Abstract of Doctoral Thesis

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Inherited Disorders of Cytochrome *c* Oxidase Biogenesis

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1. INTRODUCTION

Mitochondria

Mitochondria are localized in the cytoplasm of nearly all eukaryotic cells. They are usually depicted as stiff, elongated cylinders with a diameter of 0.5 – 1 μm. In the context of a eukaryotic cell, these dynamic organelles integrate numerous metabolic pathways and are the major producers of ATP – the universal energetic fuel. Without them, eukaryotic cells would be dependent on the relatively inefficient process of glycolysis, which yields only 2 molecules of ATP per molecule of glucose compared to 30 molecules produced, when glucose oxidation is completed in mitochondria (Alberts 2002).

The research of the past 30 years has consolidated the hypothesis of mitochondria as endosymbionts of a primitive eukaryote. The serial endosymbiosis theory postulates that a protoeukaryotic cell without mitochondria evolved first, and this organism then captured a proteobacterium by endocytosis.

Mitochondria are surrounded by the inner and outer membranes that create two separate mitochondrial compartments, the internal matrix space, and a narrow intermembrane space. The inner membrane is folded into numerous cristae, which greatly increases its total surface area. This membrane contains proteins with three major types of functions: the enzymes of the respiratory chain, ATP synthase complexes, and specific transport proteins that regulate the passage of metabolites and macromolecules across the inner mitochondrial membrane.

The Oxidative Phosphorylation Apparatus

The electrons from oxidized substrates stored in the form of redox equivalents NADH and FADH are utilized by the oxidative phosphorylation apparatus (OXPHOS) in the inner mitochondrial membrane. The OXPHOS consists of the complexes of the respiratory chain proteins - complex I, NADH-CoQ oxidoreductase; complex II, succinate-CoQ oxidoreductase; complex III, bc_1 complex, and complex IV, cytochrome *c* oxidase. These protein complexes are associated with a variety of redox-active prosthetic groups with successively increasing reduction potentials that transport electrons from NADH and FADH to oxygen (Fig 1.1). In addition to the traditional components mentioned above, the respiratory chain contains yet other dehydrogenases, e.g. the glycerophosphate dehydrogenase (GPDH) (Houstek et al. 1975) and flavoprotein-linked acetyl CoA-dehydrogenase (Liang et al. 2001). The free energy released during the electron transport is stored in the form of proton

gradient ($\Delta\mu_{H}^{+}$) across the inner mitochondrial membrane. The proton gradient is consequently used by the F_oF_1 ATPase for phosphorylation of ADP. It also drives transport of metabolites and macromolecules across the membrane and can be dissipated in the form of heat by the uncoupling proteins (Fig. 1.1).

The respiratory chain complexes have been thought to be laterally mobile in the phospholipid bilayer of the inner mitochondrial membrane, but recent studies convincingly show that the complexes are organized in supramolecular structures (Schagger 2001).

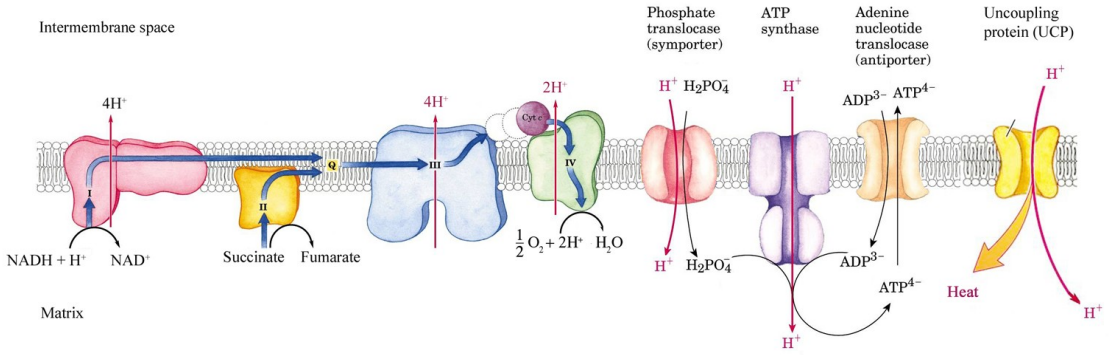

Figure 1.1 The Oxidative Phosphorylation Apparatus. *The apparatus comprises the respiratory chain enzymes (complexes I, II, III and IV), which transport electrons from NADH or FADH to oxygen and concomitantly generate proton gradient across the inner mitochondrial membrane. The proton gradient is subsequently used by F_OF_1 ATPase for ADP phosphorylation, drives the ADP/ATP exchange and transport of inorganic phosphate into mitochondria or it can be dissipated by uncoupling proteins. Adapted from (Lehninger et al. 2000).*

Mitochondrial Biogenesis and Genetics

The biogenesis of mitochondria, as the only organelle of mammalian cell, depends on the coordinated expression of two genomes, nuclear and mitochondrial. The human mitochondrial genome is a 16,569 base pairs long circular DNA (Anderson et al. 1981). Proteins encoded by mtDNA are absolutely essential for mitochondrial energetics. mtDNA has a prokaryotic character, is highly compact and contains only 37 genes: 2 genes encode ribosomal RNAs, 22 encode transfer RNAs, and 13 encode OXPHOS polypeptides. mtDNA codes for 7 subunits of Complex I (ND1-6 and ND4L), cytochrome *b* of Complex III, 3 subunits of Complex IV (COX I-III) and 2 subunits of Complex V (ATPase A6 and A8).

Most of the proteins that reside in the mitochondrion are nuclear gene products. These proteins are translated in the cytoplasmic compartment of eukaryotic cells and subsequently transported into the mitochondrion. The nuclear-encoded proteins play key roles in mitochondrial transcription and translation, mitochondrial lipid and heme synthesis, substrate

oxidation by the tricarboxylic acid cycle, and OXPHOS system. All together more than 500 different nuclear proteins are present in all four mitochondrial compartments (inner and outer mitochondrial membranes, matrix and intermembrane space) and they account for > 90 % of the protein mass of the mitochondrion.

Transcriptional regulators that act on both nuclear and mitochondrial genes have been implicated in the bi-genomic expression of the respiratory chain. Mitochondrial transcription is directed by a small number of nucleus-encoded factors (Tfam, TFB1M, TFB2M, mTERF). The expression of these factors is coordinated with that of nuclear respiratory proteins through the action of transcriptional activators and coactivators. In particular, environmental signals induce the expression of PGC-1 family coactivators (PGC-1alpha, PGC-1beta, and PRC), which in turn target specific transcription factors (NRF-1, NRF-2, and ERR alpha) in the expression of respiratory genes. This system provides a mechanism for linking respiratory chain expression coordinately from both genomes according to environmental conditions, and for integrating it with other functions related to cellular energetics (Scarpulla 2006).

Mitochondrial Diseases

Deficiencies in oxidative phosphorylation are associated with diverse array of multisystem disorders that are often referred to as mitochondrial encephalomyopathies because of the prominent involvement of the nervous system and striated muscle. Primary OXPHOS defects can be caused by mutations in mtDNA or nuclear genes.

Mitochondria follow the rules of population genetics. Six aspects of their behavior are critical for understanding the etiology and pathogenesis of mitochondrial disorders: (1) they are maternally inherited; (2) cells typically contain hundreds of organelles and thousands of mitochondrial genomes; (3) mutations can arise in a mtDNA population, resulting in the coexistence of two or more mtDNA genotypes within a single cell, organ or individual (heteroplasmy); (4) if the mutation is pathogenic, the proportion of mutated molecules in the heteroplasmic population (mutational load) affects the severity of the biochemical defect, but not necessarily in a linear fashion; (5) mtDNA replication and inheritance in lineages of somatic cells is stochastic, often resulting in changing mutational loads during the life of the patient, and in different mutational loads in different cells and tissues (mitotic segregation); (6) because different cell types have different minimal oxidative energy requirements (thresholds), the level of heteroplasmy and the dynamics of the mitotic segregation play a critical role in determining the clinical presentation and outcome.

Owing to the fact that more than 98 % of genes for mitochondrial proteins are coded in the nucleus, much attention has recently turned to the investigation of the nuclear OXPHOS gene defects. The majority of these are inherited as autosomal recessive traits, producing severe and usually fatal diseases in infants. Defects in approximately 20 different nuclear genes have now been identified which encode structural components of the OXPHOS complexes, assembly/maintenance factors and factors necessary for the maintenance of mtDNA integrity.

Cytochrome *c* Oxidase

Cytochrome *c* oxidase (COX; EC 1.9.3.1) is the terminal enzyme of the respiratory chain. It is localized in the inner mitochondrial membrane where it transfers electrons from ferrocytochrome *c* to molecular oxygen, the ultimate acceptor of all reducing equivalents. It belongs into superfamily of heme-copper oxidases which is defined by high sequence similarity within the largest subunit I and a binuclear active site consisting of a high-spin heme (heme a_3) and a closely associated copper ion (Cu_B) (Abramson et al. 2001).

