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The role of human Sco1, Sco2, Surf1 and Oxa1l in the biogenesis of the oxidative phosphorylation system

PhD Thesis

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CONTENTS

ABBREVIATIONS

2D	two-dimensional
AAA	atpases associated with a variety of cellular activities
ADP	adenosine diphosphate
Alb3	ALBINO3
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pair
CcO	cytochrome *c* oxidase, complex IV
CNS	central nervous system
CO	carbon monoxide
Coa1/2	cytochrome oxidase assembly 1/2
CoQ	coenzyme Q
COX	cytochrome *c* oxidase, complex IV
CS	citrate synthase
CxxxC	cysteine-undetermined (3x)-cysteine
DNA	deoxyribonucleic acid
FAAS	flame atomic absorption spectroscopy
FACS	fluorescent-activated cell sorting
$FADH_2$	flavin adenine dinucleotide reduced
GFP	green fluorescent protein
GIP	general import pore
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HA	hemagglutinin
HEK293	human embryonic kidney 293
HEXXH	histidine-glutamic acid-undetermined (2x)-histidine
HIF-1α	hypoxia-inducible factor 1α
HSP	heat shock protein
i-AAA	intermembrane space-AAA
IM	inner membrane

Imp1/2	intermembrane space protease 1/2
IMS	intermembrane space
kDa	kilodalton
KO	knockout
LRPPRC	leucine-rich pentatricopeptide repeat cassette
m-AAA	matrix-AAA
MDa	megadalton
MELAS	mitochondrial encephalopathy, lactic acidosis, and stroke like episodes
MERRF	myoclonic epilepsy and ragged-red fibers
Mia40	mitochondrial intermembrane space import and assembly 40
MPP	matrix processing peptidase
mRNA	messenger ribonucleic acid
mt	mitochondrial
MTS	mitochondrial targeting sequence
mt-tRNA	mitochondrial transfer ribonucleic acid
MW	molecular weight
NADH	nicotinamide adenine dinucleotide reduced
ND	NADH dehydrogenase
NO	nitric oxide
OM	outer membrane
OPA1	optic atrophy 1
ORF	open reading frame
OXA1	oxidase assembly 1
OXA1L	oxidase assembly 1-like
OXPHOS	oxidative phosphorylation system
Phb1	prohibitin 1
PNPase	polynucleotide phosphorylase
PPR	pentatricopeptide repeat
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SAM	sorting and assembly machinery
SCO	synthesis of cytochrome oxidase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

TACO1	translational activator of COX I
TCA	tricarboxylic acid
TIM	translocase of the inner membrane
TNFalpha	tumor necrosis factor alpha
TOB	topogenesis of mitochondrial outer membrane β-barrel proteins
TOM	translocase of the outer membrane
tRNA	transfer ribonucleic acid
UTR	untranslated region
VDAC	voltage-dependent anion-selective channel
YME1	yeast mitochondrial escape 1

INTRODUCTION

Mitochondria and OXPHOS

Mitochondria are highly dynamic semiautonomous organelles of endosymbiotic, α-proteobacterial descent found in virtually all eukaryotic cells. They are surrounded by two biological membranes that delimit two aqueous subcompartments, the intermembrane space and the mitochondrial matrix. They contain numerous copies of their own DNA (mtDNA) that codes for a limited number of protein products, almost exclusively subunits of the OXPHOS (Oxidative Phosphorylation System) machinery, and rRNA and tRNA components of a specific mitochondrial translation apparatus. In mammals, 13 protein products are synthesized within mitochondria, all of them being evolutionary conserved hydrophobic subunits of the OXPHOS system. The vast majority of the various ~1100 mammalian mitochondrial proteins is thus encoded in nucleus, synthesized on cytoplasmic ribosomes and subsequently sorted and imported into mitochondria by means of a specialized transport machinery (TOM/TIM translocases) (Lill et al., 1996; Voet and Voet, 1995).

Mitochondria are responsible for the conversion of energy and production of the bulk of cellular ATP via the TCA cycle and the OXPHOS machinery, serve as calcium (Ca^{2+}) stores and play a pivotal role in the mechanism of programmed cell death (apoptosis). They are the place of fatty acids oxidation, ketone body production, heme biosynthesis, cardiolipin metabolism, the biosynthesis of coenzyme Q, production of reactive oxygen species (ROS) and key steps of gluconeogenesis and the urea cycle (Voet and Voet, 1995).

The mammalian OXPHOS machinery is composed of five multi-subunit membrane-embedded enzyme complexes (the respiratory chain and the ATP synthase) build up of more than 90 protein subunits containing numerous prosthetic groups. According to nowadays widely accepted Mitchell's chemiosmotic hypothesis the electrons taken from the oxidation of NADH and succinate ($FADH_2$) undergo linear transfer through the four membrane embedded complexes of the respiratory chain, what is intimately coupled to vectorial proton pumping (translocation) across the inner membrane bilayer by three of these complexes (I, III, IV), so-called proton pumps (Mitchell, 1966; Mitchell, 1979; Voet and Voet, 1995). This results in the generation of electrochemical proton gradient (gradient of protons ΔpH and mitochondrial membrane potential $\Delta\Psi$) that might be subsequently used by the F_1F_O-ATP synthase to power

the synthesis of ATP (Voet and Voet, 1995). The individual OXPHOS complexes are further organized into higher order, supramolecular assemblies, so-called respiratory supercomplexes (Cruciat et al., 2000; Schagger and Pfeiffer, 2000). These appear to be the building blocks of a hypothetical macromolecular respiratory unit that was termed "respirasome" (Acin-Perez et al., 2008; Lenaz and Genova, 2007; Schagger and Pfeiffer, 2000).

The biogenesis of OXPHOS is complicated by the subcellular localization and intricate membrane topology of constituent complexes, the dual genetic origin of the individual subunits, the high hydrophobicity of most of them and by a plethora of prosthetic groups required for the activity and assembly/stability of the complexes. As a result, the biogenesis of OXPHOS requires coordinated expression of the nuclear and mitochondrial genome, a multilevel regulation of this process, the sorting and import of subunits and non-subunit (ancillary) proteins into the various subcompartments of the organelle, the biosynthesis and/or insertion of prosthetic groups, the membrane insertion/translocation and assembly of subunit proteins into assembly intermediates/subcomplexes and final oligomeric complexes, and the surveillance of protein quality control and proteolytic processing carried out by specific energy-dependent proteases (Fernandez-Vizarra et al., 2009; Kutik et al., 2009; Lill et al., 1996).

The quality (correct processing, folding, and topogenesis) and the abundance of proteins within the inner mitochondrial membrane are controlled by two contradictory processes: the assembly/membrane-integration and selective proteolytic degradation. The integration/assembly of proteins into the inner membrane is ensured by a number of more or less specific translocases, assembly factors and molecular chaperones that mediate the insertion of proteins or translocation of transmembrane segments from both sides of the membrane (Hildenbeutel et al., 2008; Kutik et al., 2009; Mokranjac and Neupert, 2009; Neupert and Herrmann, 2007). On the other hand, the proteolytic degradation of misfolded and/or non-assembled inner membrane proteins is conducted by two evolutionary conserved energy-dependent proteolytic machines, the m-AAA and i-AAA proteases. These ATP-dependent proteolytic complexes act at the opposite sides of the inner membrane. In addition to the metallopeptidase function, they also exert chaperone and translocase activities (Koppen and Langer, 2007; Leonhard et al., 1996; Tatsuta and Langer, 2008). Even though the processes of protein integration/assembly and degradation apparently act in a completely opposite manner, they share a common goal of maintaining the protein homeostasis within the inner mitochondrial membrane.

AIMS OF THE STUDY

- Characterize the impact of mutations in genes encoding CcO assembly factors Sco2 and Surf1 on the tissue pattern of CcO subcomplexes and the tissue levels of respective mutant proteins in order to gain new insights into the function of both factors and the process of human CcO assembly in various tissues

- Study the molecular role and biochemical properties of the human homologue, OXA1L, of yeast mitochondrial Oxa1 translocase involved in early posttranslational steps of CcO biogenesis

- Analyze the impact on CcO biogenesis and tissue copper levels of a novel mutation (G132S) in the juxtamembrane region of Sco1 metallochaperone and study the protein-protein interactions of wild-type Sco1 in order to characterize the molecular role of Sco1 metallochaperone in CcO biogenesis and cellular copper homeostasis maintenance

- Analyze the effects of mt-tRNA point mutations in various tissues from individuals affected by Leigh (8363G>A), MERRF (8344A>G), and MELAS (3243A>G) syndrome on the steady-state levels and activity of OXPHOS complexes

REVIEW OF THE LITERATURE AND DISCUSSION OF RESULTS

1. Import and membrane-integration of mitochondrial proteins

The vast majority of mitochondrial proteins, including ten of the thirteen cytochrome *c* oxidase (CcO) subunits, are encoded by nuclear genes, synthesized in cytoplasm as precursor proteins (preproteins) and further maintained in an unfolded, import-competent state by cytosolic chaperones (Hsp70, Hsp90). Subsequently, they are specifically recognized, imported and sorted into the various subcompartments of mitochondria by means of specialized translocation machinery. The mitochondrial targeting of about half of the mitochondrial proteins is ensured by a specific cleavable N-terminal amino acid presequence, so-called mitochondrial targeting sequence (MTS), often in the form of amphipathic α-helix (Neupert and Herrmann, 2007; Truscott et al., 2003). These signals are necessary and sufficient to target precursor proteins into the mitochondrial matrix, where the presequence is removed by the action of a specialized processing protease (MPP). The remaining half of mitochondrially destined proteins contains internal targeting signals as well as bipartite presequences (N-terminal MTS followed by internal hydrophobic loop signal) that can be present anywhere along the polypeptide sequence. Unlike the N-terminal MTS-containing precursors, the proteins containing internal targeting signals can be destined to all mitochondrial subcompartments (Neupert and Herrmann, 2007).

1.1. Transport across the outer membrane - the TOM complex

Depending on the protein's final destination, several multimeric membrane complexes are involved in its translocation across, and subsequent insertion into, the outer or inner mitochondrial membrane from either side.

The N-terminal targeting signals are recognized by the receptor subunits of the multimeric outer membrane TOM (Translocase of the Outer Membrane) complex that mediates the import of virtually all proteins of the mitochondria. In addition, the TOM complex is also responsible for the insertion of proteins into the outer mitochondrial

membrane. In conjunction with the TOB/SAM (Topogenesis of mitochondrial Outer membrane β-barrel proteins/Sorting and Assembly Machinery) complex, the TOM complex mediates insertion into the outer membrane of β-barrel proteins. TOM complex consists of a stable core, so-called general import pore complex (GIP complex) and loosely associated receptor proteins. The GIP complex consists of the central component Tom40 and three small associated subunits, Tom5, Tom6, and Tom7. Tom40 is a transmembrane protein that itself constitutes the import pore of the outer mitochondrial membrane. Tom40 is able to form pores in artificial membranes even in the absence of other TOM subunits. Similarly to other outer membrane channel protein porin (VDAC), Tom40 presumably forms a β-barrel membrane-spanning structure. However, it is still not clear whether the pores are formed by single or multiple Tom40 molecules. When purified using the mild detergent digitonin, the TOM complex has a molecular mass of roughly 490-600 kDa (Esaki et al., 2004; Neupert and Herrmann, 2007; van Wilpe et al., 1999; Yano et al., 2003).

1.2. Transport across the inner membrane - the TIM complexes

After crossing the outer membrane, preproteins destined to the inner membrane, IMS and matrix interact with one of the TIM (Translocase of the Inner Membrane) complexes. On the other hand, insertion into the outer membrane of β-barrel proteins, upon their release from TOM complex, is directly mediated by the TOB/SAM outer membrane-embedded complex (Neupert and Herrmann, 2007).

The mitochondrial inner membrane harbors a large variety of different membrane proteins that attain various membrane topologies, exposing active domains on one or both sides of the membrane. Although a limited number of these proteins is synthesized on mitochondrial ribosomes, the majority of protein constituents of the inner membrane is synthesized in cytosol and subsequently imported through the TOM complex. Three different import pathways were thus far described for the various inner membrane proteins. First, a specialized inner membrane transport complex TIM22 is involved in the membrane translocation of proteins (TIM subunits, metabolite carriers) with internal "hydrophobic loop" signals (TIM22 pathway). Second, some preproteins that use the inner membrane TIM23 complex are arrested at the level of the complex and then laterally inserted into the inner membrane (stop-transfer mechanism). Third, some, mostly evolutionary conserved, inner membrane destined preproteins are translocated into the matrix by the TIM23 complex and subsequently, upon cleavage of MTS, exported (inserted) into the inner membrane by

evolutionary conserved export machinery represented by the Oxa1 translocase complex (conservative sorting) (see 2.3) (Mokranjac et al., 2003; Mokranjac et al., 2005; Truscott et al., 2001).

