Supernatant, pellet and flow through fractions were loaded as controls.

The Western Blot indicated that also Sdh1p was not present in the final eluate. This suggested that neither Sdh1p nor Sdh2p interact with Sdh6p in these conditions.

In addition, in order to improve the extraction of Sdh6p, 150 mM NaCl was added to the solubilisation buffer and the purification was carried out. No changes were detected in the Western Blots performed (data not shown).

This first part of the results pointed out a fundamental role of Sdh6p in the SDH assembly. In particular these results suggested that Sdh6p absence determines a marked decrease of SDH activity which is in agreement with the decreased amount of SDH complex, seen in BN-PAGE. Moreover, Sdh6p is a rate limiting factor of SDH assembly: indeed even if all four SDH subunits were overexpressed at the same time the SDH activity could not be restored in the mutant suggesting a pivotal role of Sdh6p in cII assembly.

Both the presence of a LYR motif in the Sdh6 protein and the sequence similarity with Isd11p prompted us to investigate the putative role of Sdh6p in the insertion of Fe-S clusters on cII backbone by overexpressing *ISD11* in the *sdh6* mutant. Also frataxin was expressed in multicopy in the null mutant on the basis of the experimental evidence reported in literature.

Although from obtained results no indications have come out about a role of Sdh6p linked to the Fe-S clusters metabolism we could not exclude a role of Sdh6p in the iron metabolism.

The experiments described so far excluded any direct interaction between Sdh6p and SDH subunits suggesting that Sdh6p is not part of complex II. However these results still did not shed light on the action mechanism of Sdh6p. Therefore in order to gain more information about *SDH6* role we performed a search for multicopy suppressors.

The second part of the thesis will be focused on the results obtained with the search for multicopy suppressors and the experiments undertaken: after a brief introduction on the iron metabolism in yeast, the experiments performed will be the object of discussion.

Chapter III:

An additional role of SDH6 besides SDH assembly

3.1. Iron metabolism in yeast

Iron is essential for all organisms: it is an essential cofactor for respiration, DNA synthesis and repair, ribosome biogenesis and metabolite biosynthesis (Ihrig *et al.*, 2010). Although iron is highly abundant its bio availability is difficult: the soluble form Fe^{2+} spontaneously auto-oxidizes to Fe^{3+} which is insoluble in water at neutral pH (Kosman 2003). On the other hand iron may be toxic at higher concentrations within the cell because it is the major source of reactive oxygen species. Therefore specific mechanisms for iron uptake, storage and trafficking have been developed in the cells, in order to avoid the presence of free iron forms that would be dangerous.

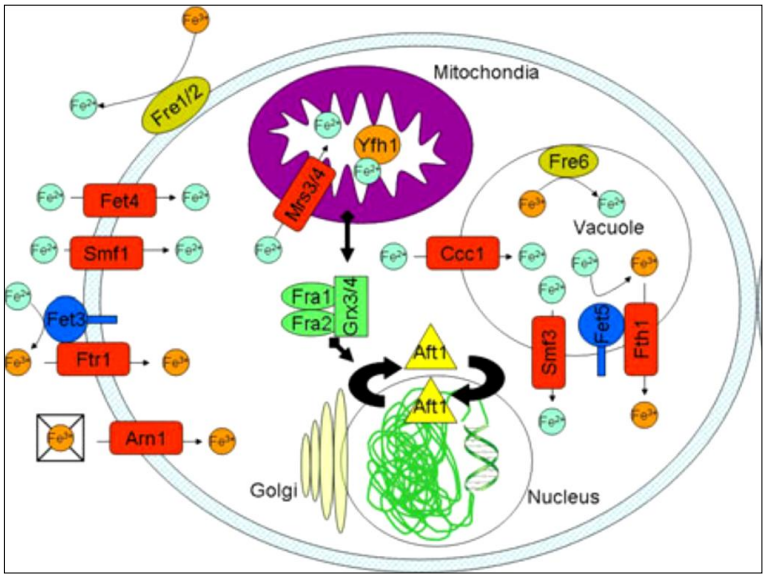


Figure 3.1. Iron homeostasis in S. cerevisiae. Transporters are in red, oxidases in blue, transcription factors in yellow, reductases in olive *(Bleackley & Macgillivray, 2011)*

In yeast *S. cerevisiae* the iron uptake occurs through the action of specific transporters that channel Fe^{2+} or Fe^{3+} within the cell (fig. 1): Fet4p and Smf1p are able to bind only the Fe^{2+} form which is produced by the action of Fre1/Fre2 reductases. This action can be bypassed by the high affinity iron transport system consisting in Fet3/Ftr1 complex which is induced in iron limiting conditions by Aft1p, transcription factor which regulates the expression of iron regulon (Yamaguchi-Iwai *et al.*, 1995). Fet3p is a oxidoreductase which oxidizes Fe^{2+} in Fe^{3+} and transports it to Ftr1p. This iron transporter is able to bind only the Fe^{3+} produced from the activity of Fet3p in a process known as iron channeling (Kwok *et al.*, 2006). Siderophore are another iron uptake mechanism which is independent by the activity of Fre reductases bound to the membrane (Bleackley and Macgillivray, 2011). These are small organic molecules able to bind Fe^{3+} secreted in iron limiting conditions by

several organisms except *S.cerevisiae* which does not produce them. However *S.cerevisiae* expresses siderophore transporters namely Arn1p, Arn2p, Arn3p, Arn4p and siderophore binding/uptake proteins Fit1p, Fit2p, Fit3p belonging to the family of cell wall mannoproteins (Protchenko *et al*., 2001).

In iron starvation conditions, *S. cerevisiae* upregulates the iron regulon which depends on the Aft1p transcription factors. In this conditions it is translocated into the nucleus where activates the transcription of more than 20 genes that are required for increasing cellular iron content (Yamaguchi-Iwai *et al*., 1995; Rutherford *et al*., 2001; Rutherford *et al*., 2003; Shakoury-Elizeh *et al*., 2004; Courel *et al*., 2005). This translocation seems to be dependent on the Fe/S clusters present in the cell.

A second iron responsive transcription factors is Aft2p which exhibits a partial overlapping function with Aft1p in activating genes in response to the iron starvation (Blaiseau *et al*., 2001; Rutherford *et al*., 2001; Rutherford *et al*., 2003; Courel *et al*., 2005; Rutherford *et al*., 2005). Indeed these two transcription factor possess two different roles in the transcriptional regulation of iron regulon with Aft2p playing a larger role in the regulation of genes involved in vacuolar and mitochondrial iron homeostasis (Courel *et al*., 2005; Rawat and Stemmler, 2011).

Within the mitochondria both the synthesis of heme and Fe/S clusters occurs and this makes the organelle a major site of intracellular iron metabolism. The iron uptake in the mitochondria is mediated by two homologous membrane transporters, Mrs3/Mrs4p, also known as mitoferrin in humans (Mühlenhoff *et al*., 2003). Once inside the mitochondria, iron is bound presumably by frataxin that shuttles iron where it is required. In the absence of frataxin iron accumulates forming inorganic precipitate and generates reactive oxygen species (Rawat and Stemmler 2011). Another important site for iron homeostasis is the vacuole.

3.2. Results

3.2.1. Identification of multicopy suppressors of *Δsdh6* mutant

As previously mentioned the experiments so far described did not elucidate the action mechanism of *SDH6*. To gain further insight into the functional role of *SDH6* an exhaustive high copy suppressors screen of the *Δsdh6* OXPHOS defect was performed. The search for multicopy suppressors is a genetic method very useful to identify genes that could functionally substitute in multicopy specific gene mutations. This approach allows to identify interactions between gene products and can reveal the existence of metabolic or cellular connections.

Therefore *sdh6* null mutant was transformed with the genomic library constructed in pSEY8 vector (kindly provided by Dott. Schatz, Basel) and after selection of Ura^+ transformants the clones able to grow on YNB-acetate (YNBA) at 28°C were identified. In this screen acetate, instead of ethanol or glycerol, was utilized because in this condition the phenotype of *Δsdh6* is more severe, condition that favours the search for multicopy suppressors.

In order to exclude the presence of the wild type gene in the recombinant plasmid, a colony PCR was performed. From suppressor colonies that did not contain *SDH6* in the vector DNA was recovered: restriction analysis with *EcoRI-HindIII* indicated that plasmids contained different fragments of yeast genomic DNA in addition to the 6000 bp pSEY8 band. An example of these different restriction patterns is reported below (fig. 2).

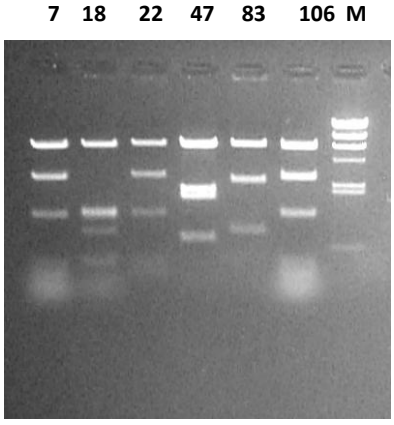


Figure 3.2.. Six different restriction patterns: the upper bands are the plasmid pSEY8 and the other bands represent the digested fragments corresponding to genomic yeast DNA.

Genomic inserts in each plasmid were sequenced at both ends and compared with the *S. cerevisiae* genome using NCBI BLAST search. The results obtained indicated that only two genes were able to rescue the *Δsdh6* OXPHOS growth phenotype: here below an example of the output of the sequence analysis is represented:

Clone 86: the size of the fragment was 3Kb, it was localized on chromosome XIII and contained the whole ORF *YAP1* and a part of *YML006C* gene (fig.3). Therefore it was probable that *YAP1* was responsible for the multicopy suppression.

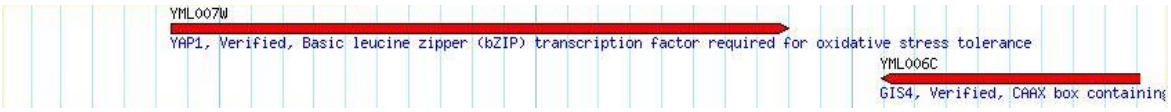


Figure 3.3. BLASTN of digested fragments

Clone 47: the identified fragment was localized on chromosome IV, was of 2,5 Kb and contained the whole ORF *YAP2*. Other genes were present but not completely and therefore the multicopy suppression was due to *YAP2* presence (Fig.4)

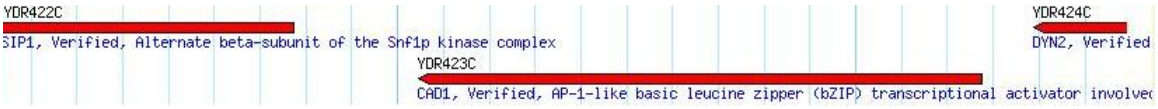


Figure 3.4.BLASTN of digested fragments.

The two genes identified were *YAP1* and *YAP2* encoding two transcription factors involved in stress response and in many other cellular processes and whose description will be discussed later. In order to evaluate whether these two genes were responsible for the rescue of OXPHOS phenotype, they were cloned in the multicopy plasmid YEplac195.

Subcloning of YAP1 and YAP2 in YEplac195

The whole *YAP1* ORF was amplified by using EYAP1F and HYAP1R primers and the amplified fragment (~2500 bp) was digested with *EcoRI* and *HindIII* and cloned in YEplac195. The same was made for *YAP2* (1700 bp) by using BYAP2F and PSYAP2R primers and digested with *BamHI* and *PstI*.