The eukaryotic cytochrome *c* oxidase is a two-face enzyme. On one hand it is extremely conservative in performing its role of transporting electrons from cytochrome *c* to molecular oxygen in essentially all aerobic organism, thus allowing them to extract a substantial portion of free energy and store it in the form of electrochemical gradient of protons. The three core catalytic subunits, the enzyme prosthetic groups, both the electron transport and proton pumping mechanisms, and the elegant way it handles such potentially toxic compound – oxygen, have virtually not changed from the simple oxidase of mitochondrion's procaryotic ancestor to the multisubunit enzyme complexes present in modern eukaryotes. It is the addition of novel subunits during the evolution of eukaryotic cytochrome *c* oxidase that represents the other, rather unconservative face of the enzyme. The new components seem to allow the eukaryotic cell to modulate the cytochrome oxidase activity according to its energy demand. In mammals these novel subunits outnumber the original ones ten to three (Fig 1.2), and they confer the enzyme many interesting features such as tissue specifity, modulation of proton transport efficiency, regulation by reversible phosphorylations and undoubtedly many other. This way cytochrome c oxidase becomes fully intertwined within the fascinating regulatory network of the cell and perhaps, as many believe, one of the key regulators of cell metabolism.

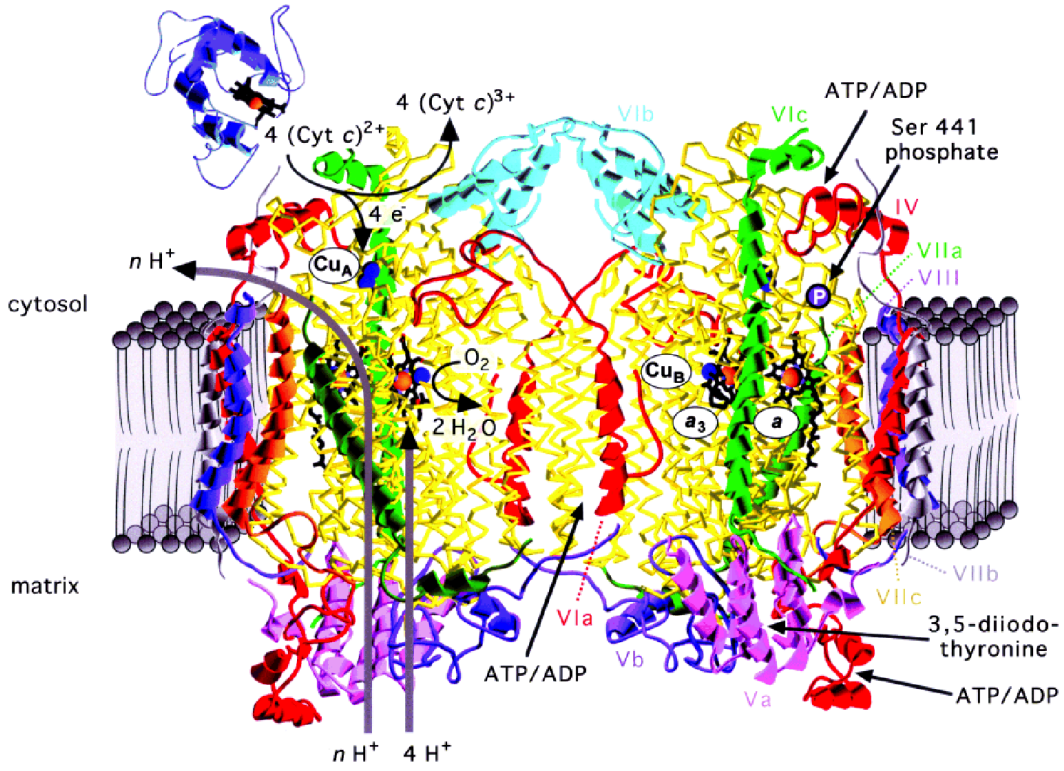

Figure 1.2 Structure of COX Dimer from Bovine Heart Mitochondria. *Indicated are: binding sites for substrates (cyt c and O_2) and allosteric regulatory factors (ATP/ADP, diiodothyronine) and phosphorylation epitope at subunit I (Ser 441). Adapted from (Ludwig et al. 2001).*

Assembly of COX Holoenzyme

Assembly of the functional multisubunit enzyme cytochrome *c* oxidase requires coordinated interplay of both nuclear and mitochondrial genome. In addition to expression of 3 structural subunits encoded by mtDNA and 10 by ncDNA, numerous "assembly factors" are necessary for successful biogenesis of the complex. These assembly factors take part in multiple levels of COX biogenesis, namely mRNA processing, mRNA translation, synthesis and incorporation of prosthetic cofactors, insertion of proteins into mitochondrial membrane and their assembly, and quality control and proteolytic degradation of misfolded proteins.

Our knowledge of the components that participate in the biosynthesis of COX is mostly based on studies of yeast respiratory defective (*pet)* mutants. Screens of nuclear *pet* mutants revealed a group of more than 30 genes that selectively affect expression of COX (Tzagoloff and Dieckmann 1990). The function of most of these genes was identified during the last ten years (reviewed in (Fontanesi et al. 2006)). Proteins encoded by genes *COX10*, *COX15* and *YAH1* are involved in the synthesis of heme *a*. Genes *COX11*, *COX17*, *SCO1* and *SCO2* code for proteins responsible for the incorporation of the copper ions into COX.

Products of genes *SHY1*, *COX14*, *COX16*, *COX20*, *COX22*, *COX23*, *COX24*, *PET100* and *PET 117* participate in subunit assembly.

Numerous homologs of these yeast genes have been found in human genome, which led to an optimistic suggestion that the COX biogenesis is evolutionary so much conserved among eukaryotes that the results from the studies in the yeast model of COX assembly provide generally relevant information. One of the original approaches used for identification of the human homologs is screening of an expressed sequence tags database (EST), which led to discovery of *COX15*, *COX11*, *SCO1* and *PET112* (Petruzzella et al. 1998).

Using two-dimensional electrophoresis, Nijtmans et al. identified four assembly intermediates (S1 – S4) that accumulate during COX assembly and most probably reflect the rate-limiting steps of the sequential incorporation of the 13 subunits of mammalian COX (Fig. 1.3) (Nijtmans et al. 1998). The intermediates could not be found in yeast mitochondria even in assembly deficient strains, probably due to efficient clearance by mitochondrial proteolytic enzymes. The assembly sequence is, however, thought to follow the scheme proposed in mammalian cells (Fontanesi et al. 2006). Since 1998, the scheme has been only slightly refined – subunit COXVa was shown to assemble at S2 level along with COXI and IV (Williams et al. 2004) and also that COXVa interacts with COXIV prior to association with COX I (Stiburek et al. 2005).

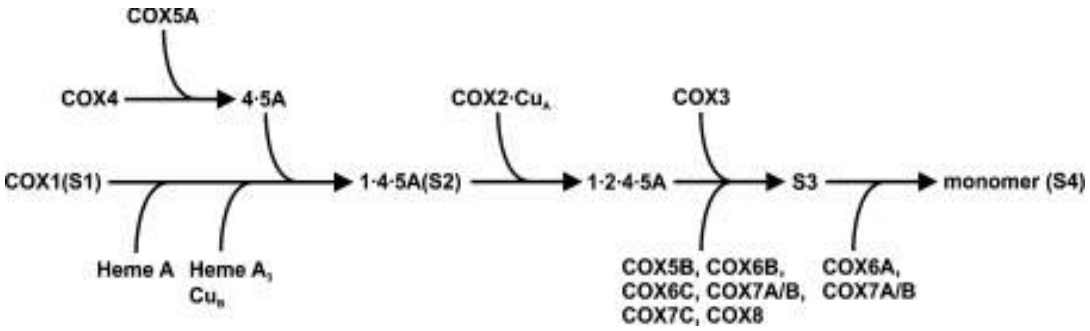

Figure 1.3 Model of the Assembly Pathway of Human COX. *The assembly intermediate S1 comprises just subunit COX I, S2 subcomplex is formed after incorporation of heterodimer of subunits IV and Va. Incorporation of subunit II starts the sequential assembly of ncDNA encoded subunits Vb, VIb, VIc, VIIb, VIIc, and VIII resulting in the S3 subcomplex. The assembly is finished with the addition of subunits VIa and VIIa, making possible the COX dimerization. Adapted from (Stiburek et al. 2005).*

COX Pathologies

Cytochrome *c* oxidase and complex I deficiencies are the most frequent cause of respiratory chain defects in humans. Patients affected with COX defects present heterogeneous clinical phenotypes. This clinical heterogeneity is probably due to a large number of genes influencing COX expression and possible tissue-specific differences existing in the cellular abundance of COX-related gene products (Barrientos et al. 2002).