Protein residents of the IMS, mostly of low molecular weight, exert essential functions in a variety of different processes, such as metabolic and bioenergetic reactions, transfer of metabolites, proteins and metal ions, and regulation of cell death. On the basis of their import characteristics, most IMS destined proteins can be attributed into one of these categories: (i) proteins with N-terminal MTS followed by a hydrophobic sorting sequence (bipartite presequence motifs) are imported via TOM and TIM23 complexes in a membrane potential, and in most cases, ATP-dependent manner. Upon import, such bipartite presequences are proteolytically removed, releasing the mature protein into IMS; (ii) the TOM complex-mediated import of small, mostly soluble IMS proteins is thought to be driven by the subsequent folding of proteins within IMS, triggered by the acquisition of cofactors or by the formation of intramolecular disulfide bridges. This so-called "folding trap hypothesis" presumes that the folded state hinders the back-translocation out of the mitochondria thereby conferring the unidirectional net import of these proteins. One example of such protein are cytochrome *c*, which is imported through the TOM complex in apo-form and subsequently locked in its folded state by cytochrome *c* heme lyase. Another example is the twin Cx_9C class of IMS proteins that are bound in precursor forms by inner membrane protein Mia40, which is able to convert its intramolecular disulfide bridges to intermolecular, covalently linking the precursors, thereby preventing their back-translocation. Lastly, (iii) the import reaction of some IMS proteins appears to be driven by affinities of these proteins to form complexes with other proteins of IMS. Examples are the import reactions of cytochrome *c* heme lyase, catalyzing the insertion of heme group to both cytochrome *c* and cytochrome c_1 in humans or creatine kinase (Brandner et al., 2005b; Herrmann and Kohl, 2007; Neupert and Herrmann, 2007).

1.3. Oxa1 translocase complex

During the evolution, most genes of α-proteobacterial descent were transferred to nucleus thus reducing the size of mitochondrial genome to the current state (Andersson and Kurland, 1999; Cavalier-Smith, 2002). However, mainly the hydrophobic nature of most mitochondrially-encoded proteins has hindered this process (Claros et al., 1995). Consequently, evolutionary conserved membrane-insertion machinery represented by the

Alb3/Oxa1/YidC protein family has evolved to ensure the cotranslational membrane insertion of hydrophobic proteins in mitochondria, chloroplasts and bacteria (Bonnefoy et al., 2009; Stuart, 2002). Hence, mitochondrial translation is thought to occur exclusively at the matrix face of the inner membrane bilayer (Liu and Spremulli, 2000). In contrast, the recognition and membrane-recruitment of translating ribosomes in the cytoplasm of eukaryotic and prokaryotic cells is mediated by signal recognition particles (Gilmore and Blobel, 1983) that appear to be absent from mitochondria (Glick and Von Heijne, 1996).

The insertion of mitochondrial translation products as well as of a subset of TIM23 conservatively sorted nuclear gene products into the inner membrane is ensured by a conserved integral inner-membrane protein Oxa1, the founding member of the Alb3/Oxa1/YidC protein family (Herrmann and Neupert, 2003; Hildenbeutel et al., 2008). Members of this family possess a hydrophobic core domain containing five transmembrane helices that facilitate the membrane export of their protein substrates (Herrmann et al., 1997; Kuhn et al., 2003). The best characterized member of this family, *Saccharomyces cerevisiae* Oxa1, is an intrinsic protein of the inner mitochondrial membrane. Unlike the bacterial homologue, YidC, mitochondrial Oxa1 proteins contain a C-terminal α-helical domain of roughly 100 residues that protrudes into the matrix (Jia et al., 2003; Preuss et al., 2005). This domain was shown in yeast to bind to the 60S ribosomal subunit protein L41, located near the polypeptide exit tunnel thereby physically recruiting the mitochondrial translation apparatus to the translocation complex (Jia et al., 2003) represented by approx. 200 kDa homooligomeric assembly of four Oxa1 subunits (Szyrach et al., 2003). In a crosslinking experiment, mitochondrially-encoded cytochrome c oxidase subunits Cox1, Cox2 and Cox3 were shown to transiently interact with Oxa1 as nascent chains (Hell et al., 2001). Although the yeast Oxa1 was shown to represent a rather general export machinery of the inner membrane, the co-translational membrane insertion of the mitochondrially encoded Cox2 precursor appears to exhibit the strictest dependency on its function. The other substrates of Oxa1, including Oxa1 itself, can be inserted independently of its function, albeit with significantly reduced efficiencies. This suggests the existence of an alternative insertion pathway(s). Recently, a novel post-translational role in the biogenesis of OXPHOS was demonstrated for yeast Oxa1. The protein was shown to stably interact in a posttranslational manner with the ATP synthase subunit c, mediating its assembly into the ATP synthase complex (Jia et al., 2007). The yeast Oxa1 null mutant is respiratory deficient, with no detectable CcO activity and markedly reduced levels of the cytochrome bc_1 complex and the F_1F_O-ATP synthase (Altamura et al., 1996; Bonnefoy et al., 1994). *Schizosaccharomyces*

pombe contains two distinct Oxa1 orthologues, both of which are able to complement the respiratory defect of yeast Oxa1-null cells. The double inactivation of these genes is lethal to this petite-negative yeast (Bonnefoy et al., 2000). Depletion of Oxa1 in *Neurospora crassa* results in a slow-growth phenotype accompanied by reduced subunit levels of CcO and NADH:ubiquinone oxidoreductase (complex I). The *N. crassa*, Oxa1 was shown to form a 170–180 kDa homo-oligomeric complex, most likely containing four Oxa1 monomers (Nargang et al., 2002).

Yeast Mba1, a protein associated with the matrix face of the inner membrane presumably functions as a ribosome receptor that cooperates with Oxa1 in the cotranslational insertion process (Ott et al., 2006). The lack of Mba1, together with the C-terminus of Oxa1, results in an association of mitochondrial translation products with mtHSP70 (Ott et al., 2006), a mitochondrial matrix chaperone known to specifically interact with unfolded polypeptides (Hartl and Hayer-Hartl, 2002). Yeast Mba1 exhibits sequence similarity with mitochondrial ribosomal L45 proteins from higher eukaryotes (Ott et al., 2006).

The human Oxa1 orthologue, referred to as Oxa1l, shares 33% sequence identity with the corresponding yeast polypeptide (Rotig et al., 1997). The human *OXA1L* cDNA was initially cloned by partial functional complementation of the respiratory growth defect of the yeast oxa1-79 mutant. It contains an open reading frame predicted to encode a protein of 435 amino acids (Bonnefoy et al., 1994). It was suggested that the ten exons of OXA1L might form an open reading frame (ORF) able to encode a precursor protein of 495 amino acids, and more recently the cDNA containing these additional 180 bp was cloned (Strausberg et al., 2002). However, this extended ORF version was shown to exhibit an even lower capacity to complement the respiratory growth defect of yeast *oxa1* cells than the original sequence. The human OXA1L mRNA was found to be enriched in mitochondria- bound polysomes from HeLa cells, and its 3′ untranslated region was shown to be functionally important when expressed in yeast cells (Sylvestre et al., 2003). We showed that human Oxa1l is a mitochondrial integral membrane protein that exists as part of a 600–700 kDa complex in mitochondria of human embryonic kidney 293 (HEK293) cells (Stiburek et al., 2007). We further demonstrated that the stable short hairpin RNA (shRNA)-mediated knockdown of human Oxa1l in HEK293 cells leads to markedly decreased protein levels and ATP hydrolytic activity of the F1Fo-ATP synthase and moderately reduced levels and activity of NADH:ubiquinone oxidoreductase (complex I), suggesting functional involvement of the protein in the assembly/stability of these two OXPHOS complexes (Stiburek et al., 2007). In sharp contrast to yeast *oxa1* mutant, the assembly/stability of cytochrome c oxidase (complex

IV) as well as of the cytochrome *c* reductase (complex III) was not negatively affected in human cells with downregulated expression of Oxa1l (Stiburek et al., 2007). These results indicate that human Oxa1l represents mitochondrial integral membrane protein required for the correct biogenesis of the F_1F_O-ATP synthase and to a lesser extent NADH:ubiquinone oxidoreductase.

A distant homologue of Oxa1, referred to as Cox18 or Oxa2, is an integral inner-membrane protein containing five predicted transmembrane helices within a conserved core domain of approx. 200 amino acid residues (Funes et al., 2004; Souza et al., 2000). Cox18 is believed to be involved in topogenesis of the C-terminal domain of Cox2. Since the HA-tagged C-terminus of Cox2, normally found exposed into IMS, becomes protease-protected in mitoplasts from Cox18 null strain (Saracco and Fox, 2002). Unlike Oxa1, Cox18 lacks the C-terminal ribosome-binding domain, and yeast Cox18 null cells exhibit isolated CcO deficiency. Overexpression of Oxa1 does not suppress Cox18 null phenotype, suggesting functional differences between both proteins (Saracco and Fox, 2002). In contrast to mammalian protein, yeast Cox2 is synthesized as a precursor with N-terminal extension of 15 amino acid residues. This presequence is removed, upon translocation into IMS, by Imp1/Imp2 protease in conjunction with Cox20 (Hell et al., 2000; Nunnari et al., 1993). Only processed Cox2 is allowed to assemble into the yeast CcO complex. Human Cox18 orthologue was identified that exhibits 25% sequence identity with yeast protein. A GFP-fused, N-terminal fragment of 210 bp of human Cox18 accumulates exclusively in mitochondria in HEK293 cells (Sacconi et al., 2005).

2. Mitochondrial translational activators - Pet309, LRPPRC and TACO1

In addition to Oxa1 translocase, another class of inner membrane proteins, so-called translational activators exists in yeast mitochondria that mediate the membrane-recruitment of translating mitochondrial ribosomes (Naithani et al., 2003; Sanchirico et al., 1998). These proteins bind specifically to sequences in 5' untranslated regions (UTRs) of mitochondrial transcripts (Green-Willms et al., 2001; Manthey et al., 1998; McMullin and Fox, 1993). Similar mechanism was not expected to be conserved in mammals, since mammalian mitochondrial mRNAs lack significant 5' UTR sequences and the majority of genes involved in translation of mitochondrially encoded proteins lack mammalian homologues. The only exception was thought to be the human homologue, LRPPRC, of yeast translation activator Pet309 involved in translation and stabilization of *COX1* mRNA (Mootha et al., 2003; Xu et

al., 2004). Both Pet309 and LRPPRC contain several PPR motifs, consisting of degenerated 35-amino acid sequences proposed to form two antiparallel alpha helices (Delannoy et al., 2007). These repeats characterize a large protein family in plants with only a few examples found among fungi, animals, and protists. PPR proteins are known to participate mostly in different steps of sequence-specific RNA metabolism (Tavares-Carreon et al., 2008). Indeed, Pet309 is the only yeast translational activator that has been found to contain PPR motifs. LRPPRC was shown to be involved in the stabilization of both *COX1* and *COX3* mRNAs. Mutations in LRPPRC lead to French-Canadian form of cytochrome oxidase deficient Leigh syndrome (Mootha et al., 2003; Xu et al., 2004).

Very recently, a nuclear gene product named TACO1 (Translational Activator of COX I) was identified as first known specific mammalian mitochondrial translational activator (Weraarpachai et al., 2009). The mature polypeptide of roughly 30 kDa was shown to exist in mitochondrial matrix as an oligomer or as part of a complex of ~74 kDa. Loss of TACO1 results in late-onset Leigh syndrome with cytochrome oxidase deficiency, very likely due to specific defect in the synthesis of mitochondrially encoded subunit Cox1. The reduced amount of newly synthesized Cox1 in *TACO1* patient fibroblasts was shown to be accompanied by increase in some of the ND subunits, and *cyt b*, probably reflecting compensatory response. The substantially decreased levels of fully assembled CcO are not accompanied by appearance of any of the previously identified subcomplexes, confirming the role of Cox1 as an initial seed of CcO assembly. Interestingly, the inactivation of *S. cerevisiae* TACO1 ortholog, YGR021w that exhibits 29% sequence identity, resulted in almost normal CcO activity of cells grown on respiratory media (Weraarpachai et al., 2009).