Following the purification and sequencing of the recombinant constructs, *YAP1*/YEplac195 and *YAP2*/YEplac195 were used to transform *Δsdh6.* Also the empty YEplac195 was used to transform *Δsdh6* as control. 3 independent clones transformed with each construct were used for spot assay on acetate carbon source. As growth controls *Δsdh6/pFL38* and *Δsdh6/SDH6* were also spotted.

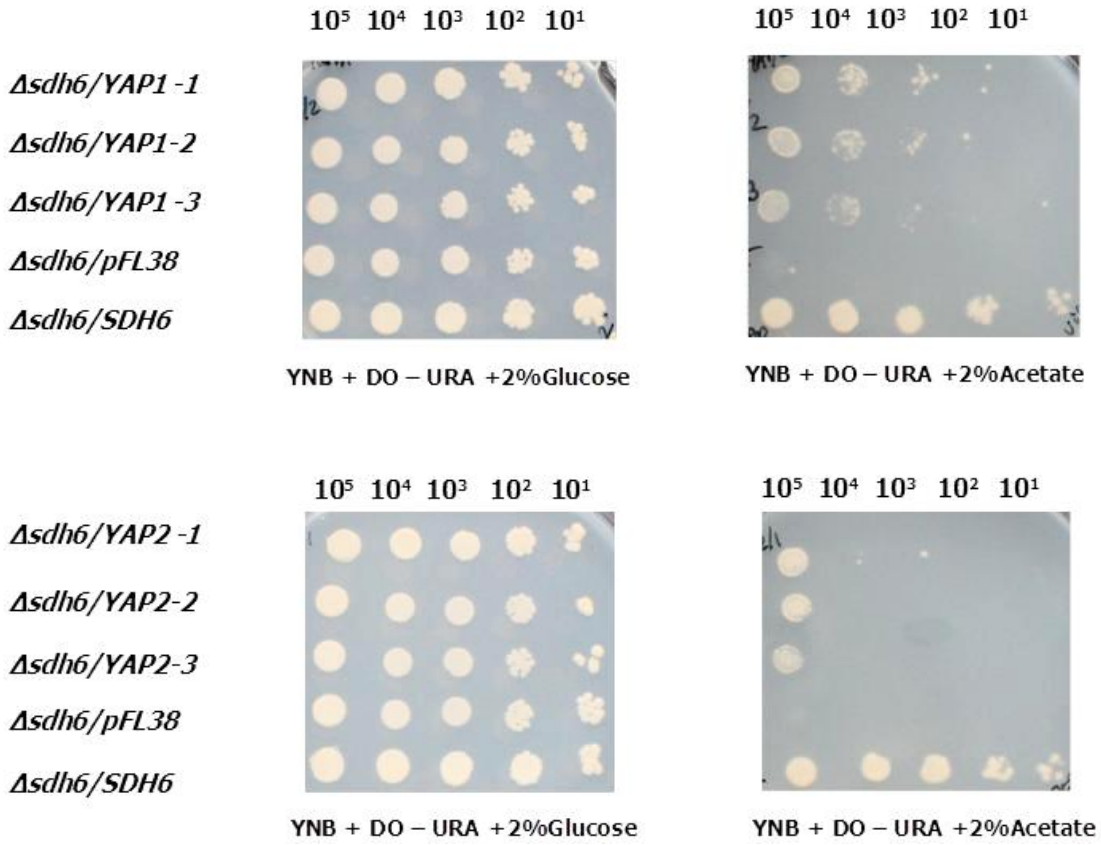


Figure 3.5. Spot assays at 28°C. 10^5, 10^4, 10^3, 10^2, 10^1cells of each clone were plated on glucose and acetate containing plates after serial dilutions. The growth was observed after 72h at 28°C.

Both *YAP1* and *YAP2* were able to rescue the OXPHOS defect on YNBA suggesting that these genes were responsible for the multicopy suppression. However, after 1 week growth at 28°C the *Δsdh6* phenotypes due to the *YAP1* or *YAP2* presence were different. The rescue dependent on *YAP1* was greater than the one due to *YAP2*. Indeed, comparing the ability of *Δsdh6* to grow on acetate-based medium, it is evident that *YAP1* allowed a growth more similar to wt than *Δsdh6* transformed with *YAP2*. These results indicate that *YAP1* and *YAP2* play different roles in the cells activating the transcription of different target genes whose effects in *Δsdh6* result in a different growth.

3.2.2. The transcription factors Yap1 and Yap2: their role in the cell.

These two genes belong to the YAP family, Yeast Activator Protein, a family of b-ZIP proteins involved in several cellular processes. As recently reviewed by Pousada *et al.*, the YAP family consists of eight proteins with sequence similarity to the conventional yeast AP-1, Gcn4, at the DNA binding-domain (Rodrigues-Pousada *et al.*, 2010). However each member of this family has key residues within the sequences that give them distinct DNA binding properties. The characteristics that distinguish the members of Yap family from

Gcn4 family are the aminoacids involved in the DNA binding. Below is reported a scheme of the structural features of the Yap family. In general, they have a cysteine-rich domain (n-CRD or c-CRD depending it is at C- or N- terminus or both) and a Nuclear Export Signal (NES) (fig.6)

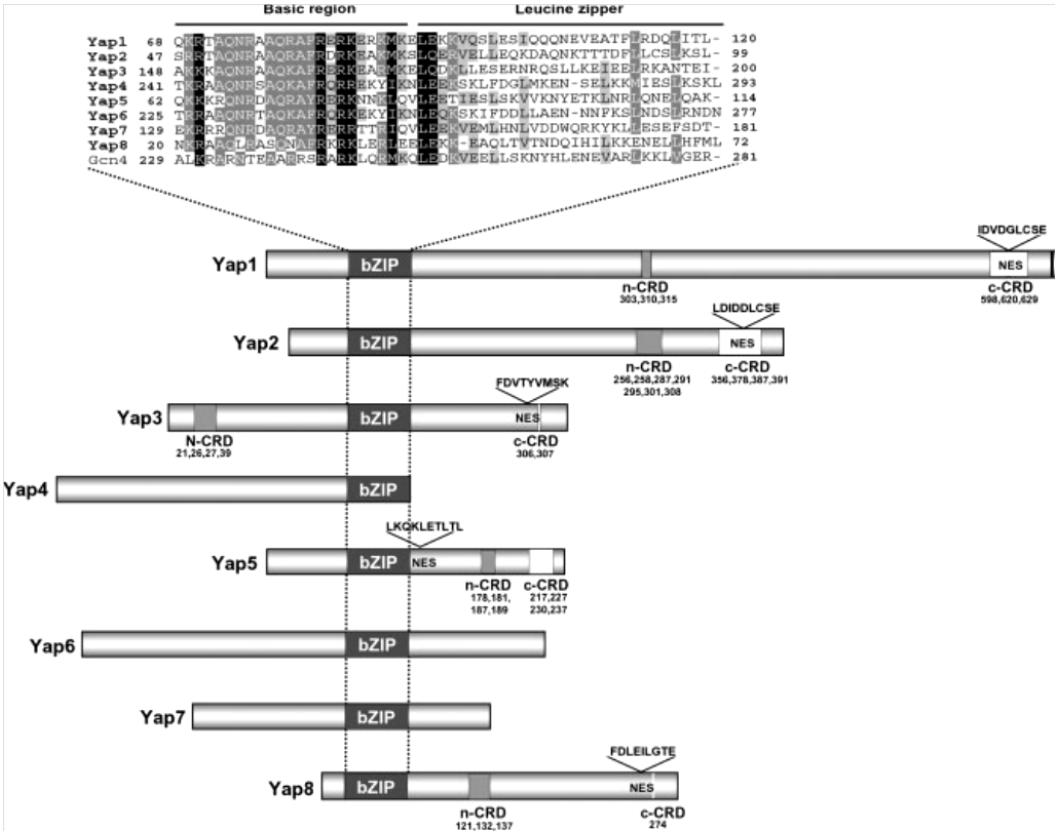


Figura 3.6. Structural features of the Yap family. In the upper part of the figure, the sequences of the eight Yap bZIP domains are compared with the equivalent region of Gcn4 (*Rodrigues-Pousada et al., 2010*)**1.**

The cysteine rich domain (CRD) guarantees the nuclear retention of the transcription factor such as Yap1p (Kuge *et al*., 1997). When this region is removed, Yap1p is constitutively localized in nucleus in an active form. This occurs because an intramolecular disulphide bond forms on Cys residues of these domains that compromise the binding of the exportin Crm1p (also known as Xpo1) to the NES (Yan *et al*., 1998).

The different DNA binding abilities confer to each Yap protein a specific role in cellular processes such as oxidative stress, cadmium stress, iron metabolism and others. A general scheme of these roles is reported in fig.7 .

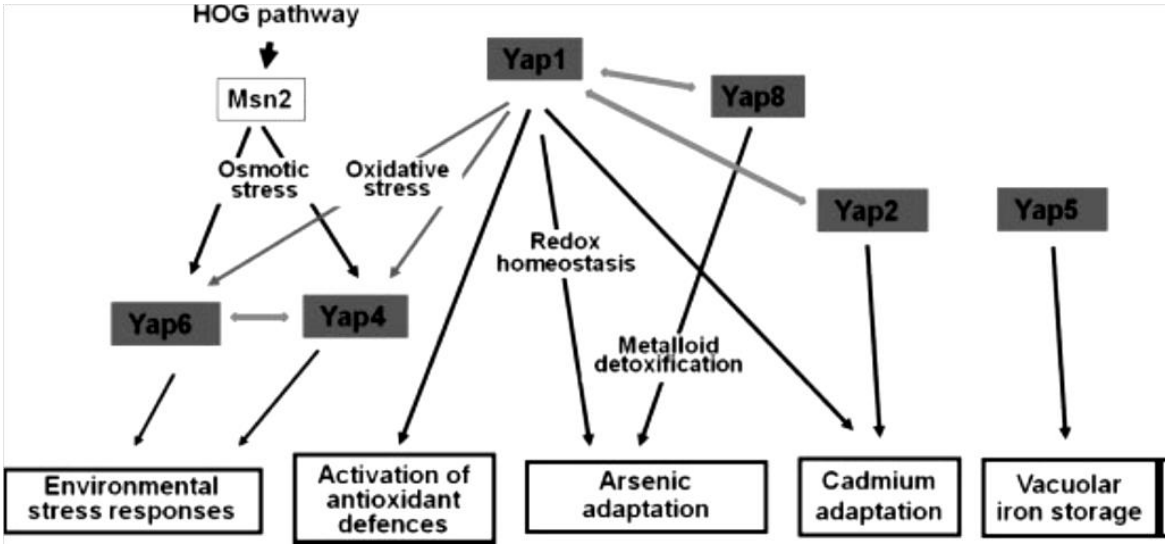


Figure 3.7. Potential interplay between members of the Yap family *(Rodrigues-Pousada et al., 2010)*

Yap1 was the first member of the YAP family to be described and was identified by its ability to bind and activate the SV-40 AP-1 recognition element (ARE: TGACTAA), functional homologue of the human *c-jun* oncogene (Moye-Rowley *et al.*, 1989). What's more, *YAP1* was found in a multicopy suppressors screen because it conferred resistance to the iron chelators 1,10-phenantroline and 1-nitroso-2-naphtol (Bossier *et al.*, 1993). In addition, also *YAP2* was able to confer resistance to 1,10 phenantroline in the aforementioned screen (Bossier *et al.*, 1993). Like Yap1, Yap2 is related to the mammalian proto-oncoproteins c-jun (Moye-Rowley *et al.*, 1989; Bossier *et al.*, 1993; Wu *et al.*, 1993). Several phenotypes associated with the disruption of *YAP1* and *YAP2* have been described. These mutants are for example more sensitive to cadmium, zinc or to oxidative agents (Wu *et al.*, 1993; Schnell and Entian, 1991).