Mutations in ncDNA Genes Encoding COX Assembly Factors

Numerous cases of isolated COX deficiency of nuclear origin could not have been associated with any pathogenic mutations for a long time. In recent years, sequencing of candidate genes encoding homologs of yeast COX assembly factors identified etiologic mutations for COX deficiencies with Mendelian inheritance. Interestingly, no mutations in any of the ten ncDNA encoded subunits have been found while mutations of assembly factors include six genes - *SURF1*, *SCO1*, *SCO2*, *COX 10*, *COX 15*, and *LRPPRC (Tab. 1).*

The Leigh Syndrome (LS) Associated with Isolated COX Deficiency (LS^{COX}) - represents the largest group of mitochondrial disorders caused by mutation in nuclear genome. LS (i.e. subacute necrotizing encephalomyopathy) is a progressive neurodegenerative disease. The severe symptoms usually have onset in less than a year after birth and are characterized by general psychomotor retardation and bilaterally symmetrical lesions in the basal ganglia region. An increased level of lactate in both blood and cerebrospinal fluid is observed. The disease is fatal in vast majority of cases, the patients usually die before five years of age (Rahman et al. 1996). In 1998, mutations in the ***SURF1*** gene were identified in LS^{COX} patients by two groups independently (Tiranti et al. 1998; Zhu et al. 1998). *SURF1* encodes an inner mitochondrial membrane protein homologous to yeast Shy1 protein which was previously shown to be necessary for proper transfer of electrons between respiratory complexes III and IV (Mashkevich et al. 1997). Recent studies on Shy1 revealed that, in cooperation with Mss51p and Cox14p, it helps regulate the translational activation of the COXI transcript according to the rate of assembly of the newly synthesized COXI into the holoenzyme (Barrientos et al. 2004). The absence of mammalian homologs of the Mss51p and the Cox14p, and the differences in translational regulation between yeast and mammals, preluded the extreme challenge of finding the role of mammalian Surf1p, which is described in the following section.

Table 1 – Mutations Causing Isolated Defects of Cytochrome c Oxidase*. The table summarizes all mutated genes causing isolated COX deficiencies that have been identified to date.*

GENE	***FUCTION***	***INHERITANCE***	***CLINICAL PHENOTYPES***
COX1	Structural subunit of the enzyme	Maternal	Multisystem mitochondrial disorder, motor neuron degeneration, myopathy with recurrent myoglobinuria
COX2	Structural subunit of the enzyme	Maternal	Encephalopathy, proximal myopathy and lactic acidosis
COX3	Structural subunit of the enzyme	Maternal	MELAS, Leigh-like syndrome, encephalomyopathy
SURF1	COX assembly – progress from S2 to S3 assembly intermediate	Autosomal recessive	Leigh syndrome
SCO1	Copper delivery to the enzyme	Autosomal recessive	Neonatal hepatic failure and encephalopathy
SCO2	Copper delivery to the enzyme	Autosomal recessive	Neonatal cardioencephalomyopathy
COX10	Heme *a* farnesyl-transferase	Autosomal recessive	Neonatal tubulopathy and encephalopathy, Leigh syndrome, cardiomyopathy
COX15	Heme *a* synthesis	Autosomal recessive	Early-onset hypertrophic cardiomyopathy, Leigh syndrome
LRPPRC	Transcriptional regulation of genes encoding mitochondrial proteins	Autosomal recessive	Leigh Syndrome, French Canadian type

Surf1p Expression and Function

The *SURF1* mRNA levels were found the same in all studied tissues with the exception of brain, where they were lower. The gene is expressed in substoichiometrical amount relative to COX subunits (Yao and Shoubridge 1999). No *SURF1* mRNA (Tiranti et al. 1999) or Surf1 protein (Surf1p) could be detected in patients with mutations resulting in premature stop codon (Tiranti et al. 1999; Yao and Shoubridge 1999). In a later study, the *SURF1* mRNA was detected at normal level in patients harboring missense point mutations, however, the Surf1 protein wasn't found either (Poyau et al. 2000).

The potential role of Surf1p was studied at all levels of COX expression. No changes could be found in the amount of both ncDNA or mtDNA encoded COX transcripts or the efficiency of their translation, suggesting that Surf1p functions at later stage of COX expression. Using two-dimensional BN-PAGE/SDS-PAGE electrophoresis, Tiranti et al. observed accumulation of early assembly intermediates of COX in LS^{COX} patients. The assembly was blocked at the level of S2 intermediate suggesting that Surf1p facilitates the incorporation of one or more of subunits COX II, COX III and majority of ncDNA encoded subunits (Tiranti et al. 1999). About 20 % of control amount of the COX holoenzyme was assembled despite the absence of Surf1p suggesting that there was a certain level of redundancy in the assembly process. Impaired COX assembly in LS^{COX} patients was confirmed in another study (Coenen et al. 1999). Also, using 2D electrophoresis, the assembly defect was confirmed in *Δshy1* yeast. In a wild-type strain, Shy1p was found in a multimeric 250 kDa complex together with COXII subunit suggesting their interaction (Nijtmans et al. 2001).

SURF1 Mutations and Changes in COX Structure and Function

More than 30 *SURF1* mutations have been reported since their discovery as the primary cause of LS^{COX}. Most of them are reviewed in (Pequignot et al. 2001), several more mutations were published since then - a splicing site mutation causing the loss of exon 8 (Williams et al. 2001), three new mutations were found in lymphoblastoid cells from Japanese Leigh syndrome patients (Ogawa et al. 2002). A novel C574→T transition changing conserved arginine 192 into tryptophane was reported from screening of Czech patients (Capkova et al. 2002). Genetic screening of 65 Chinese patients with Leigh syndrome revealed SURF1 mutations in eight of them, with major prevalence of the G604→C transition changing aspartate 202 into histidine (Yang et al. 2006). Another two novel mutations were identified in patients with distinct clinical phenotype – prominent renal symptons and muscle ragger red fibers (Tay et al. 2005). More mutations will most probably be discovered in near future thanks to routine sequencing of the *SURF1* mutations in numerous clinical laboratories all over the world. Severely decreased COX activity was reported in all LS^{COX} patients both before and after the *SURF1* mutations discovery. The spectrophotometrically measured activities ranged between 5 - 40 % of control values (Glerum et al. 1987; Lombes et al. 1991; Zimmermann and Kadenbach 1992; Possekel et al. 1996; Tiranti et al. 1998; Zhu et al. 1998; von Kleist-Retzow et al. 1999; Sue et al. 2000) Kinetic analyses revealed unchanged K_M and dramatic decrease of V_{max} of COX (Lombes et al. 1991; Zimmermann and Kadenbach 1992).

The COX complexes in LS^{COX} fibroblasts were found to be more sensitive to lauryl maltoside and increased temperature (Possekel et al. 1996).

The COX assembly defect in LS^{COX} cells is further characterized by general decrease of the steady-state levels of COX subunits (Hayasaka et al. 1989; Zimmermann and Kadenbach 1992; von Kleist-Retzow et al. 1999; Yao and Shoubridge 1999; Poyau et al. 2000; Sue et al. 2000). This fact most probably reflects decreased stability and rapid degradation of unassembled subunits. No major differences between the steady-state levels of mtDNA- and ncDNA-encoded COX subunits were reported for LS^{COX} cells.

Abnormal calcium homeostasis in LS^{COX} fibroblasts was reported and possible connection with low mitochondrial membrane potential was established (Wasniewska et al. 2001). A possible role of Surf1p in calcium homoeostasis was also suggested in a study on a model of mouse *SURF1* knock-out by group of professor Zeviani from Milano, Italy (Dell'agnello et al. 2007). The absence of Surf1p in mice results in milder COX deficiency than in humans. Therefore, the neurological phenotype is not recapitulated in this model. Paradoxically indeed, the *SURF1* knock-out animals displayed significant resistance to kainate-induced neurodegeneration compared to their control littermates. It seems that *SURF1*-lacking neurons do not face such massive calcium efflux that would lead to excitoxicity and cell death. Nearly ten years after the discovery that LS^{COX} is caused by *SURF1* mutations, its role still remains enigmatic. Solving this question will definitely require divine enlightenment or a great portion of luck.

COX Involvement in Common Disease Phenotypes

Besides classical OXPHOS disorders, dysfunction of COX associates also with other pathological states, which are currently in the spotlight of medical research. Mutations in mtDNA genes have been analyzed by an increasing number of laboratories in order to investigate their potential role as an active marker of tumorigenesis in various types of cancer (Gallardo et al. 2006). While many studies support an active role of mtDNA in tumorigenesis, there are also many caveats that make the implication of mtDNA variants a debatable issue. It is still unclear whether the somatic mtDNA mutations found in tumors are a consequence of the tumorigenic process or a predisposing condition leading towards it. Numerous investigations associating mtDNA variants with cancer may also be based on sequencing results that failed to recognize polymorphisms belonging to mtDNA haplogroups (Salas et al. 2005). In a recent study taking all possible pitfalls into account, however, a COXI mutation

G6276>A was identified in several cancer cell lines. These cell lines originated from different cancer types and carried various mtDNA haplotypes. The COXI G6276>A mutation caused a significant decrease in COX activity and mitochondrial respiration. The mutations was found in tumors but also in normal tissues of cancer patients suggesting that it is germline transmitted and plays a role in predisposing to tumorigenesis (Gallardo et al. 2006).

mtDNA variants and mitochondrial dysfunction are in the spotlight due to their potential role in common pathologies such as type2 diabetes or Alzheimer's disease (AD). The putative involvement of mitochondria in pathogenesis of diabetes and metabolic syndrome is attracting increasing attention, breakthroughs can be hopefully expected in near future (reviewed in (Taylor and Turnbull 2005). The relation between mitochondrial dysfunction and Alzheimer's disease is characterized in more detail. Amyloid beta peptide, the Alzheimer's hallmark, has been shown to accumulate inside mitochondria, where it contributes to decline of ATP production and increased ROS production, in part by associating with cytochrome *c* oxidase (reviewed in (Ohta and Ohsawa 2006). AD pathogenesis may be even more intimately intertwined with COX through MIRTD – a protein translated from an alternative transcript of dihydrolipoamide succinyltransferase (DLST) gene. The levels of the alternative transcript are decreased in brains of AD patients and its absence results in specific defect of COX assembly. The precise role of this protein in COX biogenesis is unfortunately unknown so far.