3. Cytochrome c oxidase (CcO)

Eukaryotic cytochrome *c* oxidase (CcO) is the terminal multicomponent enzyme of the energy-transducing mitochondrial electron transport chain (Capaldi, 1990; Stiburek et al., 2006). It belongs to the superfamily of heme-copper containing terminal oxidases, characterized by the presence of histidine ligands to two heme groups and to a Cu_B copper ion (Michel et al., 1998). The mitochondrial enzyme, an aa_3-type terminal oxidase, catalyzes the sequential transfer of electrons from reduced cytochrome *c* to dioxygen, coupling this reaction with electrogenic proton pumping across the inner mitochondrial membrane. Eukaryotic CcO is a heterooligomeric complex composed of 7 (*Dictyostelium discoideum*), 11 (*Saccharomyces cerevisiae*) and 13 (mammals) protein subunits embedded in the protein-rich

highly convoluted inner mitochondrial membrane. The core of the enzyme is composed of three mitochondrially encoded subunits that exhibit high evolutionary conservation. Unlike prokaryotic enzymes, mitochondrial CcOs consist of additional small peripheral subunits, encoded by the nuclear genome and synthesized in cytoplasm (Ludwig et al., 2001; Taanman, 1997). The redox-active heme and copper cofactors, directly involved in electron transfer, are coordinated by the mitochondrially encoded subunits Cox1 and Cox2 (Tsukihara et al. 1995).

The biogenesis of eukaryotic CcO complex is complicated by its subcellular location, the dual genetic origin of constituent subunits, the hydrophobic nature of most of them, and mainly by a number of prosthetic groups required for function, including two heme *a* moieties, three copper ions, and zinc, magnesium and sodium ions (Carr and Winge, 2003; Stiburek et al., 2006). Consequently, a number of specific gene products have evolved to accommodate such complex requirements. Although some of these factors act in a general manner and participate also in the biogenesis of other respiratory chain complexes, studies on yeast have identified over thirty accessory factors essential exclusively for proper biogenesis of the eukaryotic enzyme, while a number of them were shown to have human homologues (Fontanesi et al., 2008b; Herrmann and Funes, 2005). Isolated CcO deficiency represents one of the most commonly recognized causes of respiratory chain defects in humans associated with a wide spectrum of clinical phenotypes (Bohm et al., 2006; Shoubridge, 2001a). Pedigree studies suggest that the majority of genetic defects associated with fatal infantile CcO deficiency are of nuclear origin and inherited as autosomal recessive traits. To date, autosomal recessive mutations in six nuclear-encoded factors (SURF1, SCO1, SCO2, COX10, COX15, LRPPRC) required for the assembly of functional CcO complex have been identified in humans (Shoubridge, 2001a; Shoubridge, 2001b; Stiburek et al., 2006). In addition, mutations in *FASTKD2* and *ETHE1*, involved in apoptosis and sulfide catabolism, respectively, were also reported to result in severe, tissue-specific CcO defect (Ghezzi et al., 2008; Tiranti et al., 2009). However, the effects on CcO biogenesis of mutations in both of the gene products are thought to be essentially secondary.

3.1. CcO structure and function

Mammalian CcO is a heterooligomeric complex with a combined molecular weight of 205 kDa composed of thirteen structural subunits encoded by both the mitochondrial and nuclear genes (Stiburek et al., 2006; Taanman, 1997). The enzyme is embedded in the inner mitochondrial membrane, with one part extending 37 Å into the intermembrane space (IMS)

and an opposite part protruding 32 Å into the mitochondrial matrix. The three mitochondrially encoded subunits, Cox1, Cox2 and Cox3 constitute the structural core of the enzyme that incorporates all redox-active cofactors (Tsukihara et al., 1996; Yoshikawa et al., 1998). Cox1, the largest and the most evolutionary conserved subunit of the enzyme, is a highly hydrophobic protein composed of twelve transmembrane helices connected by short extramembrane loops. This subunit coordinates the catalytic site of the enzyme, and constitutes the two proton translocation pathways (D- and K-pathway) (Wikstrom, 2000). Recently, it was reported that pro-inflammatory cytokine TNFalpha inhibits oxidative phosphorylation by mediating phosphorylation of tyrosine 304 of Cox1 (Samavati et al., 2008). Cox2 is the smallest and the least hydrophobic subunit of the enzyme core. It consists of a large polar C-terminal domain that protrudes into IMS, and a transmembrane α-helical hairpin that anchors the subunit within the inner membrane. The C-terminal domain of Cox2, composed of 10-stranded β barrel, coordinates the Cu_A center and constitutes the docking site for cytochrome *c*. Similarly to Cox1, subunit Cox3 is a highly hydrophobic protein spanning the inner membrane with seven transmembrane helices. This subunit does not bear any prosthetic groups and is not directly involved in proton translocation. However, studies of the *Rhodobacter sphaeroides* aa_3-type CcO indicate that the presence of Cox3 maintains the rapid proton uptake into the D-channel at physiological pH, which presumably reduces the half-life of reactive dioxygen reduction intermediates (Gilderson et al., 2003; Hosler, 2004). This is thought to prevent the turnover-induced inactivation of the enzyme (suicide inactivation), and the subsequent loss of Cu_B site (Bratton et al., 1999; Hosler, 2004). The remaining 10 evolutionary younger subunits that associate with the surface of the complex core are encoded by the nuclear genome and are imported into mitochondria upon synthesis on cytoplasmic polysomes (Margeot et al., 2005). They include small polypeptides required for the stability/assembly of the holoenzyme, with several of them believed to be involved in regulation of its catalytic activity (Huttemann et al., 2008; Ludwig et al., 2001). It was shown that the exchange of bound ADP by ATP at the matrix domain of subunit Cox4 leads to allosteric inhibition of the bovine enzyme at high intramitochondrial ATP/ADP ratios (Kadenbach et al., 2000). Very recently, the expression of subunit Cox4 in the axons of sympathetic neurons was shown to be regulated at the posttranscriptional level by brain-specific microRNA miR-338 (Aschrafi et al., 2008). Transfection of precursor miR-338 into the axons of primary sympathetic neurons decreased Cox4 transcript and protein levels and resulted in diminished ATP levels suggestive of decreased OXPHOS activity. Some of the nuclear encoded subunits were shown to be expressed in tissue- and developmentally-specific

isoforms (Huttemann et al., 2008; Linder et al., 1995). In mammals, subunit Cox4 exists as two tissue-specific isoforms, Cox4-1 and Cox4-2 expressed ubiquitously, and at high levels in the lungs and trachea, respectively. Their expression is further regulated by oxygen availability and confers different kinetic properties to the enzyme. This regulatory circuit was shown to involve hypoxia-inducible factor 1α (HIF-1α), which is either hydroxylated under aerobic conditions and subsequently degraded by proteasome, or left unmodified. The unmodified HIF-1α accumulates in cell, which leads to transcriptional induction of both COX4-2 gene and the gene encoding mitochondrial matrix LON protease, responsible for the degradation of Cox4-1 (Semenza, 2007).

Subunits Cox5a, Cox5b and Cox6b are hydrophilic extramembrane proteins, while the rest of the nuclear encoded subunits are hydrophobic polypeptides spanning the membrane once. Subunit Cox5a is unique in that it does not interact with any of the mitochondrially encoded core subunits being held by the matrix domain of Cox4 and an extramembrane segment of Cox6c (Tsukihara et al., 1996).

Several pathogenic mutations in each of the three mitochondrially encoded CcO subunits have been reported (Shoubridge, 2001a). To date, however, only a single pathogenic mutation was found in genes encoding nuclear-encoded CcO subunits (Massa et al., 2008). It was reported that the R19H amino acid substitution in COX6B1 results in diminished assembly capacity of otherwise stable polypeptide, which leads to impairment in the late stage of CcO assembly. Affected individuals presented with severe infantile encephalopathy associated with isolated CcO deficiency (Massa et al., 2008).

The CcO complex from *S. cerevisiae*, composed of three mitochondrially encoded and eight nuclear encoded subunits closely resembles the mammalian counterpart. Yeast null mutants for the homologues of mammalian nuclear encoded subunits Cox4, Cox5a, Cox5b, Cox6c or Cox7a are respiratory deficient, lacking CcO activity and the absorption bands representing heme aa_3 (Taanman and Williams, 2001). This suggests that potential loss-of-function mutations in at least some of the human nuclear encoded CcO subunits might lead to early developmental lethality, hindering their identification.

Crystalline preparations of bovine heart CcO were shown to contain 13 lipids per enzyme dimer. These included two cardiolipins, one phosphatidylcholine, three phosphatidylethanolamines, four phosphatidylglycerols and three triglycerides. The X-ray structure resolution showed that the four phospholipids stabilize the dimeric form. The

palmitates, together with vaccenate (*cis*-Δ^{11}-octadecenoat) of phosphatidylglycerol, probably play an important role in the O_2 transfer process (Shinzawa-Itoh et al., 2007).

CcO contains several metal centers involved in electron transfer and dioxygen reduction. Besides their function in catalysis, most of these prosthetic groups confer an important structural/assembly function within the complex, since the defects in the synthesis and/or insertion of these cofactors often result in markedly reduced levels of fully assembled complex accompanied by appearance of subcomplexes (Stiburek et al., 2006). In addition to catalytic cofactors, the matrix portion-associated peripheral subunit Cox5b contains bound Zn(II) ion, while single Mg(II) ion is found at the interface of Cox1 and Cox2 subunits. Moreover, Cox1 contains single Na(I) ion in a site that can also bind Ca(II) (Yoshikawa et al., 1998). However, the functional relevance of these additional cofactors as well as their import/insertion pathways are largely unknown. The zinc atom could play a role in structural stability of the complex (Coyne et al., 2007), whereas the magnesium/manganese site located in close proximity to the water exit channel is thought to facilitate the release of water produced during the dioxygen reduction (Schmidt et al., 2003). The low-spin heme *a* and the heterobimetallic heme a_3-Cu_B center are located relatively deep within the hydrophobic interior of Cox1. In contrast, the binuclear, mixed-valent Cu_A center extends 8 Å above the surface of the inner membrane, being held by the IMS-located, C-terminal domain of Cox2. The two copper ions (Cu(I) and Cu(II)) of the Cu_A center are coordinated by two bridging cysteines of the Cx_3C motif, two histidines, one methionine and a carbonyl oxygen of the peptide backbone of Cox2 (Tsukihara et al., 1995).

The Cu_A center serves as the primary acceptor of electrons channeled through the respiratory chain. The electrons donated by cytochrome *c* are rapidly distributed between the Cu_A center and heme *a*, further continuing to the catalytic site composed of high-spin heme a_3 and an electronically coupled Cu_B ion. This part of the catalytic cycle referred to as the "reductive phase", ensures the reduction of heme a_3-Cu_B center, a prerequisite for binding of dioxygen (and CO, but not NO) to this site, and subsequent water formation ("oxidative phase") (Brunori et al., 2005; Michel et al., 1998). Protons required for the reduction of dioxygen, together with those translocated through the complex to the IMS, are taken up from the matrix and transferred via two distinct pathways to the vicinity of the heme a_3-Cu_B catalytic site. The K-pathway, named after a conserved lysine residue, is responsible for one- or two-proton supply during the reductive phase. The D-pathway, named after a conserved aspartic residue, transfers the remaining two or three "substrate" protons required for water formation, as well as four protons that are pumped to the IMS (Wikstrom, 2000). Recently,

Belevich and colleagues revealed the fundamental mechanism of coupling of electron transfer with proton translocation in CcO (Belevich et al., 2006). They showed that the electron transfer from heme *a* to the catalytic site is kinetically linked to an internal vectorial proton transfer, initiating the proton pump mechanism of the enzyme. The free energy released during the electron-transfer reactions is thus transformed into the electrochemical transmembrane gradient of protons that might be eventually utilized by the F_1F_O-ATP synthase (complex V) to power ATP synthesis.

Under mild detergent conditions and even when crystallized the majority of CcO exists as a dimer of two 13-subunit assemblies with contacts between monomers mediated merely by subunits Cox6a and Cox6b (Musatov and Robinson, 2002; Tsukihara et al., 1996). The electron-transfer activity of monomeric and dimeric form is comparable. It was suggested that the dimeric form of CcO may be required for proton translocation since this activity can be observed only with dimer. However, when reconstituted into phospholipid vesicles CcO spontaneously dimerizes, hindering the performance of proton translocation assay with monomeric enzyme. Recently, Stanicova et al. reported that the dimeric, dodecyl maltoside-solubilized complex shows high resistance to elevated hydrostatic pressure without any marked perturbations of its quaternary structure or functional activity after release of the pressure. In contrast, 3 kbar of hydrostatic pressure triggered multiple structural and functional alterations within the monomeric form. These included sequential dissociation of subunits Cox6a, Cox6b followed by Cox7a and Cox3 as well as irreversible loss of catalytic activity that dropped by as much as 60% (Stanicova et al., 2007). Thus, it appears that the dimerization of CcO plays a key structural-functional role, conferring maximal structural stability for the complex.