Taking into account the several cellular roles of Yap factors, the identification of these two genes in our multicopy suppressors screen was very intriguing. Since the two genes *YAP1* and *YAP2* were both able to rescue the OXPHOS growth defect of *sdh6* null mutant we wondered whether the phenotype suppression was accompanied by an increase of SDH activity. To answer to this question the SDH assay was carried out.

Multicopy suppressors YAP1 and YAP2 are able to rescue the growth of Δsdh6 but not to increase SDH activity

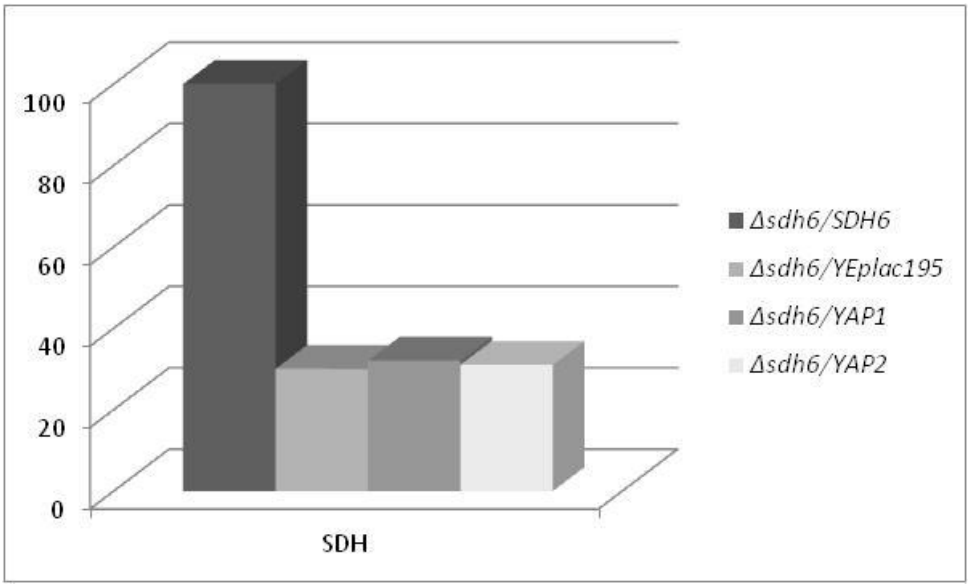


Figure 3.8. SDH assay on transformant strains *Δsdh6/YAP1* and *Δsdh6/YAP2*. *Δsdh6/pFL38 and Δsdh6/SDH6* were used as controls.

The SDH activity was measured in *Δsdh6/YAP1* and *Δsdh6/YAP2* transformant strains. The assay was carried out also on *Δsdh6/pFl38* and *Δsdh6/SDH6* as negative and positive control respectively.

As indicated in the fig.8, the suppression exerted by *YAP1* and *YAP2* was not due to an increase of SDH activity. Therefore their suppression mechanism seemed to be independent of complex II and could be ascribed to an indirect effect of these two transcriptional factors.

Moreover this result suggests that Sdh6p plays a dual role, one related to SDH functioning and the other independent from this. In this regard it may be significant and helpful to consider that mutants in SDH subunits with a similar residual SDH activity (~30/40%) are able to grow better than *Δsdh6* mutant (fig.9) (Panizza Ph.D. thesis, 2011) suggesting that the inability to grow on non fermentable carbon sources of *Δsdh6* is not only due to a SDH defect.

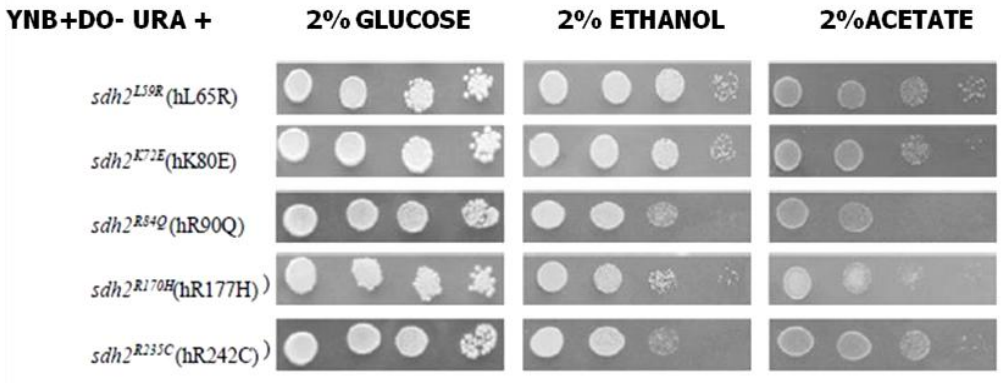


Figure 3.9. Respiratory growth of several *SDH2* mutants

Therefore, these observations were indicative of an additional role of *SDH6* besides the SDH assembly which had to be unraveled. Furthermore, the finding of *YAP1* and *YAP2* as multicopy suppressors could be the key to elucidate this role. Thus, we focused on the cellular processes which are regulated by these two transcription factors and speculated that two main processes could be involved with Sdh6p:

1. the oxidative stress response
2. the mitochondrial iron metabolism

Each hypothesis will be discussed individually.

3.2.3. The oxidative stress response

The oxidative stress response is a phenomenon by which a cell responds to alteration in its redox state due to the generation of radical oxygen species (ROS), potent oxidants capable of extensive cellular damage at the level of DNA, protein and membrane lipid content. ROS are caused by the incomplete reduction of O_2 during aerobic respiration as well as to the exposure to ionizing radiations, redox cycling chemical present in the environment or to heavy metals (Jamieson, 1998; Rodrigues-Pousada *et al.*, 2004). All the organisms, from bacteria to humans, have developed mechanisms to protect their cellular components against these reactive agents. Focusing on *S. cerevisiae* cells, there are both enzymatic and non-enzymatic defense systems to protect the cellular components and to maintain a balanced cellular redox state (Jamieson, 1998). Within the enzymatic system, one group is represented by superoxide dismutase (SOD) and catalase which act directly as ROS detoxifiers removing oxygen radicals and their products and/or repairing the damage caused by oxidative stress. The other group consists of enzymes which act as redox regulators of protein thiols and contribute to maintain the redox balance of the cell such as peroxidases which reduce inorganic and organic peroxides into the corresponding

alcohols using active site cysteine thiols (Herrero *et al.*, 2008). These two groups overlap in the cell. Besides the enzymatic protection against ROS the non-enzymatic defense systems typically consist of small molecules which are soluble in either an aqueous or, in some instances, a lipid environment such as glutathione (GSH), ascorbate or vitamin E (Herrero *et al.*, 2008). One of the mechanisms by which *S.cerevisiae* responds to the oxidative stress is altering its own transcriptional program (Ikner and Shiozaki, 2005) and Yap1, together with Skn7, is one of the transcription factors mainly involved in this kind of response (also Msn2/Msn4 are implicated in this specific stress response). In addition it is known that several mutants characterized by an increased sensitivity to the oxidative stress are unable to grow on respiratory carbon sources maybe because of the high ROS levels accumulated in the cell during respiration (Thorpe *et al.*, 2004). Therefore considering that Yap1p is the major oxidative stress regulator we wondered whether *Δsdh6* mutant could be more sensitive than a wt strain to the oxidative stress. In particular as exogenous oxidative stress were used H_2O_2 and menadione, a specific mitochondrial stress inducer that produces superoxide radicals by redox cycling in the cell damaging mtDNA (Klöhn and Neumann, 1997).

Δsdh6 mutant is not sensitive to H_2O_2 and menadione

To evaluate the sensitivity of *Δsdh6* mutant a filter-paper disk method was performed with two different concentrations of H_2O_2 and menadione. 10e7 cells of each strains were plated on YNB+DO- URA glu 0.6% as described in material and methods section. 10 µl of H_2O_2 (2% and 5%) and menadione (20mM and 40mM) were adsorbed on sterile Whatman disks (diameter 9mm) placed in the middle of the plate. After 48h of growth at 28 °C the plates were photographed as shown in fig.10 and fig.11. The strains used were the *Δsdh6* mutant and wt BY4742 in addition to *Δsdh6/YAP1* and *Δsdh6/YAP2* in order to evaluate whether these two genes could in multicopy confer more resistance to the oxidative stress.

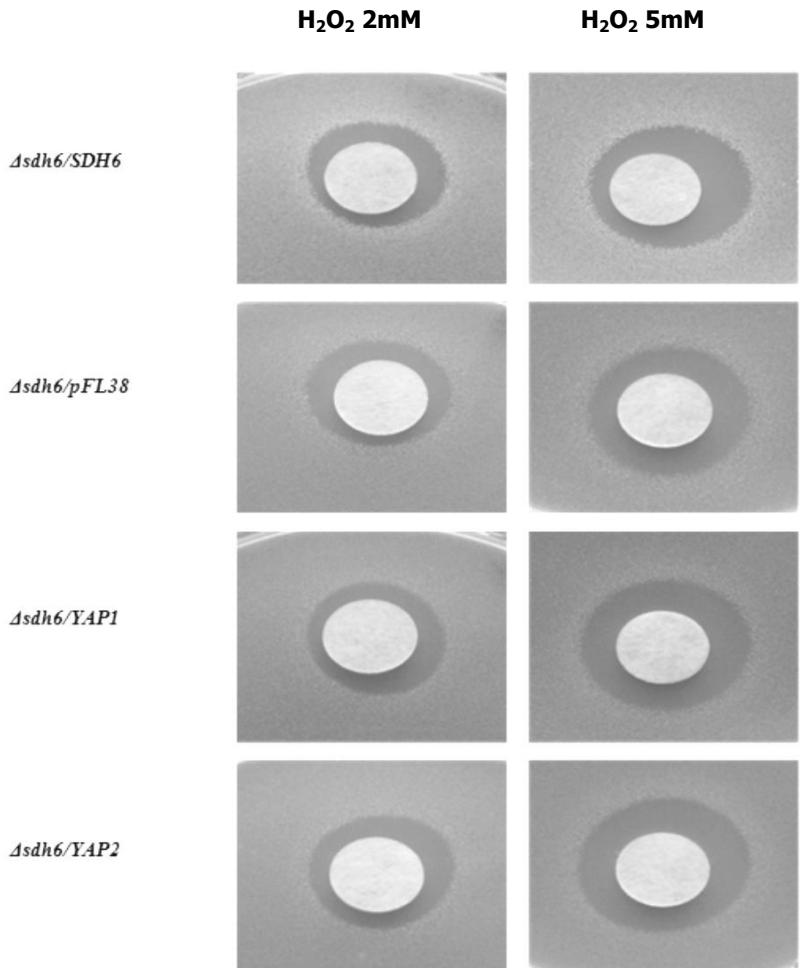


Figure 3.10. Paper disk assay with two different H_2O_2 concentrations.