2. AIMS OF THE THESIS

The present thesis was primarily directed to characterize functional consequences of cytochrome *c* oxidase deficiencies caused by defects of enzyme assembly. Thanks to author's involvement in other projects, the scope of the thesis was broadened by findings that demonstrate the role of cytochrome *c* oxidase in more general pathological states such as cancer or diabetes. The aims of the thesis were:

A) To characterize the structural and functional alterations of cytochrome *c* oxidase in mitochondrial encephalomyopathies caused by defects of enzyme assembly due to mutations in *SURF1* and other genes coding for assembly factors.

B) To determine the COX affinity for oxygen in fibroblasts harbouring *SURF1* mutations – an attempt to establish a novel relation between COX oxygen affinity and human disease.

C) To uncover the putative involvement of mitochondria and specifically cytochrome *c* oxidase in disorders of energy metabolism such as obesity, diabetes and metabolic syndrome; and in tumorigenesis of clear cell renal carcinoma (CCRC) – a hereditary type of cancer caused by deficiency of VHL protein crucial for cellular oxygen sensing

3. SUMMARY OF THE RESULTS

The thesis consists of eight publications. Five publications deal with COX deficiencies in mitochondrial diseases. Two publications are concerned with the role of COX in tumorigenesis of CCRC, and the last publication presents our findings on the involvement of COX in diabetes.

The first four publications deal with structural and functional properties of COX in defects on the basis of mutations in nuclear- encoded COX assembly factors, mainly *SURF1*.

1. **Functional alteration of cytochrome *c* oxidase by *SURF1* mutations in Leigh syndrome,** *Pecina P., Čapková M., Chowdhury S. K., Drahota Z., Dubot A., Vojtíšková A., Hansíková H., Houšťková H., Zeman J., Godinot C., and Houštěk J.; Biochimica and Biophysica Acta, 1639 (1) : 53 – 63, 2003.*

This publication represents our first encounter with fibroblasts from patients harbouring mutations in *SURF1* gene, which encodes a specific assembly factor of COX. The paper thoroughly documents the experimental basis for our hypothesis that, in addition to severely decreased content of COX holoenzyme, the patient fibroblasts accumulate incomplete unstable forms of the enzyme (Fig. 3.1) that retain their electron-transport capacity while their proton pumping ability is severely impaired (Fig 3.2).

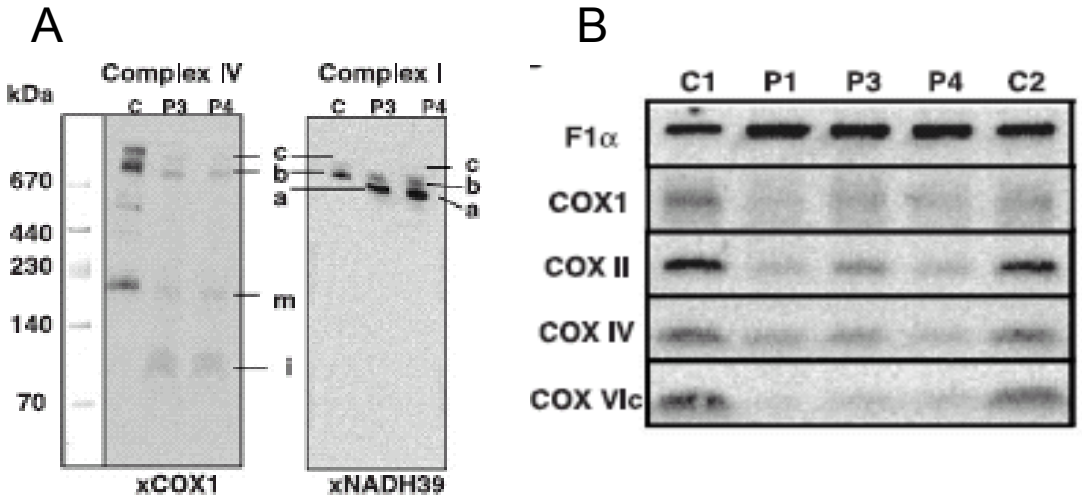

Figure 3.1. Electrophoretic analysis of COX in fibroblasts with SURF1 mutations. *(A) BN-PAGE Western blot developed with anti COX I or anti NADH39 monoclonal antibodies. Ten-microgram protein aliquots from control (C) and patients 3 and 4 (P3 and P4) were loaded. Legend to marks: i stands for incomplete COX assemblies, m stands for COX monomer, and a,*

b, and c mark the respiratory supracomplexes I_1III_2, $I_1III_2IV_1$, and $I_1III_2IV_2$, respectively. (B) SDS-PAGE Western blot of the COX subunits content. Ten-microgram protein aliquots of mitochondria from control fibroblasts (C) and from fibroblasts of LSCOX patients P1, P3, and P4 were used for analysis. Detection was done with monoclonal antibodies to the α subunit of F1-ATPase (F1 α) and COX subunits I, II, IV, and VIc.

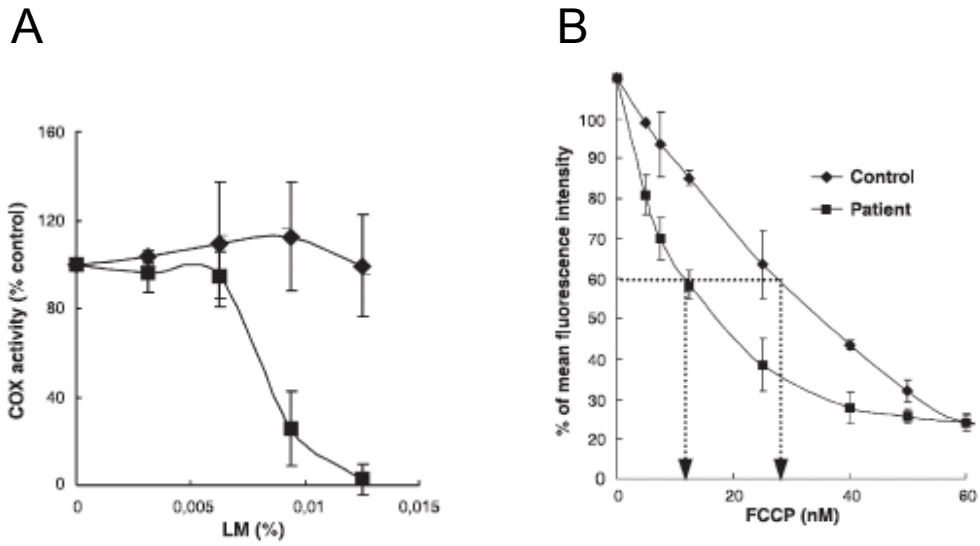

Figure 3.2. Functional alterations of COX in patient fibroblasts. (A) Decreased stability of COX after treatment with the detergen lauryl maltosidet*. COX activity measured polarographically with 5 mM ascorbate and 0.2 mM TMPD is expressed in percent of the values obtained in the absence of lauryl maltoside. Data are the means and S.D. of measurements performed in control (●, n = 7) and LS^{COX} (■, n = 5) fibroblasts.* ***(B) Impaired proton-pumping activity of COX in patient fibroblasts.*** *Digitonin-permeabilised (0.1 mg/mg protein) fibroblasts were stained with 20 nM TMRM in the presence of 10 mM succinate and various concentrations of FCCP (5 –60 nM). TMRM fluorescence is expressed as percentage of the mean fluorescence in the absence of FCCP. Data are the means and S.D. of measurements performed in control (n = 3) and LS^{COX} (n = 5) fibroblasts. The arrows point to I_{50} concentrations of FCCP.*

We proposed a possible pathogenic mechanism where the inability to maintain mitochondrial membrane potential results in severe defect of energy provision. The hypotheses originated in this publication are currently examined in our laboratory.

2. **Decreased affinity for oxygen of cytochrome *c* oxidase in Leigh syndrome caused by *SURF1* mutations ,** *Pecina P., Gnaiger E., Zeman J., Pronicka E., and Houštěk J.; American Journal of Cell Physiology – Cell Physiology, 278 (5) : C1384 – 1388, 2004.*

Our characterization of the functional consequences of the *SURF1* based COX deficiency was continued in this present study elaborated in collaboration with Erich Gnaiger from Innsbruck University. Our work was aiming to answer the question, whether the incomplete COX assemblies present in patient fibroblasts have altered affinity for oxygen. Using high-resolution respirometry, we quantified the oxygen affinity in terms of p50 (the partial pressure of oxygen at half-maximal respiration rate). Our results clearly demonstrated 3-fold increase of p50 in patient fibroblasts, which means that COX affinity for oxygen is decreased (Fig 3.3). We hypothesize that the depressed oxygen affinity may *in vivo* lead to respiratory limitation, which might represent an additional pathogenic mechanism of Leigh syndrome. Taking into consideration that central nervous system operates near maximum of its oxidative capacity and that some intercapillary regions of brain have extremely low partial pressure of oxygen, the decrease of COX affinity for oxygen may be responsible for specific degeneration of basal ganglia in Leigh syndrome. To the best of our knowledge, this study was the first to implicate an involvement of COX oxygen affinity in human pathology.