3.2. Synthesis and incorporation of heme moieties

Heme *a* is a unique heme derivative found exclusively in all eukaryotic and certain prokaryotic CcO enzymes. In contrast, many bacterial terminal oxidases utilize heme *b* or heme *o*, instead of heme *a* (Michel et al., 1998). Heme *a* differs from protoheme (heme *b* or ferroprotoporphyrin IX) in that the C2 vinyl side chain is replaced by an isoprenoid substituent and a methyl group is oxidized into a formyl group (Caughey et al., 1975). Heme represents a potentially toxic, hydrophobic iron chelate, which may facilitate harmful cellular process through ROS formation, e.g. oxidative membrane damage (Ryter and Tyrrell, 2000). Hence, the synthesis, delivery, and final incorporation of heme *a* into CcO must be carefully

regulated. Despite this fact, almost nothing is known concerning the regulation of heme *a* homeostasis. Recently, the possible role for copper as a regulator of heme *a* biosynthesis was investigated. However, no functional correlations could have been found (Morrison et al., 2005). Most of the yeast CcO accessory factors mutants characterized by blocked holoenzyme assembly and rapid turnover of intermediates is unable to accumulate heme *a*. Initially, this phenomenon was ascribed to increased turnover of free heme *a*, as a consequence of reduced Cox1 levels. However, this appears inconsistent with the fact that some of the yeast mutants retain high heme *a* levels, even when Cox1 is almost undetectable (Barros and Tzagoloff, 2002). Instead, preliminary studies have suggested that the synthesis of heme *a* is subject to either positive or negative regulation by intermediate/subunit of CcO at the level of heme *a* synthase (Cox15) (Barros and Tzagoloff, 2002).

The biosynthesis of heme *a* involves sequential conversion of heme *b*. The first step in this reaction is catalyzed by an inner membrane-associated farnesyl transferase (Cox10). It involves the conversion of C2 vinyl group on pyrrole ring A into a 17-hydroxyethylfarnesyl moiety (Tzagoloff et al., 1993). This reaction yields heme O, found as a final cofactor in some prokaryotic terminal oxidases. In the next reaction, the C8 methyl substituent on pyrrole ring D of heme O is oxidized into an aldehyde, thus generating heme *a*. This oxidation proceeds via two successive monooxygenase steps catalyzed by Cox15, an inner membrane-anchored heme *a* synthase (Brown et al., 2002). The matrix localized ferredoxin (Yah1) and ferredoxin reductase (Arh1) are thought to provide reducing equivalents during this reaction (Barros et al., 2002). Interestingly, Cox15 is itself presumably a heme-containing enzyme, employing a heme *b* cofactor at the active site (Svensson et al., 1996). Human homologues of both yeast Cox10 and Cox15 were identified sharing 33 and 42% sequence identity, respectively, with the yeast counterparts (Glerum and Tzagoloff, 1994; Petruzzella et al., 1998)). Mutations in both human genes were reported to result in isolated CcO deficiency associated with severely reduced heme *a* levels (Antonicka et al., 2003a; Antonicka et al., 2003b). In *COX10*-deficient fibroblasts and *COX15*-deficient heart mitochondria, the CcO-specific assembly defect is not accompanied by any accumulation of subassemblies (Williams et al., 2004). Muscle-specific *Cox10* knockout mice start to develop slowly progressive myopathy at the age of 3 months. Despite the fact that the CcO activity in muscle is diminished to less than 5% of control already in 2.5 months, the affected muscle retains almost normal contractile activity without any additional signs of oxidative damage or apoptosis (Diaz et al., 2005).

Two heme *a* moieties are found within the eukaryotic CcO. The bis-histidine low-spin heme *a* is a six-coordinate heme responsible for electron transfer. The second heme *a*, present

in the complex, is a five-coordinate, high-spin heme a_3, that forms a heterobimetallic site with the Cu_B ion, a place where dioxygen, CO or NO binds (Brunori et al., 2005; Michel et al., 1998). Both heme planes are oriented perpendicular to the membrane with their iron centers being 14 Å apart (Yoshikawa et al., 1998). The insertion of heme *a* moieties into the Cox1 subunit has not been characterized so far. As both metal centers are enfolded within the hydrophobic interior of Cox1, buried 13 Å below the membrane surface, their incorporation is likely to occur either on nascent Cox1 or on an early CcO subassembly (Carr and Winge, 2003). The Cox1·Cox4·Cox5a subcomplex, as structurally present in the mature CcO, constitutes an open channel through which it would be possible for both heme moieties to be incorporated into Cox1 from the IMS side (Cobine et al., 2006c).

3.3. Surf1 assembly factor

Human Surf1 is an integral protein of the inner mitochondrial membrane required for the assembly of the CcO complex. The mature form with a molecular mass of ~30 kDa is composed of two transmembrane domains with a central loop region facing the IMS (Yao and Shoubridge, 1999). Both transmembrane domains and the central loop region are required for proper insertion, but the C-terminal tail of the protein is dispensable. In contrast to yeast homologue Shy1 (Mashkevich et al., 1997), separately expressed N- and C-terminal transmembrane domains of human Surf1 are not able to form a functional protein (Yao and Shoubridge, 1999). Although the precise molecular role of human Surf1 in CcO biogenesis remains unknown, several lines of evidence indicate that the protein plays a role in some of the early events of CcO assembly, ranging from the insertion of heme a/a_3 into Cox1 to promotion/stabilization of early subunits' assembly. Human cells lacking Surf1 accumulate early assembly intermediates composed of merely Cox1, Cox4 and Cox5a, suggesting that the assembly is stalled at an initial stage (Stiburek et al., 2009; Williams et al., 2004). Surf1 orthologues are found in terminal oxidase operons of several prokaryote species, in which the mature CcO consists of only three core subunits known to associate early in the assembly process in eukaryotes (Bundschuh et al., 2008; Fernandez-Vizarra et al., 2009). Bacterial Surf1 orthologues have repeatedly been implicated in the insertion and/or stabilization of heme a/a_3, thought to occur concurrent or immediately after membrane insertion of Cox1 (Bundschuh et al., 2009; Smith et al., 2005).

Since the yeast Surf1 homologue Shy1 was repeatedly observed in high-molecular weight CcO-containing complexes, the protein is likely to have an additional role in some of

the late CcO assembly events as well (Nijtmans et al., 2001). Recently it was demonstrated that Shy1 associates with different inner membrane protein modules, including CcO subcomplexes and supercomplex species composed of partially and fully assembled forms of CcO and the cytochrome bc_1 complex. The association with complex III was observed even in the absence of the core subunit Cox2 (Mick et al., 2007). Based on these findings it was proposed that Shy1, together with another yeast CcO assembly factor Cox14 accompany transient forms of CcO, thereby maintaining their competence for the incorporation of additional subunits. This appears consistent with the previous finding that Cox13, the yeast homologue of human Cox6a seems to be assembled subsequently to formation of supercomplexes (Brandner et al., 2005a).

Yeast Shy1 was shown to genetically and/or physically interact with several factors involved in translational and early post-translational steps of Cox1 biogenesis. In *S. cerevisiae*, Cox1 synthesis is controlled by translational activators Mss51 and Pet309. The high-copy expression of Mss51, which also has a posttranslational role in Cox1 biogenesis, is able to suppress the CcO deficient phenotype of *shy1*Δ cells. Mss51 and Cox1 form a transient complex that is stabilized by Cox14. The accumulation of this complex is postulated to downregulate the translation of Cox1 under conditions of impaired CcO assembly by sequestering Mss51. Shy1 is thought to facilitate the release of Mss51 from the ternary complex, making it available for Cox1 translation (Barrientos et al., 2009). Recently, Coa1 and Coa2 were identified as new CcO assembly factors in yeast acting in early steps of enzyme assembly (Mick et al., 2007; Pierrel et al., 2007; Pierrel et al., 2008). Coa1 is an inner membrane-associated protein that exists as part of the Mss51.Cox14.Cox1 ternary complex, as well as in a complex with Shy1 alone. Coa1 appears to be involved in the transition of newly synthesized Cox1 from the Mss51 complex to a downstream intermediate involving Shy1. The respiration in both *coa1Δ* and *shy1*Δ cells is enhanced by coexpression of Mss51 and Cox10, suggesting that Coa1 may link heme incorporation and cotranslational insertion of Cox1. Coa2, which may possess a chaperone function, appears to act downstream of Coa1, likely stabilizing Cox1 during the heme a_3 insertion step. This may be accomplished by promoting Cox5a/Cox6 (human Cox4/Cox5a) association with Cox1, suggested to increase the stability of the subunit by stabilizing the loops connecting the 12 transmembrane helices (Pierrel et al., 2008). This is consistent with the fact that the HAP4 induced upregulation of subunits Cox5 and Cox6 suppresses the respiratory deficient phenotype of *shy1* cells (Fontanesi et al., 2008a).

Several lines of evidence indicate that Surf1/Shy1 may be responsible for the insertion of heme a_3 into Cox1. The *Rhodobacter sphaeroides* Surf1 homologue is required for insertion/stabilization of heme a_3 within Cox1, as the majority of CcO lacks heme a_3 in *R. sphaeroides* Surf1 null mutant (Smith et al., 2005). When coexpressed in *E. coli*, together with enzymes for heme *a* synthesis, the *Paracoccus denitrificans* Surf1 homologues Surf1q and Surf1c bind heme *a in vivo*, probably providing a protein-bound heme *a* pool for the insertion into Cox1 (Bundschuh et al., 2009). *cox11*Δ cells are peroxide sensitive showing almost 2-fold increase in protein carbonylation, consistent with enhanced ROS production (Khalimonchuk et al., 2007). The increased peroxide sensitivity of these cells is thought to stem from accumulation of a transient pro-oxidant Cox1-heme a_3 assembly intermediate. Depletion of Shy1 in *cox11*Δ background abrogates the peroxide sensitivity of these cells. Furthermore, *shy1*Δ cells remain peroxide resistant even upon overexpression of Cox15, known to markedly increase heme *a* levels in CcO assembly mutants. The identity of the pro-oxidant heme is confirmed by the fact that only heme a_3 has an open coordination site, which, when solvent exposed could catalyze formation of the hydroxyl radical pro-oxidant (Khalimonchuk et al., 2007).

Mss51, Cox14, Coa1 and Coa2 are found exclusively in fungi, lacking homologues in both higher eukaryotes and bacteria (Barrientos et al., 2009). Furthermore, yeast Shy1 possess extra C-terminal segments not conserved in Surf1 proteins of other species. Thus, it is not clear whether similar mechanisms mediated by functional homologues of these proteins exist also in higher eukaryotes.

Ubiquitous posttranscriptional silencing of SURF1 in *Drosophila melanogaster* is associated with 100% egg-to-adult lethality, underdevelopment of CNS, particularly of the optic lobes, and impaired locomotor behavior in larvae. The altered motor patterns are likely not due to structural and/or functional abnormalities of muscle fibers or reduction in contractile efficiency, but rather results from defective energy provision due to CcO deficiency. In contrast, a panneuronal knockdown led to slight impairment in locomotor behavior and photoreactivity and strikingly increased longevity when compared to controls. However, histological analysis failed to reveal any marked neurodegenerative damage in brains of these flies (Zordan et al., 2006).

In vertebrates, SURF1 is part of the very tightly organized and highly conserved surfeit gene cluster containing six housekeeping genes (SURF1–6) that encode both structurally and functionally unrelated proteins (Duhig et al., 1998). The reason for the

conservation of this structure over 250 million years of divergent evolution between birds and mammals remains obscure. Posttranscriptional silencing of Surf1 in zebrafish (*Danio rerio*) resulted in 50% reduction in CcO activity, developmental defects in endodermal tissues, cardiac function and swimming behavior. The hindbrain and neuronal tube exhibited dramatically increased apoptosis and secondary motor neurons were absent or abnormal. In contrast, the cardiac dysfunction was likely due to impaired energy metabolism, since heart was devoid of apoptotic cells, exhibiting increasingly poor performance over time (Baden et al., 2007). The described phenotype of Surf1 deficient zebrafish was almost identical to that of Cox5 deficient animals, suggesting that it can be readily attributed to general CcO deficiency rather than a specific lack of Surf1 protein (Baden et al., 2007).

Two Surf1 knockout mouse models were generated so far by using either replacement of exons 5-7 by neomycin-resistance (NEO) cassette (Agostino et al., 2003) or insertion of a *loxP* sequence in exon 7 of the gene (Dell'agnello et al., 2007). The prominent characteristic of the $SURF1^{NEO}$ KO mice was high embryonic lethality, subsequently attributed mainly to deleterious effects of the presence of the *NEO* cassette. In contrast, the later $SURF1^{loxP}$ knockouts showed, in addition to mild CcO deficiency, altered neuronal Ca^{2+} homeostasis, moderate functional and morphological abnormalities in skeletal muscle and liver and substantially prolonged lifespan (Dell'agnello et al., 2007). In sharp contrast to severe CNS involvement observed in human patients, neither mouse *SURF1* KO model exhibited spontaneous neurodegeneration at any age. The positive effects of ablation of murine Surf1 on the lifespan of knockout animals is strikingly similar to that of *D. melanogaster* CNS-wide Surf1 knockout. On this account, it was suggested that the partial suppression of respiratory chain activity might, possibly mainly due to attenuated ROS production, positively affect lifespan of (-/-) animals (Fernandez-Vizarra et al., 2009). Consistent with these findings, CcO deficiency was associated with reduced oxidative stress in CNS of *COX10* knockout mice (Fukui et al., 2007).