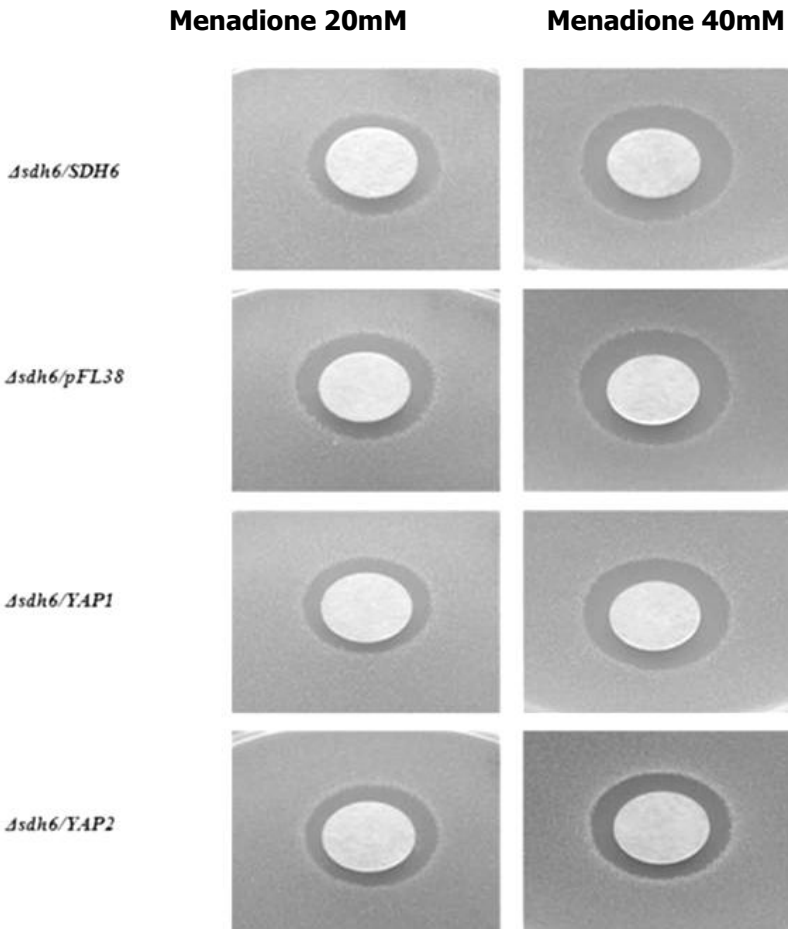


Figure 3.11. Paper disk assay with two different H_2O_2 concentrations.

As shown in the figures 10 and 11, no differences among the strains were observed with H_2O_2 or menadione treatments. These results suggested that the OXPHOS rescue by *YAP1* and *YAP2* was not due to an increased sensitivity to the oxidative stress in *Δsdh6* mutant. Furthermore these results indicated that the rescue was maybe due to the general action of all genes controlled by these transcription factors and not only to the action of a specific target since no genes were found in the multicopy suppressors research besides *YAP1* and *YAP2*. Indeed we tried also to overexpress *GLR1*, a gene encoding for the glutathione oxidoreductase known to be a target of *YAP1* and involved in the oxidative stress response and we did not observe a growth rescue (data not shown) underlining once more that:

- the single target of *YAP1* was not sufficient to compensate the OXPHOS defect of *Δsdh6* mutant;
- the rescue dependent on *YAP1* and *YAP2* was due to the action of other genes not involved in the oxidative stress response.

Therefore we focused on the second hypothesis we mentioned previously.

3.2.4. The mitochondrial iron homeostasis

Among several mechanisms in which *YAP1* and *YAP2* are involved, one is regulation of the iron metabolism. Indeed as previously described, the overexpression of these two genes confers resistance to the iron/zinc chelators 1, 10 phenantroline (Bossier *et al.*, 1993; Schnell and Entian 1991). Moreover, the addition of iron (but not zinc or copper) alleviates the growth inhibition of *Δyap1* mutant in alkaline medium (Schnell and Entian, 1991). Furthermore we performed an analysis with the online tool YEASTRACT (Yeast Search for Transcriptional Regulators And Consensus Tracking), a database of more than 48333 regulatory associations between transcription factors (TF) and target genes in *S. cerevisiae* based on bibliographic references (http://www.yeastract.com/). Noteworthy we found that several genes involved in the iron metabolism (not only mitochondrial) are controlled directly or indirectly by these two transcription factors. The following tables summarize the genes identified:

- Iron transporters controlled by YAP1

FRE1	Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels.
CCC1	Putative vacuolar $Fe2^{+}$ transporter; suppresses respiratory deficit of *yfh1* mutants, which lack the ortholog of mammalian frataxin, by preventing mitochondrial iron accumulation
FRE6	Putative ferric reductase with similarity to Fre2p; expression induced by low iron levels
FRE2	Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low iron levels but not by low copper levels
FIT1	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall
FRE7	Putative ferric reductase with similarity to Fre2p; expression induced by low copper levels
FRE4	Ferric reductase, reduces a specific subset of siderophore-bound iron prior to uptake by transporters; expression induced by low iron
MRS4	Iron transporter that mediates Fe2 transport across the inner mitochondrial membrane; mitochondrial carrier family member, similar to and functionally redundant with Mrs3p; active under low-iron conditions; may transport other cations

- Iron transporters controlled by *YAP2* and shared with *YAP1*

FRE1	Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels
MRS4	Iron transporter that mediates Fe2 transport across the inner mitochondrial membrane; mitochondrial carrier family member, similar to and functionally redundant with Mrs3p; active under low-iron conditions; may transport other cations

On the basis of these findings we investigated whether the suppression exerted by *YAP1* and *YAP2* was mediated by their role in iron metabolism. To answer to this question we evaluated whether the addition of iron to the medium could rescue the negative phenotype of *sdh6* mutant likely miming the actions carried out by *YAP1* and *YAP2*.

The addition of iron to medium rescues the OXPHOS growth defect of Δsdh6 mutant

It has been reported that *Δyap1* mutant cannot grow in alkaline medium but the addition of iron to this medium is able to restore the growth of the mutant (Schnell and Entian, 1991). To see if iron could have a similar effect also on the *sdh6* mutant a serial dilutions growth test was performed adding $FeSO_4$ to the medium. The oxidative growth of *Δsdh6* mutant and the parental strain was analyzed in a medium supplemented with three concentrations of $FeSO_4$: 0.3mM, 0.75mM and 5mM added to YNB+DO+ 2% glucose, 2% ethanol and 2% K-acetate based-plates.

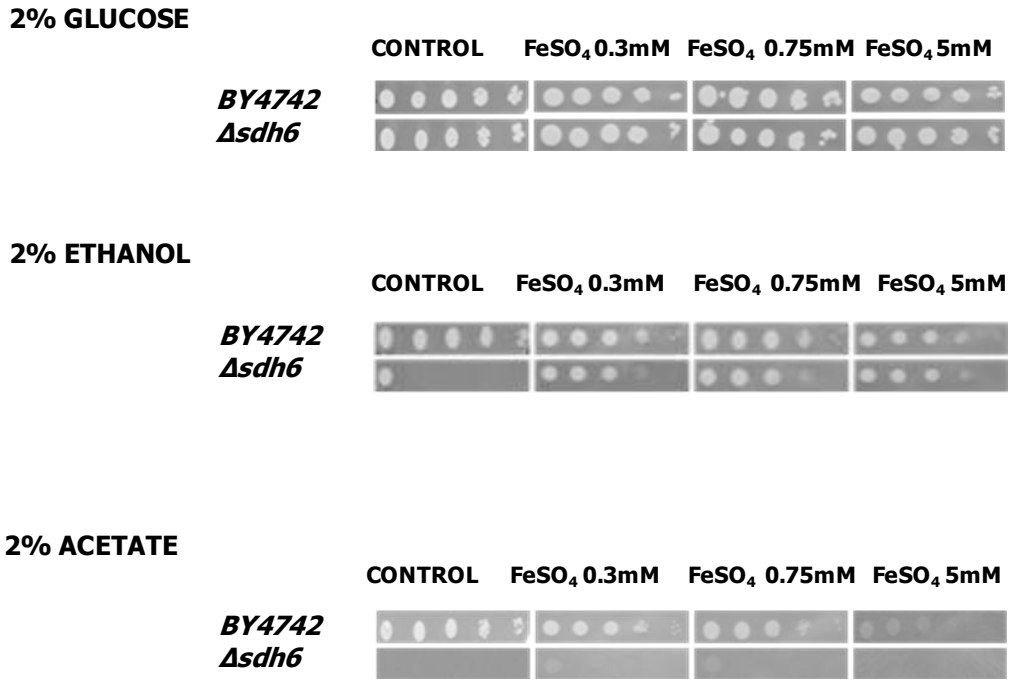


Figure 3.12. Spot assay in presence of different $FeSO_4$ concentrations.

We observed that the addition of iron was able to rescue the *Δsdh6* OXPHOS phenotype. The growth was particularly evident after 1 week of growth at 28°C and on ethanol as carbon source. This result led us to evaluate this rescue also on the mutant strains carrying *YAP1* and *YAP2*. In this experiment, another iron concentration was added (7 mM) as well as another carbon source (2% glycerol).

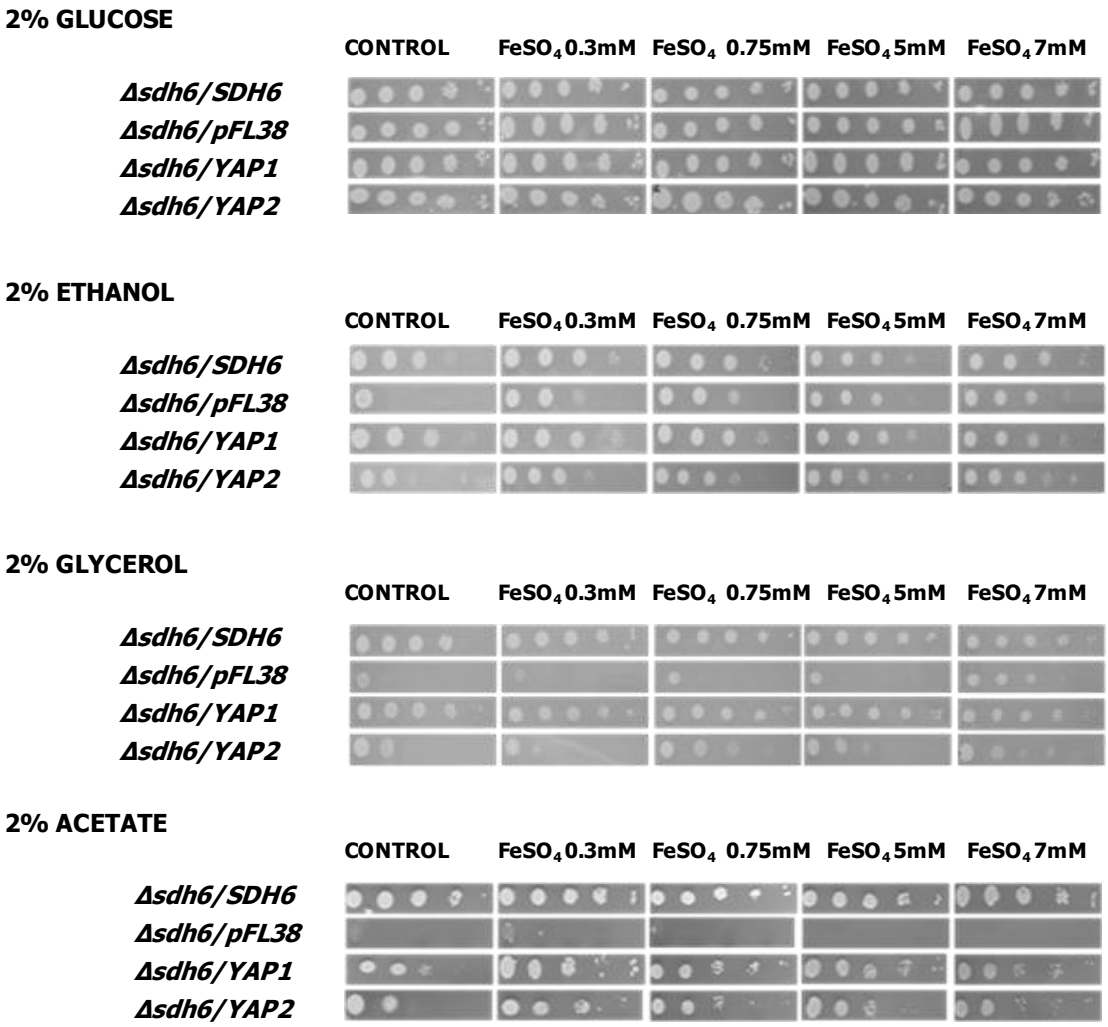


Figure 3.13. Spot assay in presence of different $FeSO_4$ concentrations on the transformants strain carrying *YAP1* and *YAP2*.