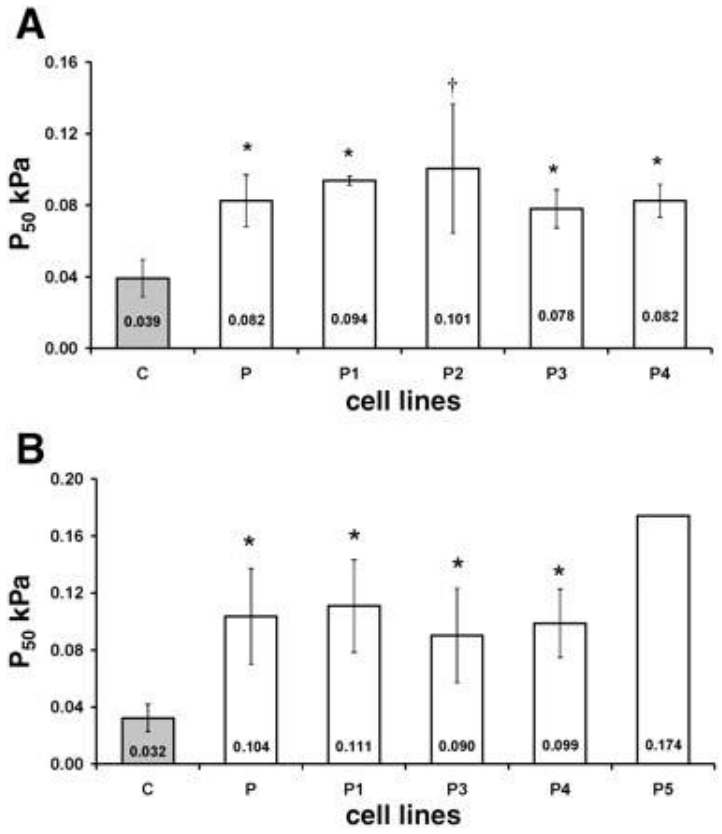

Figure 3.3. Mean p_{50} of respiration in intact cells (A) and of respiration at state 3u (B). *Data in A are displayed for all control subjects (C; n = 14), for all patients (P; n = 17), and for individual patients 1 (n = 3), 2 (n = 2), 3 (n= 7), and 4 (n= 5). Data in B are displayed for all controls (C; n = 17), for all patients (P; n = 23), and for individual patients 1 (n = 8), 3 (n = 7), 4 (n = 7), and 5 (n = 1). Mean values are shown inside columns; error bars indicate SD.*P < 0.01, †P< 0.05.*

3. **Genetic defects of cytochrome *c* oxidase assembly,** *Pecina P., Houšťková H., Hansíková H., Zeman J., and Houštěk J.; Physiological Research, 53 Suppl. 1 : S213 – 223, 2004.*

This review summarizes recent developments in elucidation of the molecular basis of mitochondrial diseases due to specific defects of COX with special focus on *SURF1*, including our achievements that have been published in the two papers discussed above. We devised a model of three distinct COX forms present in mitochondria of LS^{COX} cells (Fig. 3.4) – i) reduced content of normally- functioning COX holoenzyme, ii) accumulated COX assembly intermediate lacking several nuclear-encoded subunits with upregulated electron-transport activity and impaired proton-translocating activity, iii) inactive early COX assembly intermediates. The publication further presents "epidemiological" data on COX deficiencies in our country - among more than 60 cases of different types of COX deficiency that we have found in last 8 years in patients from the Czech and Slovak Republic, selective COX defects were present in 29 cases, 9 of which were *SURF1* mutations and 7 the *SCO2* mutations. Some of *SURF1* mutations possibly exert Slavonic prevalence, for example the 845-846delCT mutation is present in half of the cases found in Czech Republic and Poland.

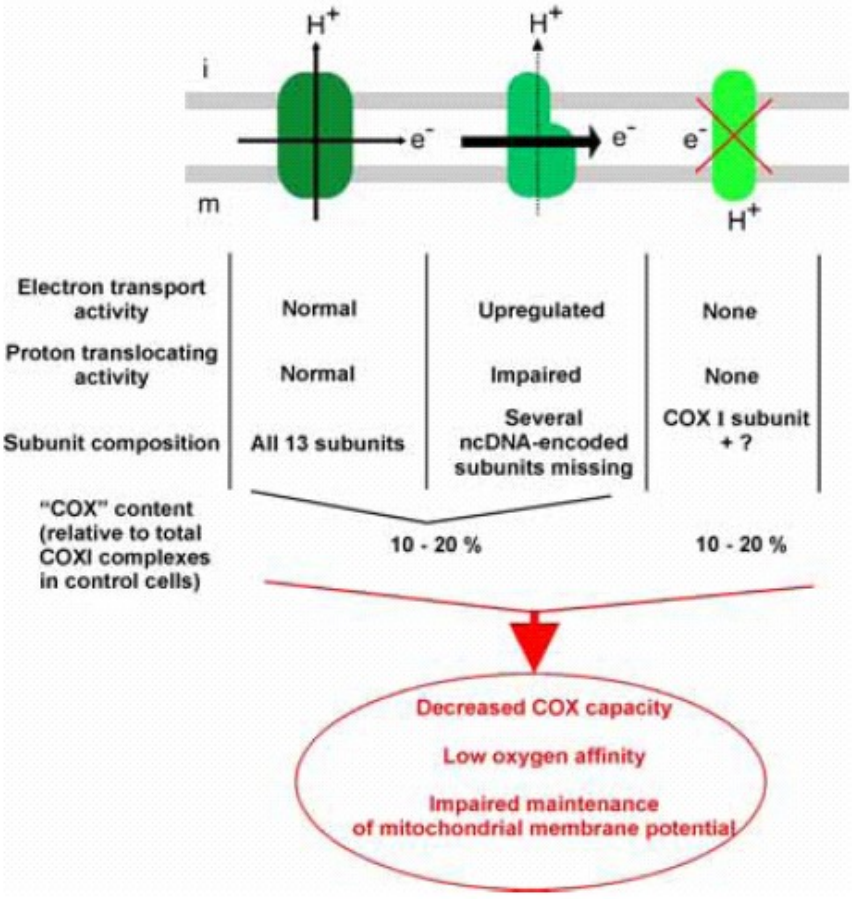

Figure 3.4. Model of COX structure and function in fibroblasts with SURF1 mutations

4. **Tissue-specific cytochrome *c* oxidase assembly defects due to mutations in *SCO2* and *SURF1*,** *Stibůrek L., Veselá K., Hansíková H., Pecina P., Tesařová M., Černá L., Houštěk J., and Zeman J.; Biochemical Journal, 392 Part3 : 625 – 632, 2005.*

The analysis of post-mortem tissue samples from patients harbouring either *SCO2* or *SURF1* mutations revealed striking differences in the extent of assembly defect between various tissues. Mutations in *SCO2*, for example, result in severe COX deficiency in heart whereas liver COX is completely unaffected. While these variations could not be correlated with the residual content of mutated *SCO2* protein, the results of this study possibly reflect the existence of tissue-specific functional differences of assembly factors, and/or tissue-specific pathways of COX biogenesis.

The following publication is a study dealing with COX deficiency caused by unique mutation in mtDNA affecting transcription and translation of ATP6/COX3 gene

5. **Diminished synthesis of subunit a (ATP6) and altered function of ATP synthase and cytochrome *c* oxidase due to the 2 bp microdeletion of TA at positions 9205 and 9206 ,** *Ješina P., Tesařová M., Fornůsková D., Vojtíšková A., Pecina P., Kaplanová V., Hansíková H., Zeman J., and Houštěk J.; Biochemical Journal, 383 Part3 : 561 – 571, 2004.*

In this study, we aimed to characterize the molecular basis for dysfunction of mitochondrial ATPase due to 2 bp deletion in stop codon of mitochondrial ATP6 gene. Eventhough our efforts were mainly occupied with the ATP synthase, the rare microdeletion impairs cleavage of polycistronic transcript between RNAs for ATP6 and COXIII, and therefore synthesis of COX subunit III is also affected. Deficiency of this subunit leads to defect of enzyme assembly, which is accompanied by accumulation of assembly intermediates (Fig. 3.5), similarly as in COX deficiency caused by *SURF1* mutations, or in a patient with a frameshift mutation in COXIII (Tiranti et al. 2000).