Mutations in *SURF1*, which account for the majority of nuclear-encoded, isolated CcO deficiencies in humans, are characterized by the development of Leigh syndrome, a subacute necrotizing encephalomyopathy (Zhu et al., 1998). Most of the identified *SURF1* mutations are predicted to lead to loss of the protein. Human as well as yeast cells lacking Surf1/Shy1 retain approx. 10-20% of the CcO activity of wild-type cells, indicating that the function of the protein is partially dispensable in both organisms (Yao and Shoubridge, 1999). In contrast to human Surf1, the lack of Shy1 leads to partially pleiotropic effects including increased levels of cytochrome *c* and elevated NADH-cytochrome *c* reductase activity (Mashkevich et

al., 1997). Furthermore, the human and yeast proteins fail to complement each. However, the proteins exhibit striking sequence similarity in some conserved domains and the elevated cytochrome *c* concentration and complex III activity might well reflect a compensatory response (Nijtmans et al., 2001). Analogous to yeast, overexpression of NF-YA, a catalytic subunit of the human homologue of the yeast HAP complex, leads to increase in CcO activity of Surf1-deficient fibroblasts. Unlike the yeast HAP complex, the human NF-Y complex is not directly involved in regulation of mitochondrial biogenesis and the molecular mechanism responsible for the suppression of the CcO defect in *SURF1* fibroblasts is not known (Fontanesi et al., 2008a).

The severe CcO deficiency of *SURF1* fibroblasts is accompanied by barely detectable changes in cellular respiratory rates under normoxic conditions (Pecina et al., 2003). The measurement of CcO oxygen kinetics by the partial oxygen pressure at half-maximal respiration rate revealed markedly attenuated affinity for oxygen of the residual enzyme. This aspect could exacerbate the respiratory defect in tissues where high energy demand meets up with very low oxygen pressure, such as in the brain, a prominent pathology site of *SURF1* patients (Pecina et al., 2004).

The fatal neurological phenotype of CcO deficient Leigh syndrome is associated with remarkable tissue pattern of CcO assembly impairment, pointing to profound tissue-specific character of regulation of CcO biogenesis (Stiburek et al., 2005). Intriguingly, various tissue samples carrying mutations in *SURF1* exhibit, similar to *SCO1* and *SCO2* tissues, marked tissue-dependent copper deficiency. This suggests that Surf1 is required, in a tissue-specific manner, to maintain proper cellular copper homeostasis (Stiburek et al., 2009). Interestingly, yeast cells lacking Shy1 are deficient in mitochondrial copper, whereas the total cellular copper content remains normal (Pierrel et al., 2007). Supplementation of *shy1*Δ cultures with exogenous copper partially rescues the respiratory capacity of these cells (Fontanesi et al., 2008a).

3.4. Delivery and insertion of copper ions

Copper ions are required in mitochondria for the formation of Cu_A and Cu_B sites in CcO and for the incorporation into IMS-located fraction of Cu/Zn-superoxide dismutase (Cobine et al., 2006a). Due to its chemical reactivity that may lead to deleterious side effects, the amount of free cellular copper is maintained at extraordinary low levels under physiological conditions (Rae et al., 1999). As a result, the delivery and compartmentalization

of copper is mediated by a specific subset of proteins termed copper metallochaperones that are thought to transfer copper ions to their target proteins via transient protein-protein interactions (ligand-exchange reaction) (Huffman and O'Halloran, 2001). Despite the recent progress in detailed structure-function characterization of several members of the mitochondrial CcO-specific copper delivery pathway, the fundamental mechanism which ensures the copper uptake into mitochondria still remains to be elucidated (Cobine et al., 2006a; Cobine et al., 2006c). Recently, it was shown that yeast mitochondria contain a significant pool of copper bound neither to proteins nor mitochondrial DNA (Cobine et al., 2004). This pool is found in matrix as a soluble, anionic, low molecular weight complex, responding to changes in cytoplasmic copper content. Although the identity of the yeast matrix copper ligand was not revealed yet (Cobine et al., 2004), a compound with the same fluorescent and chromatographic properties was found to be conserved in mouse liver (Cobine et al., 2006c). This copper pool likely serves as a reserve for metallation of mitochondrial copper metalloenzymes, since the overexpression of heterologous copper-binding proteins in yeast matrix results in respiratory growth defect, suppressible by exogenous copper supplementation (Cobine et al., 2006a). A number of proteins engaged in mitochondrial, CcO-specific copper trafficking have been identified in eukaryotes, while mutations in two of them (Sco1 and Sco2) were reported to lead to fatal neonatal CcO deficiency in human (Shoubridge, 2001b; Stiburek et al., 2006).

The small hydrophilic protein Cox17 that localizes both to the cytoplasm and the mitochondrial IMS was the first to be implicated in copper ion provision to CcO. It delivers Cu(I) to two downstream CcO copper-chaperones Sco1 and Cox11. The interaction of Cox17 with Sco1 and Cox11 is thought to be of transient nature. Based on its dual localization, Cox17 was initially proposed to act as a copper shuttle between the cytoplasm and IMS (Beers et al., 1997). The tethering of Cox17 to the inner membrane by a heterologous transmembrane domain renders the protein fully functional, suggesting that migration between the cytoplasm and IMS is not essential for its function (Maxfield et al., 2004). Deletion of *COX17* does not affect mitochondrial copper levels (Cobine et al., 2004). However, *in vitro* studies with purified proteins and the yeast cytoplasm assay have demonstrated that Cox17 is able to deliver Cu(I) to both Sco1 and Cox11 (Horng et al., 2004). In addition to the Cx_9C motif, Cox17 possess another two conserved Cys residues upstream of the twin Cys motif. The protein is functional without either of the two disulfides in the twin Cx_9C motif. Two Cu(I) conformers of Cox17 were described so far. One is a mononuclear Cu(I)-Cox17 monomer in the form of helical hairpin stabilized by two disulfide bonds with

Cys residues in the twin Cx_9C motif. The second Cu(I) conformer is an oligomeric protein complex containing reduced thiolates that is capable of binding a polycopper-thiolate cluster. The purification of Cox17 from the IMS yields a protein devoid of bound copper (Khalimonchuk and Winge, 2008).

Knockout of *COX17* in mice leads to lethality of (-/-) individuals between embryonic days E8.5 and E10, confirming the essential character of Cox17 (Takahashi et al., 2002). Human Cox17 orthologue has been identified that shares 48% sequence identity with yeast counterpart (Amaravadi et al., 1997). Overexpression of the human Cox17 rescues the CcO activity defect of human *SCO2* but not *SCO1*-deficient cells (Leary et al., 2004). HeLa cells in which the expression of Cox17 was downregulated by the use of siRNA show diminished levels of CcO and CcO-containing supercomplexes. The accumulation of a Cox1-containing subcomplex of 158 kDa devoid of Cox2 in these cells points to the role of human Cox17 in maturation of Cox2, but not Cox1 (Oswald et al., 2009).

Cox19 and Cox23 are two additional soluble Cx_9C class proteins implicated in copper transfer to CcO. Yeast cells lacking either Cox19 or Cox23 are respiratory deficient and have reduced CcO activity. Cox19 exhibits dual localization in IMS and cytoplasm, albeit only upon overexpression (Nobrega et al., 2002). Cox23 localizes to both IMS and cytoplasm (Barros et al., 2004). The CcO-specific respiratory defect of Cox19 null strain is not associated with decreased mitochondrial copper. Moreover, the mutant phenotype can not be rescued by addition of exogenous copper salts. Cox19 exists as a stable dimer, and recombinant protein was reported to bind Cu(I). The tethering of Cox19 to the inner membrane via transmembrane domain of Sco2 does not abrogate its function in CcO assembly (Cobine et al., 2006a). Cox19 is likely present within the IMS mostly in the reduced state, since when purified it contains titrable thiolates (Khalimonchuk and Winge, 2008). There is a correlation in the ability of Cox19 to bind Cu(I) and its *in vivo* function in CcO assembly (Rigby et al., 2007). The CcO-specific respiratory defect of Cox23 null mutant is rescued by increased concentrations of copper, but only when *COX17* is overexpressed simultaneously (Barros et al., 2004). However, the deletion of *COX23* does not affect mitochondrial copper level (Cobine et al., 2006c). Human orthologue of COX19 was identified that shares 40% sequence identity with yeast protein. Subcellular localization studies with full-length, GFP-fused, human Cox19 showed predominant cytoplasmic localization in HEK 293 cells (Sacconi et al., 2005).

Pet191 is another member of the twin Cx_9C class of proteins found in the IMS. In contrast to Cox17, Cox19 and Cox23, Pet191 is tightly associated with the IM facing the IMS

and its import does not rely on Mia40. Yeast cells lacking Pet191 are respiratory deficient due to isolated CcO assembly defect (Khalimonchuk et al., 2008). The protein does not appear to have a prominent function in copper delivery to CcO, as *pet191*Δ cells have normal mitochondrial copper levels. Pet191 may be involved in the maintenance of twin Cx_9C proteins within IMS (Khalimonchuk and Winge, 2008).

Human Sco1 and Sco2 are closely related inner mitochondrial membrane copper-binding proteins encoded by paralogous genes. They have been demonstrated to exert nonoverlapping, cooperative roles in copper delivery to CcO (Leary et al., 2004). In addition, they have been shown to be involved in the maintenance of cellular copper homeostasis, presumably by controlling cellular copper export (Leary et al., 2007; Leary et al., 2009b). Very recently, human SCO proteins were reported to carry out distinct, stage-specific roles during Cox2 synthesis and Cu_A site maturation (Leary et al., 2009a). Interestingly, the tumor suppressor p53 was shown to directly regulate mitochondrial respiration through transactivation of human *SCO2* transcription (Matoba et al., 2006). Mutations in both *SCO1* and *SCO2* cause severe tissue-specific CcO assembly impairment accompanied by marked copper deficiency (Leary et al., 2007; Stiburek et al., 2009; Stiburek et al., 2005). However, both genes have been shown to be ubiquitously expressed, displaying a similar expression pattern across human tissues. Mutations of *SCO1* have originally been reported in only a single pedigree, where the two patients, presenting with fatal infantile encephalomyopathy and hepatopathy, were compound heterozygotes carrying a nonsense mutation on one allele and a P174L missense mutation on the second allele (Valnot et al., 2000). Recently, we have studied a novel *SCO1* missense mutation (G132S) found in a patient with CcO deficiency and early onset hypertrophic cardiomyopathy, hypotonia, encephalopathy, and hepatopathy (Stiburek et al., 2009). Thus, the lack of an apparent cardiac involvement in the previously published *SCO1* cases, which was in sharp contrast to *SCO2* mutations, very likely resulted either from the considerably reduced survival time of both siblings or the distinct nature of the missense allele expressed in these patients. Indeed, P174L mutant Sco1 exhibits markedly altered functional properties and almost normal polypeptide levels (Cobine et al., 2006b), whereas the G132S allele appears to lead to a simple, yet almost complete loss of protein and function (Stiburek et al., 2009). In contrast to *SCO1*, mutations of *SCO2* are more common, with all reported patients carrying at least one E140K missense allele (Vesela et al., 2004). Mutations in *SCO2* cause fatal infantile encephalomyopathy and hypertrophic cardiomyopathy. *SCO2* patients homozygous for the E140K substitution have a delayed onset

and slightly prolonged course of the disease compared with compound heterozygotes (Vesela et al., 2004).

SCO proteins are integral inner membrane components consisting of a globular copper-binding domain that protrudes into the IMS (Buchwald et al., 1991). This domain exhibits a thioredoxin fold composed of a central four-stranded β sheet surrounded by four α helices (Williams et al., 2005). A single Cu(I) binding site formed by cysteinyl residues of the Cx_3C motif and a histidyl residue is found within the globular domain. The structures of the metal-free human Sco1 conformer and Cu_1Sco1 complex are similar with only loop 8 showing significant rearrangements (Banci et al., 2006). Sco1 and Sco2 are tethered to the inner membrane by a single N-terminal transmembrane helix that was shown to be functionally important in Sco1 (Beers et al., 2002). The human Sco2 conformer resembles human Sco1 with the exception of greater conformational dynamics (Banci et al., 2007). SCO proteins are thought to act downstream of Cox17 in copper delivery pathway to Cu_A site in Cox2. Sco1 was shown to be copper-metallated by Cox17 *in vitro*. It is not known whether Sco1 delivers both Cu(I) and Cu(II) ions to build the binuclear, mixed valent Cu_A center in Cox2. Consistent with the composition of Cu_A center, SCO proteins can bind either Cu(I) or Cu(II) ions (Horng et al., 2005). It was suggested that SCO proteins might form a complex in order to deliver two copper ions to Cox2 simultaneously (Leary et al., 2004). Recently, however, a sequential delivery scenario is favored (Jaksch et al., 2000; Leary et al., 2009a). The involvement of human SCO proteins in copper delivery to CcO is further supported by the fact that the missense mutations in human *SCO1* (P174L) and *SCO2* (E140K and S240F) are located in the vicinity of the conserved Cx_3C copper-binding motif (Jaksch et al., 2000; Valnot et al., 2000). Additionally, it was shown that the CcO defect of both *SCO1* and *SCO2*-deficient fibroblasts and myoblasts is at least partially rescued by exogenous copper supplementation (Jaksch et al., 2001; Leary et al., 2004). Finally, the overexpression of the human SCO proteins with conserved cysteinyl and histidyl residues substituted by alanines, fail to rescue the CcO deficiency of either *SCO1* or *SCO2*-deficient fibroblasts (Horng et al., 2005).