The results of the spot-assay analysis showed that the rescue was possible both on ethanol and glycerol (fig. 13). Moreover these results led to two additional observations:

1. the rescue on glycerol medium was possible only at 7 mM concentration;
2. the addition of iron ameliorated the growth dependent by *YAP1* and *YAP2* compared to the control without iron.

In order to exclude that the observed growth was due to the presence of sulfur in the medium, the same experiments were made with $FeCl_2$ and the spot assay is reported in fig.14.

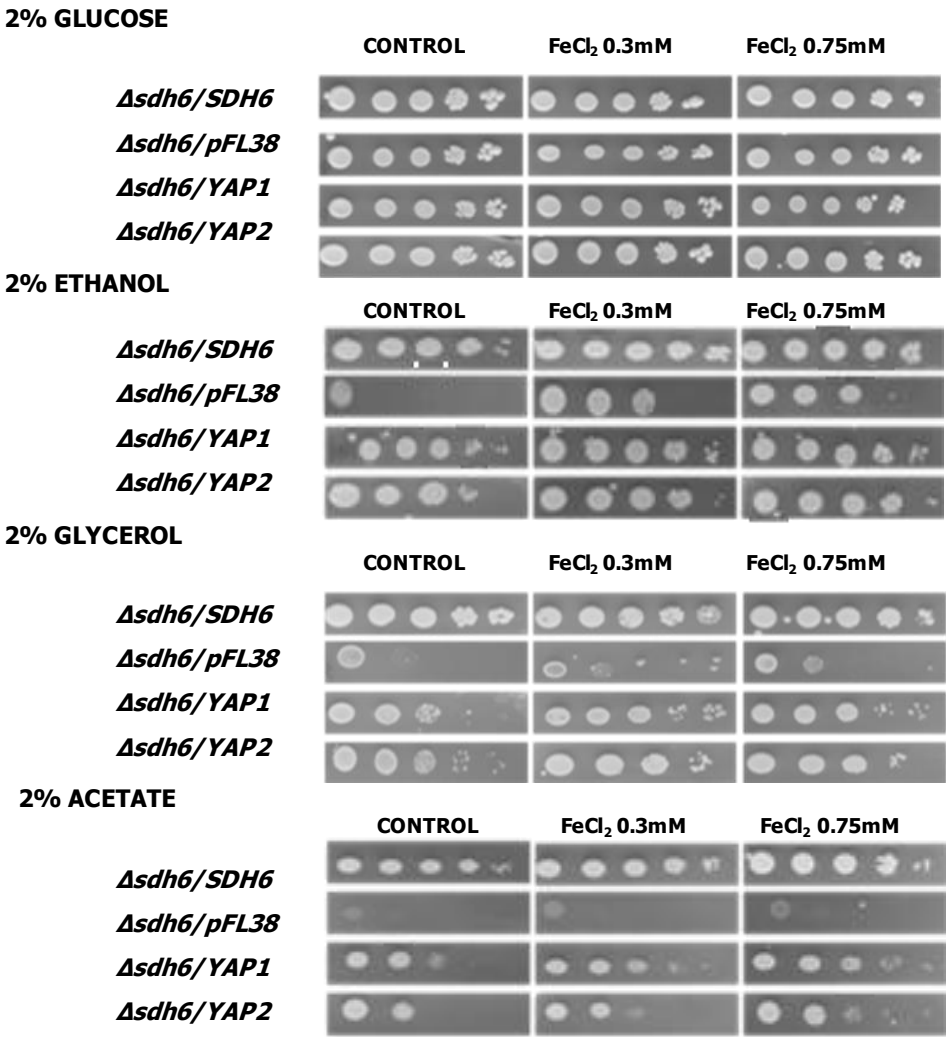


Figure 3.24. Spot assay in presence of different $FeCl_2$ concentrations on the transformants strain carrying *YAP1* and *YAP2*.

Again, we observed a rescue of OXPHOS defect either in ethanol and glycerol medium underlining that the rescue was dependent on the iron element. Furthermore, the strains transformed with *YAP1* and *YAP2* grew better than the control in presence of iron. In addition the effect of iron on the oxidative growth was specific for *Δsdh6* mutant since no amelioration of growth was observed for other *sdh* mutants (*sdh1-5*, data not shown) in presence of iron salts. The reason why the "iron rescue" is observed only in ethanol and glycerol and not in acetate medium is not clear. One reason could be the toxicity of K-acetate in *S.cerevisiae* cells. In particular, the K-acetate can be toxic for the cells and actually it is known that *Δsdh6* mutant is not able to grow when ethanol and acetate are both present in the medium (although in ethanol it should grow poorly). Another possible reason for the absence of growth could be due to the iron chelating action of organic acids such as TCA intermediates: in this mutant this action could be exerted by succinate

which is accumulated as a consequence of specific SDH impairment (Szeto *et al.*, 2007). Interesting was the work of Lemire group in which they analyzed the metabolomics of SDH mutants. They observed that the impairment of SDH activity was accompanied by large amounts of extracellular acetate: this result can lead to speculate that whatever is the effect of acetate on our mutant, this effect could be strengthened by the extracellular acetate secretion (Szeto, 2010).

The addition of iron does not increase the SDH activity in Δsdh6 mutant

These data led us to consider whether the addition of iron could partially restore SDH activity. We evaluated SDH in *Δsdh6* and BY4742 grown both in glucose 0.6% and ethanol 2% plus $FeSO_4$. The iron concentration chosen was 0.75mM and was added directly to the ethanol medium.

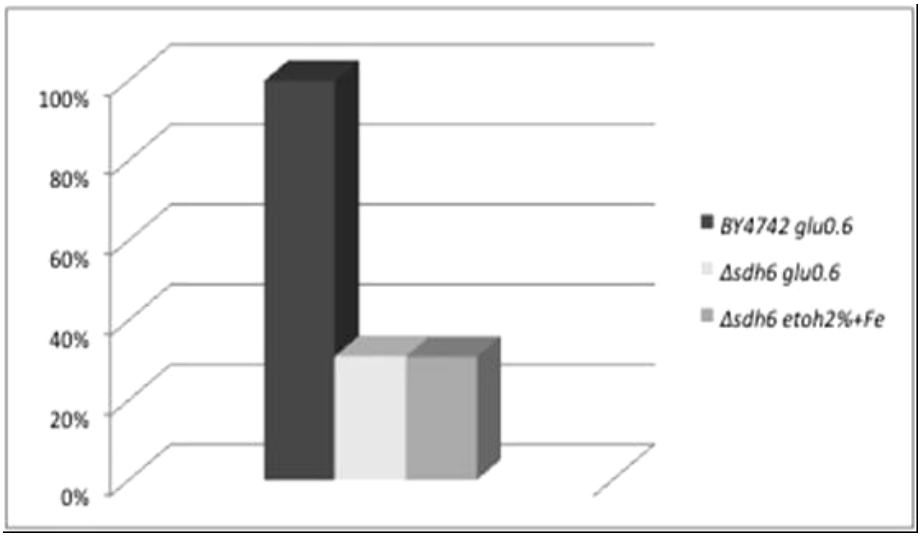


Figure3.35. SDH assay in presence of $FeSO_4$ in the medium.

As expected no increase of SDH activity was observed with the addition of iron salts to the medium. Therefore, as previously observed for the *Δsdh6* mutant overexpressing *YAP1* and *YAP2* also the rescue exerted by iron was not mediated by an increase of SDH activity.

The results so far obtained once more favor the hypothesis that Sdh6p has a second role in the cell besides the SDH assembly. It is possible to speculate that the OXPHOS negative phenotype of *Δsdh6* mutant is both due to a reduced SDH activity and to a lack of another putative cellular function. We hypothesized that this second role may be linked to iron metabolism/homeostasis since both overexpression of *YAP1* and *YAP2* and the iron supplementation are able to restore the oxidative growth without increasing SDH activity.

The observation that a residual enzymatic activity of about 30-40% is sufficient to partially support an oxidative growth (see fig 3.9) favors this hypothesis.
However the mechanism (direct or indirect) by which *YAP1* and *YAP2* can regulate iron mobilization/availability in the *Δsdh6* mutant remains to be elucidated.
Examples of growth without an amelioration of complex II activity are known in literature. A recently work of Gonzales *et al.*, showed that the *Δyfh1* strain characterized by an abnormal growth in respiratory conditions, was rescued by addition of FAD 10 μM to medium (Gonzalez-Cabo *et al.*, 2010). Strikingly, all the respiratory complexes activities which were significantly impaired in the mutant, were increased by FAD except complex II. Thus the authors suggested that FAD effect on growth improvement of frataxin-deficient cells was independent of complex II. Furthermore, they speculated that the growth capability on a respiratory medium without an intact respiratory chain able to guarantee the transport of electrons to CoQ, was due to the electron transfer flavoprotein (ETF) complex. In mammalian this system is composed by the ETF-dehydrogenase (ETF-QO) and ETF, a heterodimer made of two subunits (ETFa and ETFb), that delivers electrons coming from β-oxidation of fatty acids and amino acid catabolism to CoQ (Eaton, 2002). In *S. cerevisiae* there is a putative gene *YPR004C* as the ETFa homologue and *YGR207C* as the homologue of ETFb. The authors therefore speculated that the observed growth was due to the FAD action on complex I and ETF complex (Gonzalez-Cabo *et al.*, 2010). Although this example showed as a growth was possible without a rescue of enzymatic activity, the underlain explanation is not suitable for the *Δsdh6* mutant (FAD did not rescue the negative *Δsdh6* phenotype, data not shown). Indeed this putative ETF-complex should already support the growth of this mutant in physiological conditions. As previously suggested the observed rescue could be a consequence of the suppression exercised on a defect associated to the additional role of Sdh6p on which both *YAP* genes and iron act.

BPS treatment is detrimental for Δsdh6 mutant

Since the addition of iron caused an amelioration of *Δsdh6* growth, we wondered what could happen in a condition of deprivation of iron. Therefore we used the Fe(II) chelator bathophenantholine disulfonic acid (BPS), 40 μM and 80 μM, to prepare glucose and ethanol plates. 10e5 cells of *Δsdh6* and BY4742 were spotted for each condition indicated in fig 16 with or without BPS.