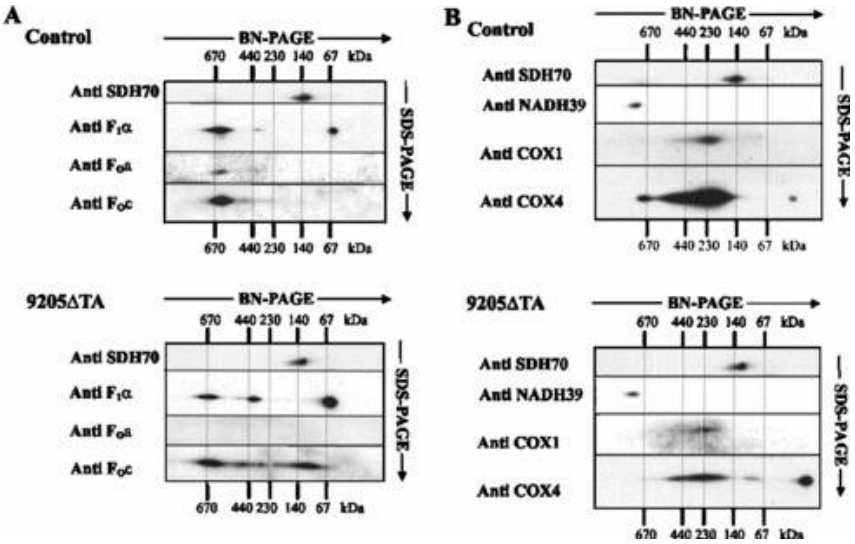

Figure 3.5 Assembly defects of ATPase and COX in patient fibroblasts. *Aliquots (15 µg of protein) of DDM-solubilized mitoplasts from 9205_TA and control fibroblasts were separated in the first dimension by BN-PAGE and in the second dimension by SDS/PAGE. WB analysis was performed with an antibody against the SDH70 subunit and with antibodies* ***(A) against ATPase subunits F1α, Foa and Fo c****, or* ***(B) against complex I subunit NADH39 and against COX subunits COX1 and COX4.***

Considering our previous findings that such incomplete assemblies have diminished proton pumping ability, the fibroblasts from this patient represent a unique model where both constitution of membrane potential, but also its discharge were affected, due to defects in COX or ATPase, respectively. While the measurements of mitochondrial membrane potential indicate that ATPase defect has a dominant effect on this bioenergetic parameter (Fig 3.6), both enzyme deficiencies underlie the decrease of mitochondrial energy provision that leads to the severe multisystemic disorder of the patient.

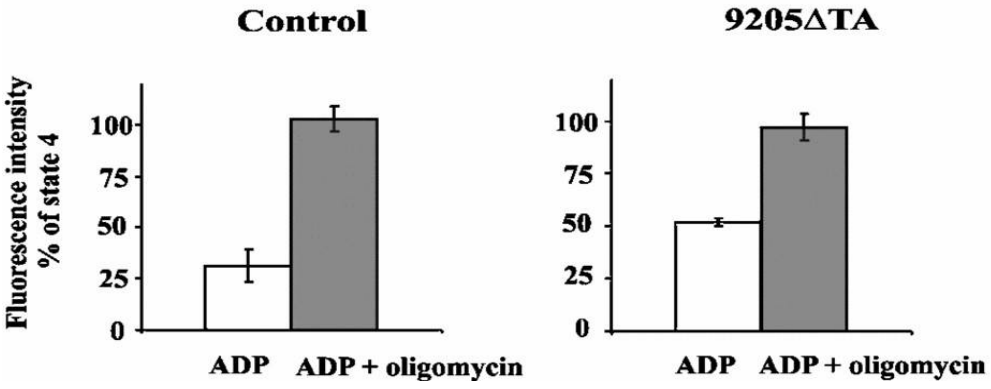

Figure 3.6 Decreased discharge of mitochondrial membrane potential. *Cytofluorimetric analysis was performed in digitonin-treated fibroblasts (0.1 mg of digitonin/mg of protein) and stained with 20 nM TMRM in a KCl medium containing 10 mM succinate. TMRMfluorescence of state 3-ADP and effect of oligomycin is expressed as percentage of the state 4 signal. The $\Delta\Psi_m$ -independent signal (after addition of FCCP) was subtracted from all data. The data represent means ± S.D. for four independent experiments.*

The role of mitochondrial dysfunction in tumorigenesis of clear cell renal carcinoma is described in the two following publications.

6. **A new role for the von Hippel-Lindau tumor suppressor protein: stimulation of mitochondrial oxidative phosphorylation complex biogenesis,** *Hervouet E., Demont J., Pecina P., , Vojtíšková A., Houštěk J., Simmonet H., and Godinot C.; Carcinogenesis, 26 (3) : 531 – 539, 2005.*

This is a first publication resulting from our long-lasting collaboration with the group of Catherine Godinot from Lyon University focused on study of clear cell renal carcinoma (CCRC). This particular type of cancer is caused by inherited mutations of *VHL* gene, which encodes a protein responsible for downregulation of hypoxia inducible factor (HIF-1α) under normoxia. pVHL deficient cells are therefore adapted to "eternal hypoxia", in this sense the CCRC perhaps represents the very essence of Warburg effect. Our results indicate that downregulation of mitochondrial proteins is one of the most crucial steps in CCRC tumorigenesis. The mitochondrial downregulation is most evident in severe decrease of COX content.

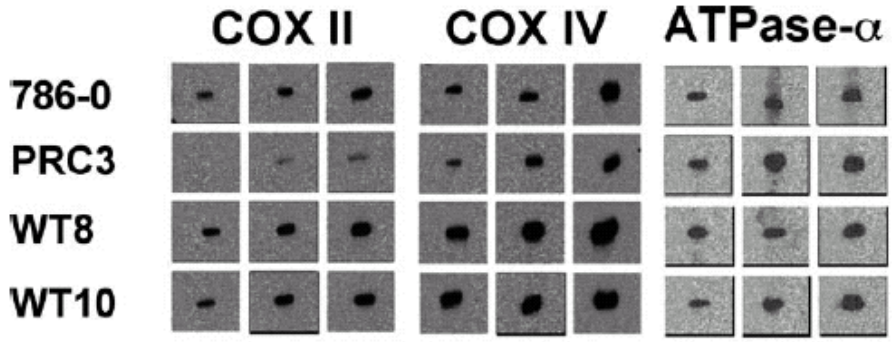

Figure 3.8 Increase in OXPHOS protein contents in 786-0 cells transfected with VHL. *Western blot analysis of OXPHOS subunits and of pVHL in 786-0 parental, 786-0-PRC3, 786-0-WT8 and 786-0-WT10 cells. Increasing amounts (10--30 μg) of proteins were used to estimate the proportionality between antibody signal and protein amounts. Antibodies directed against complex IV subunits: Cox II (mtDNA-encoded) and Cox IV (nuclear origin) and against complex V: ATPase F1 alpha subunit (nuclear origin), were used.*

7. **Inhibition of cytochrome *c* oxidase subunit 4 precursor processing by the hypoxia mimic cobalt chloride,** *Hervouet E., Pecina P., Demont J., , Vojtíšková A., Simmonet H., Houštěk J., and Godinot C.; Biochemical Biophysical Research Communications, 344 (4) : 1086 – 1093, 2006.*

Our tackling with CCRC was followed by this study, which primarily aimed to uncover the mechanism of mitochondrial downregulation in this cancer type. Tumor cell transfected with wild type VHL were treated with cobalt chloride, frequently used as a

hypoxia mimic in cell culture, due to its ability to inhibit enzymes responsible for hydroxylating HIF -1α in order to destine it for degradation. Cobalt treatment, unfortunately, had quite pleiotropic effect on CCRC cells. However, we considered one of the side effects very intriguing – namely the impairment of COX subunit IV precursor processing. This was due to cobalt inhibition of mitochondrial intermediate peptidase (MIP), which is used only for several mitochondrial proteins. Our unexpected result indicates that COX IV processing requires special care. This event may likely represent the rate-limiting step of S2 intermediate formation during COX assembly.

Our work on CCRC is still running, we are trying to identify the mechanism of mitochondrial enzymes downregulation. The preliminary results indicate that increased production of reactive oxygen species may be responsible for the observed changes in mitochondria of CCRC cells.

The last publication of the thesis presents an evidence for mitochondrial involvement in obesity

8. **Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat,** *Flachs P., Horáková O., Brauner P., Rossmeisl M., Pecina P., Franssen-van Hal N., Růžičková J., Šponarová J., Drahota Z., Vlček C., Keijer J., Houštěk J., and Kopecký J.; Diabetologia, 48 (11) : 2365 – 2375, 2005.*

We were invited to participate in this study aiming to characterize the mechanism, how dietary n-3 polyunsaturated fatty acids (PUFA) contribute to white adipose tissue reduction. Mice were fed with high-fat diet, one group received increased proportion of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids. The EPA + DHA uptake resulted in upregulation of mitochondrial proteins in white adipose tissue that was associated with increased rate of beta-oxidation. The increased expression of mitochondrial enzymes was due to 3-fold upregulation of PGC1 and NRF1, which are master transcriptional activators of mitochondrial genes. COX seemed to be upregulated to similar extent as complex II, and therefore had probably no specific role in the observed phenomenon.