Yeast cells lacking Sco1 are devoid of CcO activity and show markedly diminished protein levels of Cox2. Although yeast also encode a Sco2 protein, capable of binding copper ions (Cobine et al., 2006c), this has no apparent function in CcO assembly (Glerum et al., 1996). Both yeast Sco1 and Sco2 were shown to physically interact with Cox2, albeit only upon overexpression (Lode et al., 2000). Recently, we have reported that human Sco1 physically interacts with the fully assembled CcO complex in both skeletal muscle and

HEK293 cell mitochondria (Stiburek et al., 2009). Originally, based on sequence similarity of Sco1 with the peroxiredoxin protein family, the protein was proposed to be involved in the maintenance of Cu_A site cysteines in reduced state (Chinenov, 2000). Furthermore, on the basis of high-resolution structural data, human Sco1 has been implicated to function as a redox switch in the IMS (Williams et al., 2005).

We have demonstrated that human Sco2 acts in a highly tissue-specific manner at an early stage of CcO assembly, very likely during the maturation of Cox2 subunit (Stiburek et al., 2005). Furthermore, we showed that the E140K substitution leads to severely diminished Sco2 levels in all probed tissues. Since this substitution was shown to slightly perturb copper-binding of Sco2 it was speculated that the stability of Sco2 may depend on it being copper-loaded. The previously identified missense mutations in *SCO1* (P174L) and *SCO2* (E140K and S240F) are located in the vicinity of the conserved Cx_3C motif and the essential histidyl residue, suggesting that the loss-of-function may relate to perturbed copper-binding of the protein. However, both *SCO2* missense mutations are associated with severely impaired stability of the protein and the E140K mutant was shown to retain appreciable residual function in terms of Cu(I) binding (Foltopoulou et al., 2004). Furthermore, the overexpression of the E140K mutant Sco2 in the corresponding mutant background led to rescue of the CcO defect. In contrast, the P174L mutation does not affect the ability of Sco1 to bind and retain copper ions, however, its ability to be copper loaded by Cox17 is severely compromised. Based on our inability to detect the residual G132S mutant Sco1 in the dimeric form on blue-native gels and the fact that the mutation lies in a protein region shown to be required for dimerization, we proposed that the dimerization is required to stabilize Sco1. The G132S Sco1 skeletal muscle mitochondria accumulated two Cox2-containing subcomplexes, whereas corresponding Sco2-deficient samples are characterized by the complete absence of such species. This suggests that Sco1 is very likely responsible for a different posttranslational aspect of Cox2 biogenesis than Sco2 (Stiburek et al., 2009). This appears further supported by the fact that the steady-state level of Sco2 was virtually unaffected in Sco1-deficient background. Very recently, Leary and colleagues have reported that the synthesis of Cox2 is diminished in human *SCO2*, but not *SCO1* cells. On the other hand, the newly synthesized Cox2 exhibited increased stability in *SCO2* cells when compared to controls. It was concluded that Sco2 is required for the synthesis of Cox2, in a manner that depends on its ability to bind copper, acting upstream of Sco1 during the biogenesis of Cox2. It was further proposed that the association of Sco2 with Cox2 is required immediately following its synthesis, possibly to recruit Sco1 to the subunit. Otherwise the nascent Cox2 is rapidly degraded. Thus, the

maturation of Cox2 appears to require formation of a complex that contains both SCO proteins, each with a functional Cx_3C copper-coordinating motif (Leary et al., 2009a). Leary and colleagues further demonstrated that a fraction of total Sco2 acts as a thiol-disulfide oxidoreductase, oxidizing the copper-coordinating cysteines in Sco1 during Cox2 maturation. Under physiological conditions the cysteines in Cx_3C motif of Sco1 exists as a mixed population of oxidized disulfides and reduced thiols. In contrast, Sco1 molecules from either SCO background exhibited altered ratio of oxidized to reduced cysteines. This ratio was shown to be shifted towards disulfides upon overexpression of wild-type Sco2, and towards thiols upon knockdown of mutant Sco2, in *SCO2* background (Leary et al., 2009a).

Tissues and/or fibroblasts cultures harboring mutations in *SCO1*, *SCO2*, *COX10* or *COX15* were found to exhibit marked copper-deficient phenotype, consistent with involvement of the corresponding gene products in regulation of cellular copper homeostasis. Importantly, the copper deficiency phenotypes of *SCO1*, *SCO2* and *COX15* mutant fibroblasts were shown to be fully dissociable from the respective CcO defects. Kinetic labeling studies using ^{64}Cu indicated that the copper defect of *SCO1* and *SCO2* patient fibroblasts is caused by a defect in cellular copper retention, rather than copper uptake. Although overexpression of *SCO2* was shown to suppress the copper defect, this rescue was only partial in *SCO1* background. This was attributed to aberrant signaling of the P174L Sco1 variant (Leary et al., 2007). Based on these findings, it was postulated that Sco1 and Sco2 are bifunctional proteins that interplay in order to generate a signal that modulates the rate of copper efflux from the cell. Since it was not possible to alter the copper content of control fibroblasts by changing the levels or relative ratio of SCO proteins, it is unlikely that similar mechanism could be involved under physiological conditions. On the other hand, it was observed that the cysteinyl residues of Sco1, but not those of other CcO assembly factors, shows altered oxidized-to-reduced ratio in copper-deficient mutant *COX10*, *COX15* and *SCO2* backgrounds relative to controls. And based on the observation that the cysteinyl residues of the P174L Sco1 variant are completely oxidized in the *SCO1* background, it was proposed that the mutant protein elicits a signal equivalent to that of the wild-type copper-loaded conformer, thereby signaling a state of cellular copper overload. Thus, it was concluded that the copper-related signaling of SCO proteins is modulated through changes in the redox state of Sco1's cysteine thiols (Leary et al., 2009a). Recently, we have shown that also affected tissues harboring mutations in *SURF1* exhibit marked tissue-dependent copper deficiency, further expanding the list of CcO assembly factors with possible role in cellular copper homeostasis maintenance. The observed association of human Sco1 with the fully assembled CcO prompted us to hypothesize that the

regulation of cellular copper homeostasis may involve cytochrome oxidase, as an important cellular copper recipient (Stiburek et al., 2009).

The inner-membrane protein Cox11 represents another mitochondrial CcO-specific copper metallochaperone. Similarly to SCO proteins, Cox11 is thought to function downstream of Cox17 in copper delivery to CcO, presumably inserting Cu(I) into Cox1 to build the Cu_B site. Yeast Cox11 null mutant have diminished CcO activity and reduced levels of Cox1 (Tzagoloff et al., 1990). The role for Cox11 in Cu_B site formation was implicated by the observation that CcO isolated from *R. sphaeroides* Cox11 null mutant lacked Cu_B site, along with diminished magnesium content and altered heme a_3 environment, but contained both heme moieties (Hiser et al., 2000). Like Sco1 and Sco2, yeast Cox11 is tethered to the inner membrane by a single N-terminal transmembrane helix, while the soluble C-terminal domain harboring three copper-binding cysteinyl residues protrudes into the IMS (Carr et al., 2002). These cysteinyl residues are responsible for both Cu(I)-binding and *in vivo* activity. Truncation of the transmembrane domain leads to soluble protein that dimerizes upon Cu(I) binding (Carr et al., 2002). Cox11 was shown to be copper-metallated by Cox17 (Horng et al., 2004). Two *COX11* homologues have been identified in human genome, however only one represents active gene predicted to encode protein with 55% sequence identity with yeast polypeptide (Petruzzella et al., 1998).

3.5. Assembly of the mammalian CcO complex

The spatiotemporal assembly of mammalian CcO within the inner mitochondrial membrane is a sequential, tissue-specific and relatively slow process (Nijtmans et al., 1998; Wielburski and Nelson, 1983). The half-life of the holoenzyme is thought to be about three days (Leary et al., 2002). Little is known about the sequential order in which prosthetic groups are delivered/synthesized and inserted, and constituent subunits are assembled to form the mature membrane-embedded complex. The fact that CcO subcomplexes are allowed to accumulate in human mitochondria, have permitted the *bona fide* definition of several key stages of this intricate process (Stiburek et al., 2006). In contrast, yeast CcO subcomplexes are difficult to detect as they likely undergo rapid proteolytic degradation (Horan et al., 2005). The nuclear encoded CcO subunits are imported into mitochondria upon synthesis on free cytoplasmic polysomes (Margeot et al., 2005). It is not known whether all of these subunits undergo conservative sorting or whether a subset of them is inserted from the IMS side. In contrast, most of the CcO accessory proteins are translated on outer membrane-attached

polysomes, and might be imported through the TOM machinery in a cotranslational manner (Margeot et al., 2005). The intramitochondrial steady-state levels of various unassembled CcO subunits differ considerably. Significant pools of free Cox1 and Cox5a appear to exist in mitochondria of various human tissues, whereas the levels of unassembled Cox4, and in particular of Cox2 are substantially lower (Stiburek et al., 2005). Cox1 appears to stably interact with several nonsubunit proteins before it associates with Cox4 and Cox5a, since it is readily detected as part of three 60-100 kDa complexes that apparently lack other CcO subunits. Subsequently, upon membrane insertion, Cox1 associates with the Cox4·Cox5a heterodimer, forming Cox1·Cox4·Cox5a subassembly (Stiburek et al., 2005). This subcomplex readily accumulates under conditions of blocked/retarded assembly, pointing to its high intrinsic stability (Nijtmans et al., 1998; Stiburek et al., 2005; Williams et al., 2004).

Two lines of evidence suggest that the insertion of heme *a* occurs either on unassembled Cox1 or during the formation of Cox1·Cox4·Cox5a subassembly. First, both heme moieties are buried deep within the transmembrane interior of Cox1, making the incorporation at the later stages unlikely (Tsukihara et al., 1996). Second, human cells deficient in heme *a* synthesis do not accumulate Cox1·Cox4·Cox5a subassembly (Antonicka et al., 2003b; Williams et al., 2004). The later finding also suggests that the presence of heme *a* within Cox1 might stabilize the binding of Cox4·Cox5a heterodimer to this subunit. In contrast, heme *a* is not required for the assembly of the core subunits in *R. sphaeroides* CcO (Hiser and Hosler, 2001). The insertion of active site heme might require the inner-membrane protein Surf1, since a significant fraction of CcO isolated from *R. sphaeroides* Surf1 null mutant was devoid of heme a_3 (Smith et al., 2005). Owing to the location of Cu_B site, its formation is likely to occur more or less concurrently with the insertion of heme groups. However, the presence of Cu_B ion within Cox1 does not seem to be essential for stable incorporation of heme a_3 (Hiser et al., 2000). The intrinsic inner-membrane protein Cox11 might be responsible for the formation of Cu_B site (Hiser et al., 2000). Upon assembly of heme moieties and formation of the Cu_B center, the Cu_A-containing Cox2 is believed to join the Cox1·Cox4·Cox5a subcomplex. Since the diminished formation of Cu_A site apparently leads to an accelerated turnover of Cox2 (Stiburek et al., 2005; Williams et al., 2004), formation of the Cu_A site in Cox2 appears to constitute a prerequisite for an efficient association of this subunit with Cox1·Cox4·Cox5a subcomplex. The increased proteolytic degradation of such Cox2 might result either from the lowered intrinsic stability of the protein or its reduced binding to Cox1. Conversely, the proper assembly of Cox2 appears indispensable for subsequent association of Cox3, and hence for the stable binding of most of

the remaining nuclear-encoded subunits. Indeed, a transmitochonrial cell line (cybrid) with 100% mutant load of a large C-terminal truncation in Cox3 was shown to lack the holoenzyme complex and accumulate subcomplex composed of Cox1, Cox2, Cox4 and Cox5a (Tiranti et al., 2000). Cox2 might be required to secure the incorporation of heme a_3, or whole active site, via capping the proposed heme-insertion channel formed in Cox1·Cox4·Cox5a subassembly (Cobine et al., 2006c). Upon assembly of Cox2 and Cox3 the remaining nuclear encoded subunits, with the exception of Cox6a and Cox7a or Cox7b, are thought to join the complex (Nijtmans et al., 1998). The resulting assembly intermediate S3 represents a ubiquitous, although minor form of CcO in lauryl maltoside preparations. Subsequent association of the rest of the subunits completes the assembly of the holoenzyme complex. In the next, maturation step a covalent bond is formed on assembled Cox1 bridging His^{240}, one of the three histidine ligands of Cu_B, with conserved Tyr^{244} located at the end of the proton translocation K-channel (Yoshikawa et al., 1998). This posttranslational modification is thought to secure the Cu_B ion in a certain configuration and distance from heme a_3, thus preventing the coordination of Cu_B via histidine ligands of the active site heme (Pinakoulaki et al., 2002). Finally, the mature holoenzyme complex associates with complex I and dimeric complex III, to form the 1.7-MDa respiratory supercomplex (Schafer et al., 2006; Schagger and Pfeiffer, 2000). The role of cardiolipin in final maturation of CcO and the function of cytochrome *c* during CcO assembly remains elusive.