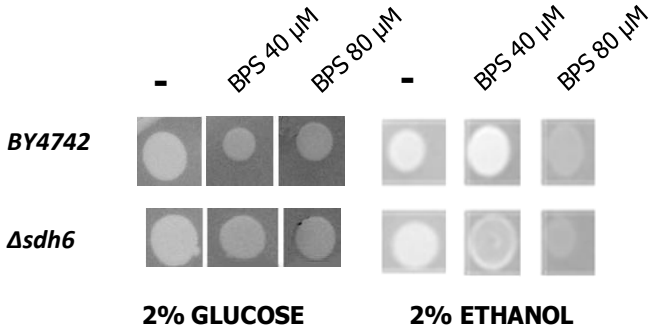


Figure 3.16. Treatments with two different BPS concentrations

As depicted in fig. a marked decrease of *Δsdh6* growth on ethanol was visible in presence of BPS. In particular if we compare the spots on 40 µM BPS it is evident how the *sdh6* mutant is more sensitive than wt despite the same amount of cells were spotted as indicated in the control spot. Conversely, on glucose medium no differences between *Δsdh6* and *BY4742* were visible. Therefore it is possible to speculate that the absence of iron is deleterious for the viability of *Δsdh6* mutant in respiratory medium. We evaluated the growth only in ethanol as carbon source since here it is possible to appreciate a growth of this mutant.

Considering that the iron supplementation was positive for the growth on respiratory carbon sources of *sdh6* mutant and that on the contrary, its absence was detrimental, we investigated whether and how the *SDH6* expression was modulated by addition or depletion of iron.

SDH6 expression is induced by iron

In order to evaluate the expression of *SDH6* in presence or absence of iron, total RNA from BY4742 grown in several conditions were extracted. In particular BY4742 was grown in:

- 2%Glucose
- 2%Glucose + Fe 0.75 mM
- 2%Ethanol
- 2% Ethanol + Fe 0.75 Mm
- 2%Glucose + BPS

As described in the material and methods section, a Retro-Transcription reaction was carried out to obtain cDNAs to be processed with a standard PCR. The PCR products were run in a 2% agarose gel and the results are represented in the following figure.

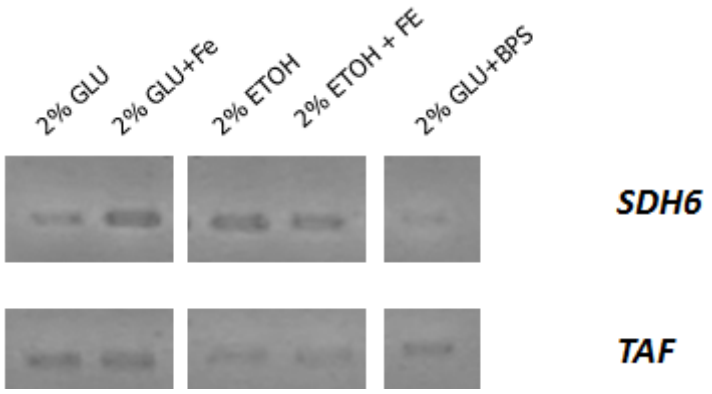


Figure 3.17. Expression of *SDH6* in presence or absence of iron. Transcripts of *SHD6* were detected by RT-PCR of total RNA from BY4742 grown in YP added with the different carbon sources. RT-PCR control for all samples is represented by the amplification of *TAF10*. The cells were stocked at 1 OD.

The expression pattern shown in the fig 17 indicated that *SDH6* is induced by iron. Indeed, in a medium containing glucose plus iron we can observe an increased *SDH6* signal while *TAF10* remains constant. In ethanol we cannot appreciate the induction by iron since, as described in the chapter II, ethanol itself determines a *SDH6* upregulation. Furthermore, we evaluated the expression of *SDH6* in a medium with BPS. A marked decrease of *SDH6* signal was observed despite the loading control assured the presence of RNA. This result supports the hypothesis that Sdh6p can be involved in the metabolism of iron and not in its transport within the cell because in this case in absence of iron its upregulation should be expected. These speculations require further investigations.

Despite the great knowledge on the function and structure of the SDH complex, little is known about its assembly intermediates, factors involved, the timing as well as the mechanism of the addition of the various prosthetic groups. Only in the 2009 two assembly factors specific for SDH complex have been identified: *SDH6/YDR379C-A* and *SDH5* in yeast, in human *SDHAF1* and *SDHAF2* respectively.

While it is clear that Sdh5p is involved in flavination of Sdh1p subunit, nothing is known about the action mechanism of Sdh6p.

The work presented in this thesis has given more insights into the knowledge of *SDH6* through genetic and biochemical studies performed in the yeast *S. cerevisiae.*

In particular the results described in the first part were focused on understanding the role of Sdh6p in the assembly and full functioning of SDH complex. Through 2D-BNGE analysis a marked reduction of complex II was observed in *Δsdh6*. This result definitely demonstrated that defective SDH activity is caused by a reduced number of enzyme units rather than by qualitative alterations of complex II. Furthermore both the fact that Sdh6p is required for a full assembly of complex II and that it is a rate limiting step in this process are supported by experimental evidence. The decrease of SDH activity indeed cannot be bypassed over-expressing the four SDH subunits in the mutant. Moreover the steady state levels of SDH subunits 1 and 2 are decreased in *Δsdh6* as pointed out in SDS-PAGE analysis. An interesting contribute was given by the biochemical analyses performed with the recombinant variants of Sdh6 tagged protein. Indeed the results obtained performing a 2D-BNGE analysis on the strain expressing Sdh6p-HA indicated that Sdh6p does not establish stable physical contact with complex II nor it takes part to other molecular complexes. In addition affinity purification analyses demonstrated that Sdh6p is not an interactor of subunit 1 and 2 of SDH complex, since these two proteins are not eluted together with Sdh6p.

The results obtained in this work suggested that *SDH6* plays a different role from that played by *SDH5*, the other conserved SDH assembly factor. Indeed neither the overexpression of *SDH6* rescues the OXPHOS defect of *Δsdh5* and nor the expression in multicopy of *SDH5* is able to restore the respiratory growth of *sdh6* mutant. Therefore the two SDH specific assembly factors are not interchangeable.

To evaluate whether Sdh6p was necessary for the insertion or assembly of Fe-S clusters into complex II we performed a genetic interaction study between *SDH6* and *ISD11*.

ISD11 encodes for an essential protein of the ISC biogenesis and as well as *SDH6* contains a LYR motif that is a signature for proteins involved in Fe-S metabolism. Moreover the two proteins show a significant similarity. As already discussed, SDH complex contains three Fe-S centers but nothing is known about the mechanism of insertion of these clusters in the backbone of CII. The overexpression of *ISD11* did not rescue the SDH defect due to the *sdh6* mutation. The experiments performed so far, albeit not exhaustive, do not favor an involvement of Sdh6p in Fe-S centers biogenesis.

To further investigate the functional role of *SDH6* a search for multicopy suppressors was performed. This analysis identified *YAP1* and *YAP2*, two transcription factors involved in several cellular processes. Although *YAP1* and *YAP2* are able to rescue the OXPHOS defect of *sdh6* mutant, their action is not exerted by increasing the SDH activity. Therefore their suppression mechanism seems to be independent of complex II and could be ascribed to an indirect effect of these two transcriptional factors. *YAP1* and *YAP2* might suppress the OXPHOS negative phenotype by compensating some other cellular defects which are impaired in the *sdh6* mutant.

Thus Sdh6p should have an additional role besides the SDH assembly: in this case the OXPHOS negative phenotype of the null *sdh6* mutant would be due to both a reduced SDH activity and to a lack of the other putative function. The observation that several *sdh* mutant with a 30-40% of residual SDH activity are able to grow on oxidative carbon sources much better than *sdh6* mutant strengthens this hypothesis.

In order to understand the mechanism of suppression exerted by *YAP1* and *YAP2* we focused on two main functions linked to these genes: the oxidative stress and the iron metabolism. The first was chosen since Yap1p is a master oxidative stress gene regulator in the yeast cells. The second because among targets of Yap1p and Yap2p there are genes encoding for iron transporters. In addition it is worth mentioning that *YAP1* and *YAP2* were identified in a multicopy suppressors research since they were able to confer resistance to the iron chelators 1,10-phenantroline and 1-nitroso-2-naphtol. On the basis of the results obtained we favor the hypothesis that the suppression mechanism exercised by *YAP1* and *YAP2* could be mainly ascribed to their role in iron metabolism/homeostasis. Moreover when the effect of iron supplementation on the oxidative growth of the *Δsdh6* strain was tested a rescue of the OXPHOS phenotype was observed without any increase of SDH activity. The results obtained support the view that the second role of Sdh6p could be related to iron metabolism/homeostasis. Further analysis will be necessary to unravel

this hypothesis and the measurement of the iron intracellular content could be for instance a useful tool to investigate in the *sdh6* mutant a possible imbalance of the iron homeostasis.

In order to dissect the two possible functions of *SDH6*, the one linked to cII assembly from that related to the iron metabolism response and in order to identify which amino acid residues within Sdh6p are critical for its function *in vivo* two approaches will be pursued. First, a search for multicopy suppressors of the strains carrying the two pathological mutations will be carried out. To this purpose the pathological alleles $SDH6^{R55P}$ and $SDH6^{G57R}$ have been integrated at *SDH6* locus and the two mutants strains have been constructed. This analysis could provide different information respect to that obtained with the search for multicopy suppressors previously performed. Indeed, the suppression of the null mutation generally creates a bypass, whereas the suppression of missense mutations may identify interactors. Second a collection of random *sdh6* point mutants will be generated and analyzed. The intragenic and extragenic suppressors identified being able to restore the respiratory competence of the negative mutant will be then selected and characterized.

All together these analyses will allow to identify putative *SDH6* interactors which might clarify the role of this gene within the cell.

Material and Methods

Miscellanea

Strains used

- *S.cerevisiae strains*

NAME	GENOTYPE	REF
BY4741	*Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	(Brachmann et al., 1998)
Δsdh6	*BY4741 ydr379c-a::KANMX4*	(Brachmann et al., 1998)
Δsdh5	*BY4741 yol071w::KANMX4*	(Brachmann et al., 1998)
Δtcm62	*BY4741 ybr044c::KANMX4*	(Brachmann et al., 1998)
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(Brachmann *et al.,* 1998)
Δsdh6	*BY4742 ydr379c-a::KANMX4*	2009, our laboratory
W303-1a	*MAT*a *leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Brachmann *et al.,* 1998)
W303-1b	*MAT*α *leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Brachmann *et al.,* 1998)
Δsdh6	*W303-1a ydr379c-a::KANMX4* *W303-1b ydr379c-a::KANMX4*	This study
isd11BY4743	*Mat a/α, his3Δ1/ his3Δ1; leu2Δ0/ leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YER048w-a::kanMX4/YER048w-a*	(Brachmann*et al.,* 1998)
nfs1 BY4743	*Mat a/α, his3Δ1/ his3Δ1; leu2Δ0/ leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YCL017c::kanMX4/YCL017c*	(Brachmann *et al.,* 1998)
jac1 BY4743	*Mat a/α, his3Δ1/ his3Δ1; leu2Δ0/ leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YGL018c::kanMX4/YGL018c*	(Brachmann *et al.,* 1998)
yah1 BY4743	*Mat a/α, his3Δ1/ his3Δ1; leu2Δ0/ leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YPL252c::kanMX4/YPL252c*	(Brachmann *et al.,* 1998)

- *Bacterial strains*

NAME	GENOTYPE	REF
DH10B	*Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	(Brachmann et al., 1998)

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 NH_4SO_4 1,9 g/L, NH_4SO_4 5 g/L). Minimum media was enriched with drop-out powder (Kaiser et *al.*, 1994) . If necessary singles aminoacids could be excluded from complete drop-out to maintain selective pressure .
- 5-FOA YNB: YNB ForMedium™ with 1 g/l *5-Fluoroorotic Acid* (Melford), 50 mg/l uracile with aminoacids necessary to complement the auxotrophies (Boeke et *al.*, 1984).
- Sporification Medium: 2% potassium acetate, 0.25% yeast extract ForMedium™.