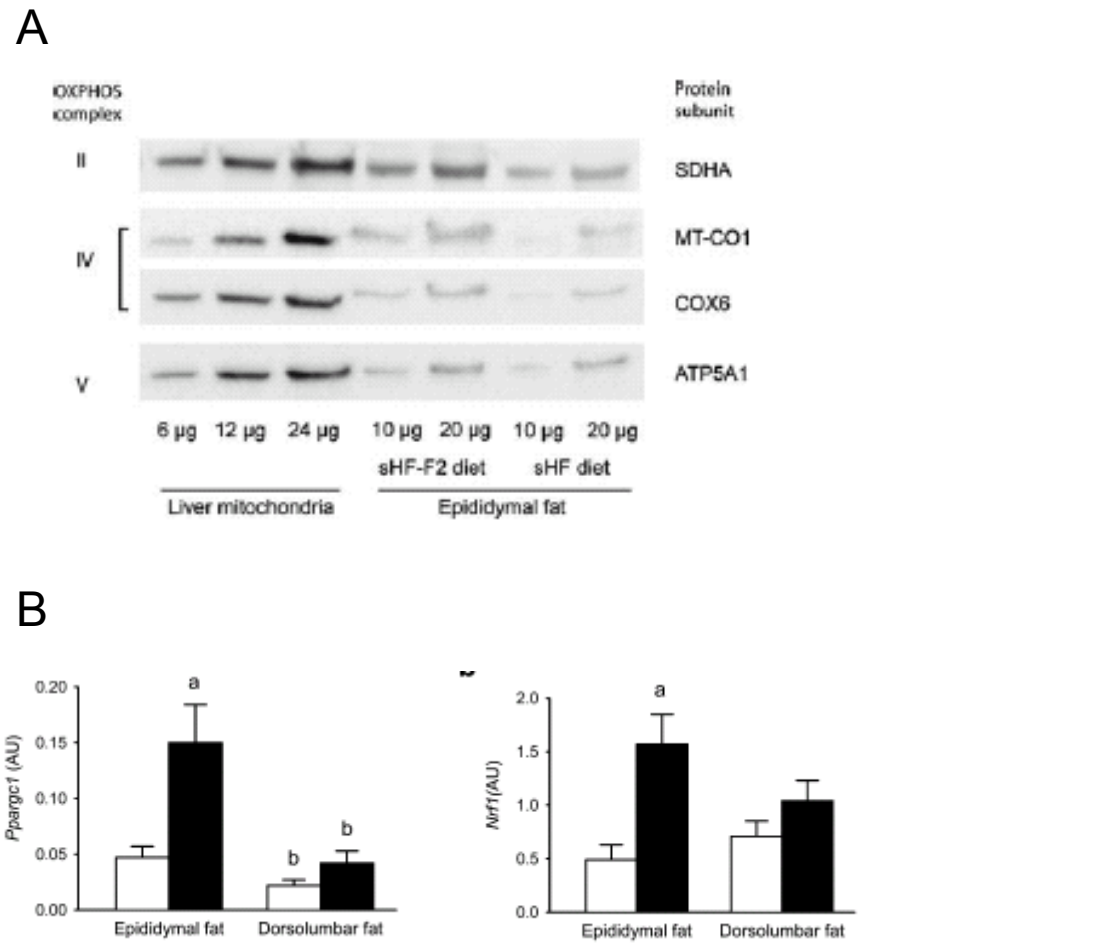

Figure 3.9 (A) Immunoblotting of mitochondrial proteins in epididymal fat of the mice fed sHFf and mice fed sHFf-F2 diets. *The analysis as performed using crude cell membranes isolated from adipose tissue. The amount of protein in each lane is indicated. Isolated mouse liver mitochondria were used as a standard.* ***(B) Expression of transcriptional regulators of mitochondrial genes in adipose tissue****. a–c Using qRT-PCR, transcript levels were evaluated in total RNA isolated from adipose tissue depots of mice fed the sHFf diet (white bars) or the sHFf-F2 diet (black bars). For all transcripts, the effect of diet in fat depots was different (two-way ANOVA). Significant differences between diets; significant differences between fat depots. Data are means ± SEM (n=9–15).*

4. CONCLUSIONS

The results of the thesis clearly demonstrate that mutations of *SURF1* gene result in profound alterations of cytochrome *c* oxidase structure and function, which underlie a pronounced defect of mitochondrial energetics. Deficiency of COX seems to be one of the hallmarks of frequent pathologies such as cancer or metabolic syndrome.

A) Accumulation of incomplete forms of cytochrome *c* oxidase was found in fibroblasts from patients with *SURF1* mutations. These subassemblies retain their electron-transport capacity, while their proton-pumping ability is severely impaired. These functional alterations result not only in serious decrease of energy provision, but underlie even more complex disorder of cellular physiology that ultimately manifests as Leigh Syndrome.

B) The affinity for oxygen of COX assembly intermediates is decreased. This finding implicates that COX deficiency in Leigh syndrome results in even more pronounced impairment of mitochondrial energy provision in tissues with limited oxygen supply.

C) Decrease of cytochrome *c* oxidase content and activity was shown to be involved in metabolic alterations that lead to tumorigenesis of clear cell renal carcinoma caused by inherited mutations of VHL protein. General disorders of energy metabolism such as obesity seem to be associated with cytochrome *c* oxidase decrease. In such cases, however, this phenomenon is not specific and probably reflects downregulation of mitochondrial function in general.

To conclude, I would like to remark that alterations of cytochrome *c* oxidase are most likely involved in vast majority of defects of energy metabolism, which underscores the COX position as one of the key regulatory enzyme of the cell. Extrapolating our findings on cytochrome *c* oxidase function towards a comprehensive model of subsequent changes at the level of cell physiology remains a challenge for future.

5. REFERENCES

Abramson, J., M. Svensson-Ek, et al. (2001). "Structure of cytochrome c oxidase: a comparison of the bacterial and mitochondrial enzymes." Biochim Biophys Acta **1544**(1-2): 1-9.

Alberts, J., Lewis, Raff, Roberts, Peter Walter (2002). Molecular Biology of the Cell. New York, Garland Science.

Anderson, S., A. T. Bankier, et al. (1981). "Sequence and organization of the human mitochondrial genome." Nature **290**: 457-465.

Barrientos, A., M. H. Barros, et al. (2002). "Cytochrome oxidase in health and disease." Gene **286**(1): 53-63.

Barrientos, A., A. Zambrano, et al. (2004). "Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in Saccharomyces cerevisiae." Embo J **23**(17): 3472-82.

Capkova, M., H. Hansikova, et al. (2002). "[A new missense mutation of 574C>T in the SURF1 gene--biochemical and molecular genetic study in seven children with Leigh syndrome]." Cas Lek Cesk **141**(20): 636-41.

Coenen, M. J., L. P. van den Heuvel, et al. (1999). "SURFEIT-1 gene analysis and two-dimensional blue native gel electrophoresis in cytochrome c oxidase deficiency." Biochem Biophys Res Commun **265**(2): 339-44.

Dell'agnello, C., S. Leo, et al. (2007). "Increased longevity and refractoriness to Ca2+-dependent neurodegeneration in Surf1 knockout mice." Hum Mol Genet **16**(4): 431-44.

Fontanesi, F., I. C. Soto, et al. (2006). "Assembly of mitochondrial cytochrome c-oxidase, a complicated and highly regulated cellular process." Am J Physiol Cell Physiol **291**(6): C1129-47.

Gallardo, M. E., R. Moreno-Loshuertos, et al. (2006). "m.6267G>A: a recurrent mutation in the human mitochondrial DNA that reduces cytochrome c oxidase activity and is associated with tumors." Hum Mutat **27**(6): 575-82.

Glerum, M. D., B. H. Robinson, et al. (1987). "Abnormal kinetic behavior of cytochrome oxidase in a case of Leigh disease." Am.J.Hum.Genet. **41**: 584-593.

Hayasaka, K., G. K. Brown, et al. (1989). "Cytochrome c oxidase deficiency in subacute necrotizing encephalopathy (Leigh syndrome)." J Inherit Metab Dis **12**(3): 247-56.

Houstek, J., B. Cannon, et al. (1975). "Glycerol-3-phosphate shuttle and its function in intermediary metabolism of hamster brown adipose tissue." Eur. J. Biochem. **54**: 11-18.

Lehninger, A. L., D. Nelson, et al. (2000). Lehninger Principles of Biochemistry, W H Freeman & Co.

Liang, X., W. Le, et al. (2001). "Impact of the intramitochondrial enzyme organization on fatty acid oxidation." Biochem Soc Trans **29**(Pt 2): 279-82.

Lombes, A., H. Nakase, et al. (1991). "Biochemical and molecular analysis of cytochrome c oxidase deficiency in Leigh's syndrome." Neurology **41**: 491-498.

Ludwig, B., E. Bender, et al. (2001). "Cytochrome C oxidase and the regulation of oxidative phosphorylation." Chembiochem Europ J Chem Biol **2**(6): 392-403.

Mashkevich, G., B. Repetto, et al. (1997). "SHY1, the yeast homolog of the mammalian SURF-1 gene, encodes a mitochondrial protein required for respiration." J Biol Chem **272**(22): 14356-64.

Nijtmans, L. G., M. Artal Sanz, et al. (2001). "Shy1p occurs in a high molecular weight complex and is required for efficient assembly of cytochrome c oxidase in yeast." FEBS Lett **498**(1): 46-51.

Nijtmans, L. G., J. W. Taanman, et al. (1998). "Assembly of cytochrome-c oxidase in cultured human cells." Eur.J.Biochem. **254**(2): 389-94.