4. Respiratory supercomplexes

Growing body of experimental evidence suggests that the interactions among the complexes of the mitochondrial oxidative phosphorylation system, and in particular of the respiratory chain, are not limited to their functional interplay during electron transfer reactions, mediated merely by the electron carriers coenzyme Q (CoQ) and cytochrome *c*, but extend well beyond that point to their stable and organized physical association within the membrane and their interdependence during assembly/biogenesis. Nowadays, it became widely accepted that the respiratory complexes are found within the inner membrane in the form of stable high order, supramolecular assemblies, so-called respiratory supercomplexes, the building blocks of functional association that was termed respirasome (Lenaz and Genova, 2007; Schagger and Pfeiffer, 2000). It was suggested that the functional relevance of supramolecular associations of respiratory complexes resides in facilitating the electron flux

between the complexes by reducing the distance of diffusion of mobile carriers, and by substrate channeling (Lenaz and Genova, 2007; Schagger and Pfeiffer, 2000).

Although stable assemblies of complexes III and IV were isolated in past from several prokaryote species including *P. denitrificans* and *Sulfolobus sp*., they were recognized as being characteristic of these bacteria (Berry and Trumpower, 1985; Iwasaki et al., 1995). More recently, mainly the development of BN-PAGE enabled detailed quantitative analysis of this phenomenon in both yeast and mammals (Schagger and Pfeiffer, 2000). In *S. cerevisiae*, which lacks complex I, the major supramolecular assemblies of respiratory complexes were found to be two species with apparent masses of 750 and 1,000 kDa containing merely subunits of complexes III and IV (Cruciat et al., 2000; Schagger and Pfeiffer, 2000). The smaller supercomplex was shown to consist of a complex III dimer and a complex IV monomer (III_2IV_1), whereas the larger supercomplex (III_2IV_2) contained a complex III dimer associated with two complex IV monomers. In contrast, supercomplex composed of complex I monomer and complex III dimer (I_1III_2) appears to be the most stable assembly of respiratory complexes found in mammalian mitochondria. On the other hand, supercomplex consisting of complex I monomer, complex III dimer and of one to two copies of complex IV monomer ($I_1III_2IV_{1-2}$) appears as the most physiologically relevant. However, only <10% of total mitochondrial complex IV was found to exist in supercomplex form in mammalian mitochondria (Schagger and Pfeiffer, 2000). The molecular architecture of both I_1III_2 and $I_1III_2IV_1$ supercomplexes was characterized using electron microscopy single particle image analysis (Schafer et al., 2006). Digitonin-solubilized mammalian mitochondria contain various additional minor stoichiometric variants of these two major species. Very recently, Acín-Pérez and colleagues demonstrated that some of the previously identified mammalian supercomplexes contain also complex II, and complex V, as well as electron carriers CoQ and cytochrome *c* (Acin-Perez et al., 2008). Importantly, the authors were also able to demonstrate that the supercomplexes containing complexes I, II, III, and IV, and the mobile carriers are capable of transferring electrons from NADH or succinate to oxygen, exhibiting properties of true respirasome.

In addition to the functional relevance with respect to electron transfer activity, supramolecular organization of respiratory complexes appears to play a role in the assembly/stability of the individual complexes, particularly of complex I. It has been repeatedly demonstrated in various systems that in the absence of appreciable amounts of either complex III or complex IV, the amount of fully assembled complex I is severely diminished. Human and mouse cells with complex III deficiency due to mutations in

mitochondrial gene encoding cytochrome *b* showed dramatic loss of complex I (Acin-Perez et al., 2004). Attempts to isolate complex I from *P. denitrificans* mutant strains lacking complexes III or IV led to complete dissociation of complex I under the conditions of BN-PAGE (Stroh et al., 2004). Mouse cells with severely reduced complex IV due to either downregulated expression of nuclear encoded subunit Cox4 or chromosomal deletion of *COX10* showed markedly diminished levels of complex I (Li et al., 2007). Finally, it was reported that the knockdown of Cox4 and Cox5a in *Caenorhabditis elegans* leads to enzymatic defect of complex I with otherwise normal amount of the assembled complex (Suthammarak et al., 2009). While the dependence of complex I biogenesis on the presence of complex III is relatively easy to reconcile due to ubiquitous character of I_1III_2 supercomplex, the contingence of complex I biogenesis upon complex IV remains obscure.

In yeast it was shown that complex IV associates with complex III already in the form of incomplete subcomplexes, with some of the late assembled subunits being likely added directly to the III/IV supercomplex (Mick et al., 2007). An example of such subunit was shown to be Cox13, the yeast homologue of human Cox6a (Brandner et al., 2005a). However, since complex IV assembly does not depend on complex III in yeast (Mick et al., 2007), the physiological relevance of this observation remains to be elucidated.

5. Degradation of proteins within the inner mitochondrial membrane

In contrast to most of the constituents of mammalian cellular proteome that were shown to exhibit half-lives of about 0.5 to 2 hours (Yen et al., 2008), most mitochondrial proteins are quite stable with half-lives of several days. A number of more or less specific proteases, including processing peptidases, ATP-dependent proteases, and oligopeptidases are found within the various subcompartments of mitochondria that mediate the selective proteolysis/degradation of mitochondrial proteins. However, if mitochondria are severely damaged or become superfluous due to altered physiological demands, they can be removed by a non-selective process, the autophagy (Mijaljica et al., 2007; Yorimitsu and Klionsky, 2005). Two ATP-dependent proteolytic machines, the *m*- and *i*-AAA metalloproteases that expose their catalytic domains to the opposite sides of the inner membrane are known to mediate the surveillance of protein quality control and eventually the selective degradation of non-assembled and damaged proteins in the inner mitochondrial membrane (Koppen and Langer, 2007).

5.1. i-AAA protease - Yme1

Yme1l1 is the human homologue (42% sequence identity, MW of processed protein ~69 kDa) of the yeast ATP-dependent i-AAA metalloprotease subunit Yme1 (*Y*east *m*itochondrial *e*scape 1) (Aldridge et al., 2007; Coppola et al., 2000; Shah et al., 2000). The yeast i-AAA protease is an inner membrane-anchored homooligomeric complex of approx. 1MDa with the active domain protruding into the IMS. It was shown to conduct protease, chaperone and translocase functions on the IMS side of the inner membrane (Leonhard et al., 1996; Leonhard et al., 1999). The yeast i-AAA protease complex belongs, along with the bacterial FtsH and mitochondrial m-AAA protease, to the group of so-called AAA (*A*TPases *A*ssociated with a variety of cellular *A*ctivities) proteases characterized by the presence of (i) a highly conserve Walker type ATPase module, (ii) a conserved HEXXH metal-binding module and (iii) membrane localization. Subunits of these complexes share a similar domain structure: they are anchored to the membrane by one or two membrane spanning segments at their N-terminal end, which are followed by one AAA domain and a metallopeptidase domain (Koppen and Langer, 2007). Inactivation of yeast Yme1 leads to temperature-sensitive respiratory growth defect, an increased frequency of DNA escape from mitochondria to nucleus, a petite-negative (ρ^-) phenotype and accumulation of mitochondria with aberrant morphology (Thorsness et al., 1993; Weber et al., 1996). Thus far, the yeast Yme1 was, either directly or indirectly, connected with the following protein substrates: Cox2, Phb1, Nde1, Atp4, Atp6, Atp17, Yme2, Inh1 and Yta10. It was shown that the ectopic overexpression of human Yme1l1 partially complements the termosensitive respiratory growth defect of the yeast *yme1* mutant, suggesting at least partial functional conservation of the human orthologue (Shah et al., 2000). Using a conserved *S. cerevisiae* import system, it was demonstrated that yeast Yme1 is required for mitochondrial translocation of ectopically expressed human polynucleotide phosphorylase (PNPase) (Rainey et al., 2006). On the other hand, the human Yme1l1 was so far indirectly connected only with the constitutive proteolysis of several splice variants of the intermembrane space dynamin GTPase Opa1 involved in mitochondrial fusion and cristae remodeling during apoptosis (Griparic et al., 2007; Song et al., 2007).

5.2. m-AAA protease - paraplegin (Spg7) and/or Afg3l2

In addition to the *i*-AAA complex, inner mitochondrial membrane harbors another homologous protease complex of the AAA type, the m-AAA protease. This oligomeric proteolytic machine with ATP-dependent metallopeptidase activity is active on the matrix side of the inner membrane. It exists in mammalian mitochondria in two forms: as a heterooligomer of paraplegin (Spg7)/Afg3l2 subunits and as a homooligomeric complex composed of solely Afg3l2 subunits (Koppen et al., 2007). In contrast to human i-AAA protease Yme1l1, much experimental attention was given to the human m-AAA complex in the past decade. This was mainly due to the fact that mutations in SPG7, which encodes the subunit paraplegin, are responsible for one form of Hereditary Spastic Paraplegia, a genetically heterogeneous neurological disorder characterized by a selective degeneration of long axons of the corticospinal tract and fasciculus gracilis (Atorino et al., 2003; Nolden et al., 2005; Rugarli and Langer, 2006). In yeast, the use of proteolytically inactive m-AAA protease as a substrate trap has led to the identification of mitochondrial ribosomal protein MrpL32 as a substrate of the m-AAA protease (Nolden et al., 2005). Unexpectedly, the protease does not affect the overall stability of the protein but cleaves off its N-terminal targeting sequence upon import of the precursor into mitochondria. The processing of MrpL32 is a prerequisite for the assembly of the protein with the mitochondrial ribosome, substantiating the mitochondrial translation defect and respiratory incompetence of yeast m-AAA protease deficient cells (Nolden et al., 2005). The yeast ROS-scavenger protein Ccp1 is another protein whose processing was shown to depend on m-AAA protease. Interestingly, it was demonstrated that m-AAA protease is not directly responsible for the cleavage of the bipartite presequence of Ccp1, but that it is required for ATP-dependent vectorial dislocation of Ccp1 precursor from the membrane bilayer. This activity mediated by the AAA domain of m-AAA protease is thought to ensure correct positioning of Ccp1 relative to membrane, most likely allowing intramembrane cleavage of the presequence by the rhomboid protease (Tatsuta et al., 2007).

CONCLUSIONS

- The loss-of-function of human Sco2 and Surf1 leads to highly tissue-specific patterns of CcO assembly impairment in terms of both diminished holoenzyme levels and accumulation of incomplete CcO subcomplexes. Together with the lack of pronounced tissue-specific differences in the expression of Sco2 and Surf1, it suggests tissue-dependent functional differences of both proteins that likely evolved to accommodate the profound tissue-specific requirements of metazoan mitochondrial function and biogenesis

- The dissection of subunit composition of CcO subcomplexes from Sco2 deficient mitochondria argues for the role of Sco2 in a distinct posttranslational step of Cox2 biogenesis, most probably in Cu_A site maturation. The accumulation of early CcO assembly intermediates in mitochondria with loss of Surf1 point to the role of the protein in some of the early stages of the process. The interdependence of association of Cox4 and Cox5a subunits with Cox1 during the assembly process brings new important addition to the current knowledge of human CcO assembly pathway

- The biochemical characterization of human OXA1L gene product showed that the processed form is a 42-kDa integral mitochondrial membrane protein that exists as part of a 600-700 kDa complex in mitochondria of HEK293 cells. The RNAi knockdown of OXA1L in HEK293 cells showed that the protein plays an important role in the biogenesis of F_1F_O-ATP synthase and respiratory complex I. In sharp contrast to the yeast orthologue, the loss of human Oxa1l does not lead to any impairments of assembly of CcO or the cytochrome bc_1 complex, suggesting functional divergence during evolution.