As solidifying agent 2% agar ForMedium™ 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), Potassium Acetate (A).

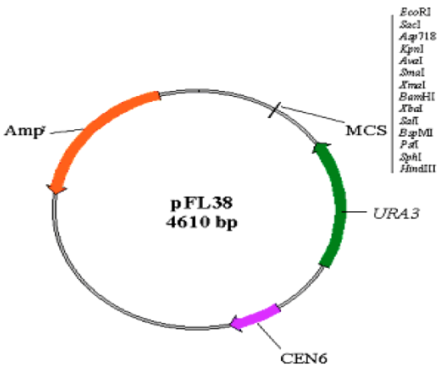
S. cerevisiae was cultured at 28°C, in constant shacking 120 rpm if liquid media was used.

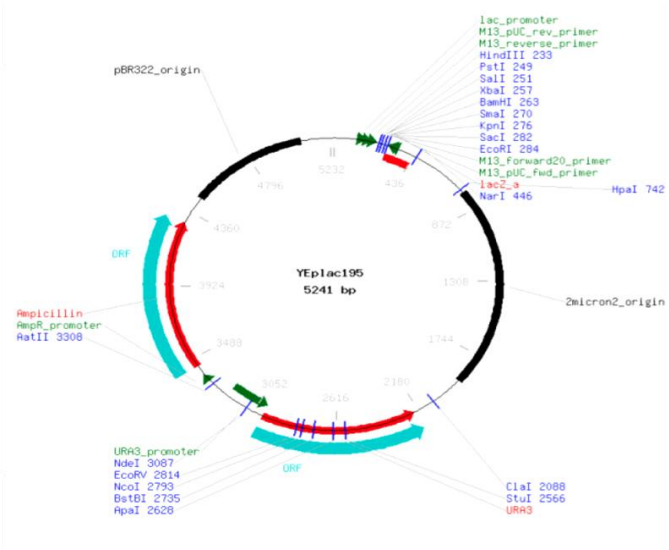
For *E. coli* LB media was used (1% bacto tryptone Difco™, 0.5% yeast extract Difco™, 0.5% NaCl, pH 7.2-7.5). Agar 2% and ampicillin (Sigma-Aldrich®) 100mg/ml were added if needed. For α-complementation selection 80µl of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Xgal) 2% (dissolved in dimethilformamide) and 40µl isopropyl-beta-D-thiogalactopyranoside (IPTG) 23,8 mg/ml were added.

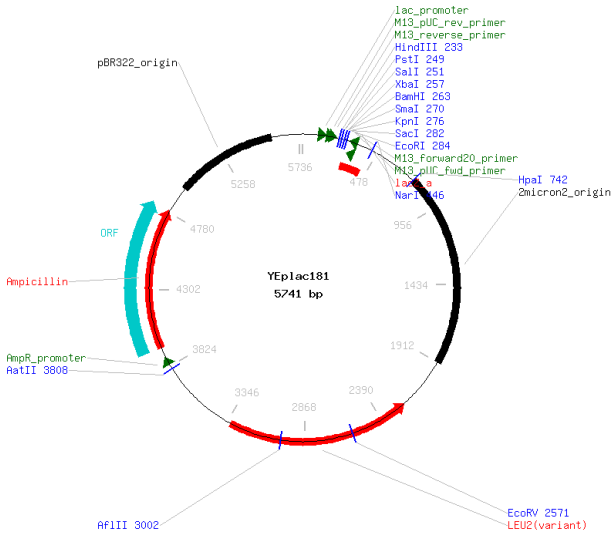
E.coli cultures were incubated at 37°C in constant shacking if necessary.

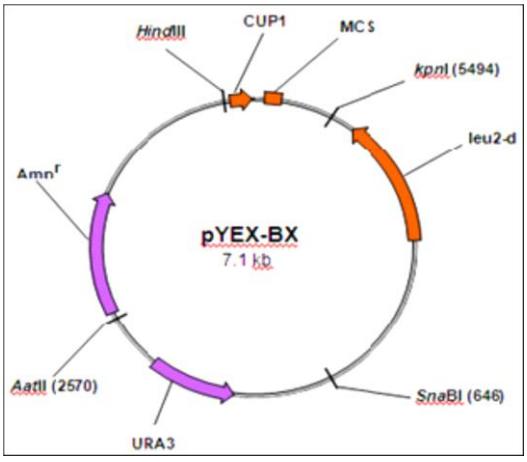
Plasmids

In particular for this study the following plasmids were used:









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 were performed with a high fidelity polymerase. KOD HiFi DNA polymerase was used (Novagen®).

For the yeast colony PCR cells were suspended in 10 µl H_20 after 24h of growth in selectable medium. 3 µl were utilized for the PCR reaction and after 15' at 95 °C the standard PCR reaction was carried out adding GoTaq® DNA polymerase (©Promega).

The primers utilized are the following:

- *Cloning primers*

Target gene	**Oligo sequence**	**Restriction site at 5' end**
ISD11	Fw: GGATCCATGTCCACAGTAAAGTAGAT Rv: AAGCTTCTTATAGTCCCATGTGG	*BamHI* *HindIII*
GLR1	Fw: CCCCGAATTC CAGCATTGAAAGCGTGGAAG Rv: CCCCAAGCTT GAGTCCACAGTAGCTAATAG	*EcoRI* *HindIII*
SDH5	Fw: CCCCGGATCCCGCGCAAACTGTTTCCCACAG Rv: CCCCAAGCTTGATGGTGCAACAACAATAATA	*BamHI* *HindIII*
SDH6	Fv: GAATTCCGGTAAATGACGGCTATCCGCG Rv: CTGCAGGCAAGCATAATACAGATTTGC	*BamHi* *PstI*
TCM62	Fw: CCCCGAATTCCCGCTATCTGTGGGAGTGA Rv: CCCCCTGCAGAGGATACAAGGACACAG	*EcoRI* *PstI*
YAP1	Fw: CCCCGAATTCGCTGATCTTACCCTGTTGCA Rv: CCCCAAGCTTCCGAGGTGATACAATCTACCT	*EcoRI* *HindIII*
YAP2	Fw: CCCCGGATCCGCTCTGGACAAGTACCAGTT Rv: CCCCCTGCAGAGGTATCCACTTGTATCGGA	*BamHI* *PstI*
YFH1	Fw:GGGGGGATCCCCGTATAGATTCATGCTCCGGAC Rv:CCCCCTGCAGCTTCAGTGCAGGTATTGCGTGG	*BamHI* *PstI*

- *Tag primers*

Target gene	**Oligo sequence**
SDH6-HA	Rv:CTGCAGAAGTAAGAAAATAGTTCAAGCGTAATCTGGAA CATCGTATGGGTAATGTATATTCGTCAGTTCAGGGTGTGA
SDH6-HIS	Rv:GGGGCTGCAGAGTAAGAAAATAGTTCAGTGGTGGTGGT GGTGGTGATGTATATTCGTCAGTTCAGGGTGTG

- *Control primers (for colony PCR)*

Target gene	**Oligo sequence**
SDH6	Fw:GTCTTACACCTGTATAGGGC Rv: CGTCATTCAGGGTGTGAAAAG

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 2KV, 25 µF 200 Ω.

Alternatively, $CaCl_2$ competent cells were prepared and transformed with standard techniques (Maniatis *et al.,* 1982).

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 the cells were regenerated in two ml of YPAD for 2 h and then plated on plates added with G418.

Integration of mutant alleles R55P and G57R at SDH6 locus

The integration at *SDH6* locus requires two principal genetic steps:

- the substitution of *KanMX* cassette with *URA3* cassette in *Δsdh6* mutant (Fig.2.A) (Voth *et al.*, 2003).
- the subsequent substitution of URA3 cassette with the *R55P* and *G57* alleles (Fig.2.B)

This strategy was possible thanks to the plasmid M4758 (fig.1) which contained the *URA3* cassette flanked both ends by parts of *KanMX* cassette.

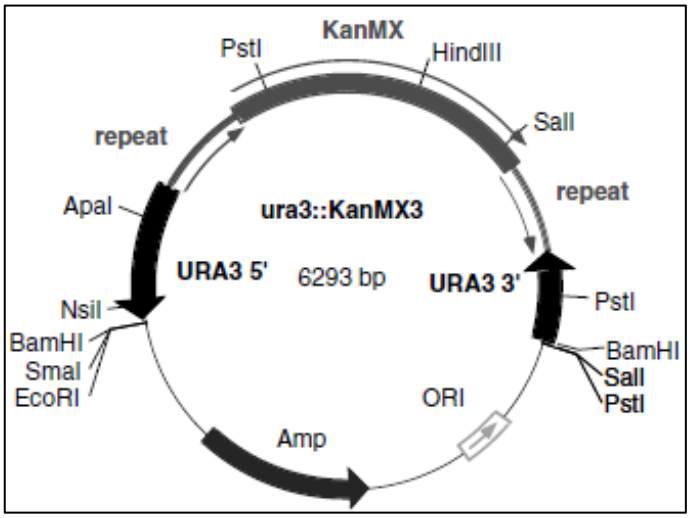


Figure4.1. M4758 plasmid

A scheme of this genetic approach is reported below:

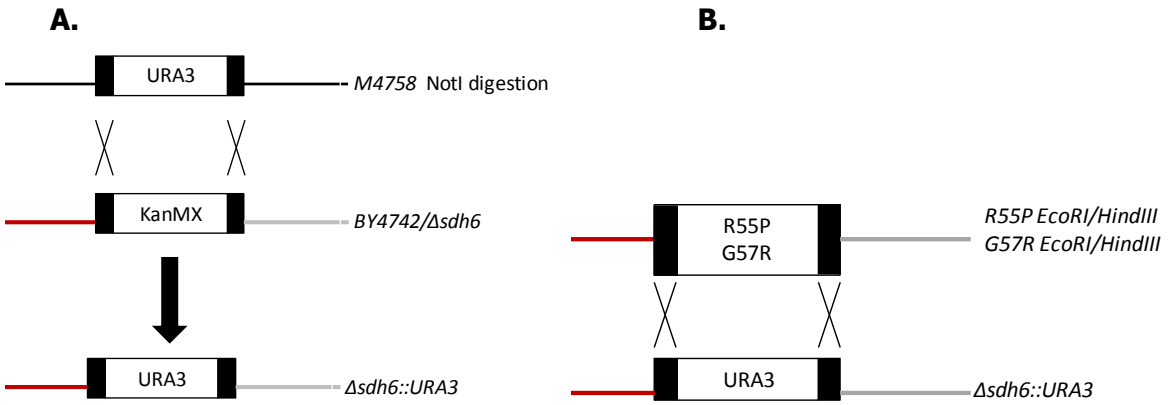


Figure 4.2. Scheme of mutant alleles integration at SDH6 locus. (A.) The first step substitutes KanMX cassette with URA3. (B). Integration of *SDH6*^{R55P} and *SDH6*^{G57R} cassette.