Ogawa, Y., E. Naito, et al. (2002). "Three novel SURF-1 mutations in Japanese patients with Leigh syndrome." Pediatr Neurol **26**(3): 196-200.

Ohta, S. and I. Ohsawa (2006). "Dysfunction of mitochondria and oxidative stress in the pathogenesis of Alzheimer's disease: on defects in the cytochrome c oxidase complex and aldehyde detoxification." J Alzheimers Dis **9**(2): 155-66.

Pequignot, M. O., R. Dey, et al. (2001). "Mutations in the SURF1 gene associated with Leigh syndrome and cytochrome c oxidase deficiency." Hum Mutat **17**(5): 374-381.

Petruzzella, V., V. Tiranti, et al. (1998). "Identification and characterization of human cDNAs specific to BCS1, PET112, SCO1, COX15, and COX11, five genes involved in the formation and function of the mitochondrial respiratory chain." Genomics **54**(3): 494-504.
Possekel, S., C. Marsac, et al. (1996). "Biochemical analysis of fibroblasts from patients with cytochrome c oxidase-associated Leigh syndrome." Biochim Biophys Acta **1316**(3): 153-9.
Poyau, A., K. Buchet, et al. (2000). "Missense mutations in SURF1 associated with deficient cytochrome c oxidase assembly in Leigh syndrome patients." Hum Genet **106**(2): 194-205.
Rahman, S., R. B. Blok, et al. (1996). "Leigh syndrome: clinical features and biochemical and DNA abnormalities." Ann Neurol **39**(3): 343-51.
Salas, A., Y. G. Yao, et al. (2005). "A critical reassessment of the role of mitochondria in tumorigenesis." PLoS Med **2**(11): e296.
Scarpulla, R. C. (2006). "Nuclear control of respiratory gene expression in mammalian cells." J Cell Biochem **97**(4): 673-83.
Schagger, H. (2001). "Respiratory chain supercomplexes." IUBMB Life **52**(3-5): 119-28.
Stiburek, L., K. Vesela, et al. (2005). "Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1." Biochem J.
Sue, C. M., C. Karadimas, et al. (2000). "Differential features of patients with mutations in two COX assembly genes, SURF-1 and SCO2." Ann Neurol **47**(5): 589-95.
Tay, S. K., S. Sacconi, et al. (2005). "Unusual clinical presentations in four cases of Leigh disease, cytochrome C oxidase deficiency, and SURF1 gene mutations." J Child Neurol **20**(8): 670-4.
Taylor, R. W. and D. M. Turnbull (2005). "Mitochondrial DNA mutations in human disease." Nat Rev Genet **6**(5): 389-402.
Tiranti, V., P. Corona, et al. (2000). "A novel frameshift mutation of the mtDNA COIII gene leads to impaired assembly of cytochrome c oxidase in a patient affected by Leigh-like syndrome." Hum Mol Genet **9**(18): 2733-42.
Tiranti, V., C. Galimberti, et al. (1999). "Characterization of SURF-1 expression and Surf-1p function in normal and disease conditions." Hum Mol Genet **8**(13): 2533-40.
Tiranti, V., K. Hoertnagel, et al. (1998). "Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency." Am J Hum Genet **63**(6): 1609-21.
Tzagoloff, A. and C. L. Dieckmann (1990). "PET genes of Saccharomyces cerevisiae." Microbiol Rev **54**(3): 211-25.
von Kleist-Retzow, J. C., E. Vial, et al. (1999). "Biochemical, genetic and immunoblot analyses of 17 patients with an isolated cytochrome c oxidase deficiency." Biochim Biophys Acta **1455**(1): 35-44.
Wasniewska, M., E. Karczmarewicz, et al. (2001). "Abnormal calcium homeostasis in fibroblasts from patients with Leigh disease." Biochem Biophys Res Commun **283**(3): 687-93.
Williams, S. L., J. W. Taanman, et al. (2001). "A Novel Mutation in SURF1 Causes Skipping of Exon 8 in a Patient with Cytochrome c Oxidase-Deficient Leigh Syndrome and Hypertrichosis." Mol Genet Metab **73**(4): 340-3.
Williams, S. L., I. Valnot, et al. (2004). "Cytochrome c oxidase subassemblies in fibroblast cultures from patients carrying mutations in COX10, SCO1, or SURF1." J Biol Chem **279**(9): 7462-9.
Yang, Y. L., F. Sun, et al. (2006). "Clinical and laboratory survey of 65 Chinese patients with Leigh syndrome." Chin Med J (Engl) **119**(5): 373-7.
Yao, J. and E. A. Shoubridge (1999). "Expression and functional analysis of SURF1 in Leigh syndrome patients with cytochrome c oxidase deficiency." Hum Mol Genet **8**(13): 2541-9.
Zhu, Z., J. Yao, et al. (1998). "SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome." Nat Genet **20**(4): 337-43.
Zimmermann, P. and B. Kadenbach (1992). "Modified structure and kinetics of cytochrome c oxidase in fibroblasts from patients with Leigh syndrome." Biochim.Biophys.Acta **1180**: 99-106.

6. LIST OF PUBLICATIONS

1. Pecina P, Capkova M, Chowdhury SK, Drahota Z, Dubot A, Vojtiskova A, Hansikova H, Houst'kova H, Zeman J, Godinot C, Houstek J (2003): **Functional alteration of cytochrome *c* oxidase by *SURF1* mutations in Leigh syndrome,** *Biochimica et Biophysica Acta*, 1639: 53-63.
2. Dubot A, Godinot C, Dumur V, Sablonniere B, Stojkovic T, Cuisset JM, Vojtiskova A, Pecina P, Jesina P, and Houstek J. (2004): **GUG is an efficient initiation codon to translate the human mitochondrial ATP6 gene,** *Biochemical and Biophysical Research Communication*, 313(3):687-93.
3. Pecina P, Houstkova H, Hansikova H, Zeman J, Houstek J (2004): **Genetic defects of cytochrome *c* oxidase assembly,** *Physiological Research,* 53(Suppl 1): S213-233.
4. Mayr JA, Paul J, Pecina P, Kurnik P, Forster H, Fotschl U, Sperl W, Houstek J. (2004): **Reduced respiratory control with ADP and changed pattern of respiratory chain enzymes as a result of selective deficiency of the mitochondrial ATP synthase,** *Pediatric Research,* 55(6):988-94.
5. Jesina P, Tesarova M, Fornuskova D, Vojtiskova A, Pecina P, Kaplanova V, Hansikova H, Zeman J, Houstek J. (2004): **Diminished synthesis of subunit a (ATP6) and altered function of ATP synthase and cytochrome *c* oxidase due to the mtDNA 2 bp microdeletion of TA at position 9205 and 9206**, *Biochemical Journal*, 383: 1-11.
6. Pecina P, Gnaiger E, Zeman J, Pronicka E, Houstek J. (2004): **Decreased affinity for oxygen of cytochrome *c* oxidase in Leigh syndrome caused by *SURF1* mutations,** *American Journal of Physiology – Cell Physiology,* 287: C1384-1388
7. Hervouet E, Demont J, Pecina P, Vojtiskova A, Houstek J, Simonnet H, Godinot C. (2005): **A new role for the von Hippel-Lindau tumor suppressor protein: stimulation of mitochondrial oxidative phosphorylation complex biogenesis**, *Carcinogenesis*, 26(3): 531-539.
8. Stiburek L, Vesela K, Hansikova H, Pecina P, Tesarova M, Cerna L, Houstek J, Zeman J. (2005): **Tissue-specific cytochrome *c* oxidase assembly defects due to mutations in *SCO2* and *SURF1***, *Biochemical Journal*, 392: 625-632.
9. Flachs P, Horakova O, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N, Ruzickova J, Sponarova J, Drahota Z, Vlcek C, Keijer J, Houstek J, Kopecky J. (2005): **Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat,** *Diabetologia,* 48: 2365-2375.
10. Mracek T, Pecina P, Vojtiskova A, Kalous M, Sebesta O, Houstek J. (2006): **Two components in pathogenic mechanism of mitochondrial ATPase deficiency: energy deprivation and ROS production,** *Experimental Gerontology,* 241(7):683-7
11. Hervouet E, Pecina P, Demont J, Vojtiskova A, Simonnet H, Houstek J, Godinot C. (2006):**Inhibition of cytochrome *c* oxidase subunit precursor processing by the hypoxia mimic cobalt chloride,** *Biochemical and Biophysical Research Communications*, 344: 1086-1093.
12. Houstek J, Pickova A, Vojtiskova A, Mracek T, Pecina P, Jesina P. (2006): **Mitochondrial diseases and genetic defects of ATP synthase,** *Biochimica and Biophysica Acta - Bioenergetics,* 1757(9-10):1400-5
13. Pravenec M, Hyakukoku M, Houstek J, Zidek V, Landa V, Mlejnek P, Miksik I, Dudova-Mothejzikova K, Pecina P, Vrbacký M, Drahota Z, Vojtiskova A, Mracek T, Kazdova L, Oliyarnyk O, Wang J, Ho C, Qi N, Sugimoto K, and Kurtz T (2007): **Direct linkage of mitochondrial genome variation to risk factors for type 2 diabetes in conplastic strains**, *Genome Research*, in press