- Analysis of protein-protein interactions of wild-type Sco1 demonstrated that a fraction of the protein associates with the fully assembled CcO complex in both human muscle and HEK293 cell mitochondria. The immunoblot analysis of skeletal muscle expressing mutated (G132S) version of Sco1 showed that the mutation, which lies in a region required for protein dimerization, compromises stability of the protein, presumably by hindering its dimerization, leading to impairment of CcO assembly. The dissection of CcO

subcomplex pattern in Sco1-deficient muscle mitochondria suggests that the protein functions in a different posttranslational stage of Cox2 biogenesis than its paralogue Sco2.

- The severe copper defect of tissues harboring loss-of-function mutations in Sco1, Sco2 and Surf1 indicates either that each of these proteins plays an important role in cellular copper homeostasis maintenance or that the marked secondary CcO deficiency *per se* leads to highly tissue-specific pattern of cellular copper impairment in human. The former suggestion is supported by the association of a fraction of Sco1 with the CcO holoenzyme complex.

- Mutations that affect mt-tRNA^{Lys} (8363G>A, 8344A>G) lead to combined deficiency of complexes I and IV, compared to an isolated defect of complex I in the 3243A>G sample with impaired mt-tRNA$^{Leu(UUR)}$. The patterns of OXPHOS deficiencies in frontal cortex mitochondria of 8363G>A and 3243A>G patients differed substantially from those of other tissues. Particularly, in the frontal cortex mitochondria of the 3243A>G patient the assembly of complex IV appeared to be hindered by some factor other than the sole availability of mtDNA-encoded subunits.

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LIST OF ORIGINAL ARTICLES

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. *Biochem J,* 392, 625-32.

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Stiburek, L., Vesela, K., Hansikova, H., Pecina, P., Tesarova, M., Cerna, L., Houstek, J. and Zeman, J.

Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1

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In this paper we addressed the roles of human Sco2 and Surf1 in the assembly of cytochrome *c* oxidase as well as the sequence of early events in the CcO assembly pathway. We analyzed the assembly state of CcO and the levels of Sco2 protein in various tissues of six patients carrying previously identified mutations in *SCO2* and *SURF1*.

We used fibroblast cell culture, spectrophotometric enzyme activity assays, isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, blue-native (BN), two-dimensional (2D) BN/SDS and SDS-PAGE with downstream immunoblotting using battery of monoclonal and polyclonal antibodies against various mitochondrial proteins.

The biogenesis of eukaryotic CcO requires several accessory proteins in addition to structural subunits and prosthetic groups. SCO2 is a copper-binding protein presumably involved in formation of the CuA centre of the COX2 subunit. The function of SURF1 is unknown. Immunoblot analysis of native gels demonstrated that CcO holoenzyme is reduced to 10–20% in skeletal muscle and brain of *SCO2* and *SURF1* patients and to 10–30% in heart of *SCO2* patients, whereas liver of *SCO2* patients' contained normal holoenzyme levels. The steady-state levels of mutant SCO2 protein ranged from 0 to 20% in different *SCO2* patient tissues. In addition, eight distinct CcO subcomplexes and unassembled subunits were found, some of them identical with known assembly intermediates of the human enzyme. Heart, brain and skeletal muscle of *SCO2* patients contained accumulated levels of the COX1·COX4 ·COX5A subcomplex, three COX1-containing subcomplexes, a COX4·COX5A subcomplex and two subcomplexes composed of only COX4 or COX5A. The accumulation of COX1·COX4·COX5A subcomplex, along with the virtual absence of free COX2, suggests that the lack of the CuA centre may result in decreased stability of COX2. The appearance of COX4·COX5A subcomplex indicates that association of these nucleus-encoded subunits

probably precedes their addition to COX1 during the assembly process. Finally, the consequences of SCO2 and SURF1 mutations suggest the existence of tissue-specific functional differences of these proteins that may serve different tissue-specific requirements for the regulation of CcO biogenesis.

I contributed to this study by designing the research, performing part of the mitochondrial isolations, carrying out the vast majority of electrophoretic and immunoblot analyses, and by writing the manuscript. The work on this project was done in part in collaboration with Department of Bioenergetics, Institute of Physiology, ASCR.

Stiburek, L., Fornuskova, D., Wenchich, L., Pejznochova, M., Hansikova, H. and Zeman, J.

Knockdown of human Oxa1l impairs the biogenesis of F1Fo-ATP synthase and NADH:ubiquinone oxidoreductase.

Journal of Molecular Biology *2007*; *374*: 506-16

In this paper we addressed the molecular role and biochemical properties of human OXA1L gene product in the biogenesis of oxidative phosphorylation system.

We used human HEK293 cell culture, stable shRNA-mediated RNA interference approach, expression cloning, eukaryotic transfections and bacterial transformations, immunocytochemistry, confocal microscopy, subcellular and submitochondrial fractionation and localization, co-immunoprecipitation, spectrophotometric enzyme activity assays, oxygen consumption analysis, FACS analysis, isolation of crude mitochondrial fraction by differential centrifugation, rabbit OXA1L antibody design and preparation, BN, 2D BN/SDS and SDS-PAGE with downstream immunoblotting using battery of monoclonal and polyclonal antibodies against various mitochondrial proteins.

The Oxa1 protein is a founding member of the evolutionarily conserved Oxa1/Alb3/YidC protein family, which is involved in the biogenesis of membrane proteins in mitochondria, chloroplasts and bacteria. The predicted human homologue, Oxa1l, was originally identified by partial functional complementation of the respiratory growth defect of the yeast oxa1 mutant. We demonstrate that both the endogenous human Oxa1l, with an apparent molecular mass of 42 kDa, and the Oxa1l-FLAG chimeric protein localize exclusively to mitochondria in HEK293 cells. Furthermore, human Oxa1l was found to be an integral membrane protein, and, using two-dimensional blue native/denaturing PAGE, the majority of the protein was identified as part of a 600–700 kDa complex. The stable short hairpin (sh) RNA-mediated knockdown of Oxa1l in HEK293 cells resulted in markedly decreased steady-state levels and ATP hydrolytic activity of the F1Fo-ATP synthase and moderately reduced levels and activity of NADH:ubiquinone oxidoreductase (complex I). However, no significant accumulation of corresponding sub-complexes could be detected on

blue native immunoblots. Intriguingly, the achieved depletion of Oxa1l protein did not adversely affect the assembly or activity of cytochrome *c* oxidase or the cytochrome bc1 complex. Taken together, our results indicate that human Oxa1l represents a mitochondrial integral membrane protein required for the correct biogenesis of F1Fo-ATP synthase and NADH:ubiquinone oxidoreductase.

I contributed to this study by designing the research, maintaining the HEK293 cell culture, carrying out expression cloning, transfections and transformations, subcellular and submitochondrial fractionation and localization studies, electrophoretic and immunoblot analyses, immunocytochemical staining, co-immunoprecipitation assays, and by writing the manuscript.

Stiburek, L., Hansikova, H., Tesarova, M., Cerna, L. and Zeman, J.

Biogenesis of eukaryotic cytochrome c oxidase

Physiological Research *2006*; *55*, Suppl 2, S27-41.

In this paper we reviewed recent advancements in the understanding of the biogenesis of cytochrome *c* oxidase, with a focus on mammalian enzyme, and presented several unpublished results on the same subject.

Eukaryotic cytochrome *c* oxidase (CcO), the terminal component of the mitochondrial electron transport chain is a heterooligomeric complex that belongs to the superfamily of heme-copper containing terminal oxidases. The enzyme, composed of both mitochondrially and nuclear encoded subunits, is embedded in the inner mitochondrial membrane, where it catalyzes the transfer of electrons form reduced cytochrome *c* to dioxygen, coupling this reaction with vectorial proton pumping across the inner membrane. Due to the complexity of the enzyme, the biogenesis of CcO involves a multiplicity of steps, carried out by a number of highly specific gene products. These include mainly proteins that mediate the delivery and insertion of copper ions, synthesis and incorporation of heme moieties and membrane insertion and topogenesis of constituent protein subunits. Isolated CcO deficiency represents one of the most frequently recognized causes of respiratory chain defects in humans, associated with severe, often fatal clinical phenotype.

I contributed to this study by writing the manuscript and performing all the mentioned studies including 2D BN/SDS-PAGE immunoblotting of Surf1 in Sco2-deficient heart mitochondria as well as expression cloning and immunocytochemistry of OXA1L-FLAG fusion protein in HEK293 cells.

Stiburek, L., Vesela, K., Hansikova, H., Hulkova, H. and Zeman, J.

Loss of function of Sco1 and its interaction with cytochrome c oxidase

American Journal of Physiology - Cell Physiology *2008*; *296*(5):C1218-26.

In this paper we assessed the impact on CcO assembly and tissue copper levels of a G132S mutation in the juxtamembrane region of SCO1 metallochaperone associated with early onset hypertrophic cardiomyopathy, encephalopathy, hypotonia, and hepatopathy, assessed the total copper content of various *SURF1* and *SCO2*-deficient tissues, and investigated the possible physical association between CcO and Sco1.

We used spectrophotometric enzyme activity assays, HEK293 cell culture, isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, BN, 2D BN/SDS and SDS-PAGE with downstream immunoblotting using battery of monoclonal and polyclonal antibodies against various mitochondrial proteins, co-immunoprecipitation and copper content analysis using flame atomic absorption spectroscopy (FAAS).

Sco1 and Sco2 are mitochondrial copper-binding proteins involved in the biogenesis of the CuA site in the cytochrome *c* oxidase (CcO) subunit Cox2 and in the maintenance of cellular copper homeostasis. Human Surf1 is a CcO assembly factor with an important but poorly characterized role in CcO biogenesis. The steady-state level of mutant Sco1 was severely decreased in the muscle mitochondria of the *SCO1* patient, indicating compromised stability and thus loss of function of the protein. Unlike the wild-type variant, residual mutant Sco1 appeared to migrate exclusively in the monomeric form on blue native gels. Both the activity and content of CcO were reduced in the patient's muscle to ~10-20% of control values. *SCO1*-deficient mitochondria showed accumulation of two Cox2 subcomplexes, suggesting that Sco1 is very likely responsible for a different posttranslational aspect of Cox2 maturation than Sco2. Intriguingly, the various *SURF1*- deficient samples analyzed showed a tissue-specific copper deficiency similar to that of *SCO*-deficient samples, suggesting a role for Surf1 in copper homeostasis regulation. Finally, both blue native immunoblot analysis and coimmunoprecipitation revealed that a fraction of Sco1 physically associates with the CcO

complex in human muscle mitochondria, suggesting a possible direct relationship between CcO and the regulation of cellular copper homeostasis.

I contribute to this study by designing the research, performing part of the mitochondrial isolations, all electrophoretic and immunoblot analyses, human cell culture, and by writing the manuscript.

Fornuskova, D., Brantova, O., Tesarova, M., Stiburek, L., Honzik, T., Wenchich, L., Tietzeova, E., Hansikova, H., Zeman, J.

The impact of mitochondrial tRNA mutations on the amount of ATP synthase differs in the brain compared to other tissues

Biochimica et Biophysica Acta-Molecular Basis of Disease *2008*; *1782*: 317-25

In this paper we compared deficiency patterns of the individual OXPHOS complexes of various tissue samples of patients with Leigh (8363G>A), MERRF (8344A>G), and MELAS (3243A>G) syndromes due to mutations in mt-tRNA genes.

We used spectrophotometric enzyme activity assays, isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, blue-native and two-dimensional blue-native/SDS-PAGE immunoblotting with battery of monoclonal and polyclonal antibodies against various mitochondrial proteins and oxygen consumption analysis.

The impact of point mutations in mitochondrial tRNA genes on the amount and stability of respiratory chain complexes and ATP synthase (OXPHOS) has been broadly characterized in cultured skin fibroblasts, skeletal muscle samples, and mitochondrial cybrids. However, less is known about how these mutations affect other tissues, especially the brain. Both mutations that affect mt-tRNA^{Lys} (8363G>A, 8344A>G) resulted in severe combined deficiency of complexes I and IV, compared to an isolated severe defect of complex I in the 3243A>G sample (mt-tRNA$^{Leu(UUR)}$). Furthermore, we compared obtained patterns with those found in the heart, frontal cortex, and liver of 8363G>A and 3243A>G patients. In the frontal cortex mitochondria of both patients, the patterns of OXPHOS deficiencies differed substantially from those observed in other tissues, and this difference was particularly striking for ATP synthase. Surprisingly, in the frontal cortex of the 3243A>G patient, whose ATP synthase level was below the detection limit, the assembly of complex IV, as inferred from 2D-PAGE immunoblotting, appeared to be hindered by some factor other than the availability of mtDNA-encoded subunits.

I contributed to this study by assisting in research design, performing part of the electrophoretic and immunoblot analyses, and by helping to write the final manuscript.