M4758 plasmid was digested with *NotI* and two fragments were obtained: one of 1700 bp containing URA3 cassette and the other of 2400 bp with the rest of plasmid. Following the digestion, *Δsdh6* (*Δsdh6::KanMX)* was transformed with the long protocol described by Woods and Gietz. The selection of transformants was carried out on selectable medium without uracile. Transformants were also controlled on G418 containing plates. The transformants able to grow both on uracile and G418 based-medium in which the homologous recombination was correctly occurred, were transformed with the whole *SDH6*^{R55P} and *SDH6*^{G57R} cassettes obtained by PCR. The protocol was the same used before. The cells were plated on YPD and replicated on 5-FOA containing plates after 24h at 28°C. A PCR on genomic DNA extracted was performed to assure the correct cassette integration.

Subsequently, spot assays were carried out to evaluate the transformants phenotypes.

Multicopy suppressors research

The *sdh6::KanMX* strain was transformed with the standard LiAc method (Gietz *et al.,* 2002) and the pSEY8 (URA3) genomic library was used (300ng). Transformants were plated on YNB+ DO- URA added with 2%glucose. After growth at 28 °C the cells were replicated on YNBAcetate plates and the growth of suppressor clones was observed. All the clones were subsequently analyzed by PCR to exclude the clones able to grow thanks to the wt gene. Then the plasmids were recovered from the Ace^{+} transformants and were sequenced at both ends with universal primers. The genes were identified through a BLAST research. A plasmid loss was carried out to correlate the growth on YNBA to the plasmids contained in the cells.

Plasmid loss

Each clone Ace^+ was inoculated in 10 ml of YPD 2% in order to induce the loss of plasmids. After 24h of growth, 200 cells were plated on YPD plates and then were replicated on YNBA - uracile. So the growth on acetate based medium was linked to the presence of the plasmids in the cells.

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).
- ✓ RNA extraction was perfomed as described by Ausubel *et al.* (Ausubel et *al.*, 1994) and quantified.

RT-PCR

Probes

Target gene	Oligo sequence
SDH6	Fw:GTCTTACACCTGTATAGGGC Rv: CGTCATTCAGGGTGTGAAAAG
TAF10	Fw:ATATTCCAGGATCAGGTCTTCCGTAG Rv: GTAGTCTTCTCATTCTGTTGATGTTGT

Reaction I:

Material needed:

3 µg total RNA
3 µl DNAse I Buffer (Invitrogen™)
3 µl DNAse I (Amp Grade Invitrogen™)
H_20+DEPC 1% (diethyl pyrocarbonate treated)
to a final volume of 30 µl

Protocol:

For each sample, 3 µg RNA were utilized.

1. Add 3 µl DNAseI (Invitrogen™) and incubate the samples 15' at room T.
2. Add 3 µl EDTA 25 mM to inactivate DNAse I and incubate the samples 10' at 65 °C.
3. Precipitate the reactions with 1/10 vol NaAc 3M pH 5.2 and 2.5 vol of ETOH96%. Freezing with N_2 before storing at -80 °C overnight.

Reaction II:

Solutions needed: H_20+DEPC 1%
Oligo dT [Oligo(dT)15 Primer]
RT BUFFER 10X (NEB Biolabs)
5mM dNTPs
RNAse-OUT (Invitrogen™)
M-MuLV Reverse transcriptase (NEB Biolabs)

Protocol:

1. After precipitation, resuspend RNA pellets in 16 µl H_20+DEPC 1% and the samples are split in two eppendorf tubes: one will be for the control without Reverse Transcriptase.
2. Add 1 µl oligo-dT and incubate the samples 10' at 70°C.
3. In ice, add the following components in a final volume of 20 µl:

RT-BUFFER (10X)	2	µl
dNTP (2,5 mM)	4	µl
Rnasi-OUT	0,5	µl
Reverse Transcriptase	1	µl
H_2O + DEPC 1%	3,5	µl

Transcriptase is added to all samples except for the control

4. Incubate the samples at 42°C for 2h and stop reactions with heat inactivation (70°C for 15')
5. Process the samples with PCR or stock them at -20°C.

PCR Reaction

cDNAs are diluted 1:10 and 2 µl are utilized for a normal PCR reaction with GoTaq® DNA polymerase (©Promega). The PCR reactions were performed following manufacturer indications. PCR products were run on a 2% agarose gel and controlled.

Protein separation with SDS-page

Protein separation with SDS-page was performed with classical Laemmli system (Laemmli, 1970). Separating gels were prepared at 12% or 15% polyacrylamide (36:1 acrylamide-bis), stacking gel at 5%. Running was performed for 1hr 30' at 100-120volts in a Mini Trans-Blot cell (BIORAD).

Blue Native Gel Electrophoresis (BNGE)

Isolated mitochondria were treated as described by Nijtmans et al. (2002). 150 µg were solubilized with 1% Lauryl maltoside and the extracts were loaded and run into a 5-13% gradient non-denaturing 1D-BNGE. For denaturing 2D-BNGE, the 1D-BNGE lanes were excised and treated for 1h30' at room temperature with 1% SDS, 1% β-mercaptoethanol and then run through a 16.5 % tricine-SDS-polyacrylamide gel using a 10% spacer gel (Schagger and von Jagow, 1987).

Western Blotting and Ig-detection

Separated proteins were transferred to nitrocellulose membrane (GE Healthcare) by electroblot for 1hr 15' at 100V or overnight at 20V. The electroblotting system was used. Quality of the blot was assessed with Ponceau S staining.

Membranes were blocked 1hr with 5% non-fat dry milk prepared in washing buffer (TBS 1X+ Tween 0.1%) and incubated o/n with appropriate primary antibody (mono or polyclonal).

Blocked membranes were washed 3 times for 15 min with washing buffer prior incubation with suitable secondary antibodies (Anti-Rabbit, Anti-Mouse and Anti-Rat) conjugated with horseradish peroxidase (1:5000 in 5% milk).

The antibodies used in this study were:

- Anti-Sdh2 against the subunit 2 of SDH complex (a kind gift from Dott. B.Lemire)
- Anti-Atp4 against the subunit 4 of Complex V (a kind gift from Prof. J. Velours)
- Anti-HA against the hemagglutinin epitope of Sdh6p-HA fusion protein (Roche Applied Science)

- Anti-His against the SDH6-HIS fusion protein (Bethyl Laboratories inc)

After 1hr incubation membranes were washed as above and developed with *ECL™ Western Blotting Detection Reagents* (GE Healthcare) commercial kit. If greater sensitivity was necessary the *ECL™ Advance Western Blotting Detection Reagents* (GE Healthcare) kit was utilized.

Whole cell analyses

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 OD_{600} 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.

Filter paper disk-method

This assay allows to evaluate the strain sensibility to specific substances. 20 ml of 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 were added on the disk and after an incubation at 28 °C for 3-4 days, the halos were observed.

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.

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).

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 37°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 poor medium supplemented with 0.2% glucose and 2% ethanol. Incubate at 28°C for 3-4 days and count the cells.

0.2% 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. 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.

Mitochondrial Analysis

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 KPO_4 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.

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.

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 NaN_3

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).

Cytochrome c oxidase (COX) enzymatic activity

This assay measures the rate of cytochrome c oxidation by complex IV in yeast as described by Warthon and Tzagaloff, 1967.

Solutions needed: 10mM K-phosphate pH 7
80 µM cytochrome c
1 mg/ml BSA (in EDTA 10 mM, pH 7.4)

COX activity was recorded at 550 nm.

Specific activity: COX activity is calculated with cytochrome c ε of 21 and the following formula (ΔOD*ε/min mg).

Mitochondrial Protein Localization

Based on Glick procedure

Prepare mitochondria with intact outer membrane as previously described.

1. Centrifuge 10000rpm, 10 minutes 4°C to pellet mitochondria. Set up two different tubes with 250µg of total proteins
2. Wash sample 1 and 2 with 0.6M sorbitol, 20mM HEPES pH 7.4
3. Set up the following conditions:

	1	2
Mitochondria	250µg	250µg
0.6M sorbitol, HEPES 20 mM pH 7.4	250µl	250µl
Proteinase K (1mg/ml)	-	5µl

4. Incubate samples on ice for 60 minutes
5. Add 5µl of 0.1M PMSF to stop reaction of Proteinase K
6. Centrifuge 15300 rpm, 22 minutes 4°C. Remove and keep supernatants
7. Pellet is resuspended in 0.1ml of 0.6M sorbitol, 20mM HEPES pH 7.4
8. Centrifuge 14000rpm, 5 minutes 4°C
9. Dissolve pellets in 100µl of 1X SDS-page loading buffer (LB) and add 2µl of 0.1M PMSF
10. Load 20 in 15% SDS-page and perform western against different mitochondrial proteins. Ideally use a control for each mitochondrial compartment

Solubilization of mitochondrial proteins

To solubilize mitochondrial tagged proteins (for following biochemical characterization) different extracting conditions were tested to find the mildest one. The following test protocol was used:

1. Pellet 0.25 mg of total mitochondrial proteins centrifuging 10000rpm 4°C
2. Discard supernatant and resuspend in 25µl of Extraction buffer

   Extraction buffer: 20mM HEPES
   0.5mM PMSF
   + detergent (digitonin, laurylmaltoside)
   + salt (NaCl)
   water to 100µl
3. Incubate 10 min on ice
4. Centrifuge 15300 rpm, 30 minutes 4°C
5. Transfer 25 µl of supernatant (+5 µl H_2O) in a new tube and add 10 µl of LB4X
6. Wash the pellet twice with 10mM HEPES without resuspending
7. Resuspend pellet in 40µl of LB1X

Equivalent µg from pellet and supernatant are loaded in SDS page to evaluate the extraction efficiency for protein of interest.

Affinity purification analyses with Ni-NTA

For affinity purification analyses a Ni-NTA agarose (Qiagen) affinity matrix was used, consisting of nickel-nitrilotriacetic acid coupled to Sepharose beads.

General protocol can be briefly summarized as follows.

Use 60µl of resin for 2 mg of mitochondrial proteins, solubilized with the opportune extraction buffer supplemented with 1/10 vol Complete Protease Inhibitor Cocktail (Roche) and 1/10 vol PMSF 10mM .

Solutions needed:

Buffer A: 50mM Na-Phospate pH 8.0
15mM Imidazole
50mM NaCl
15% glycerol

1. Pellet Ni-NTA beads 1000 rpm 1min 4°C
2. Wash three times with 10 volumes of Buffer A
3. Add supernatant from mitochondrial protein solubilization (2-4mg)
4. Incubate o/n 4°C in gently rotation
5. Centrifuge 1min 1000 g 4°C
6. Recover the supernatant (Flow Through fraction) and keep the pellet
7. Wash 5 times with 1.5ml of Buffer A
8. Eluition with SDS loading buffer 1X.

Volumes and timing have to be optimized. A standard condition can be 20-30 minutes in gently rotation at room temperature. Load equivalent quantities of FT and ELUATE fractions on SDS-page along with extraction control.

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