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PhD thesis summary

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

Mgr. Lukáš Stibůrek

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and Academy of Sciences of the Czech Republic

Section:	Biochemistry and pathobiochemistry
Section chairman:	Prof. MUDr. Jiří Kraml, DrSc.
Workplace:	Laboratory for study of mitochondrial disorders Department of Pediatrics 1st Faculty of Medicine, Charles University Ke Karlovu 2, 128 08 Praha 2
Author:	Mgr. Lukáš Stibůrek
Supervisor:	Prof. MUDr. Jiří Zeman, DrSc.

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CONTENTS

ABBREVIATIONS

2D	two-dimensional
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
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
GFP	green fluorescent protein
HA	hemagglutinin
HEK293	human embryonic kidney 293
HSP	heat shock protein
IM	inner membrane
IMS	intermembrane space
kDa	kilodalton
KO	knockout
MDa	megadalton
MELAS	mitochondrial encephalopathy, lactic acidosis, and stroke like episodes
MERRF	myoclonic epilepsy and ragged-red fibers
mRNA	messenger ribonucleic acid
mt	mitochondrial
MTS	mitochondrial targeting sequence
mt-tRNA	mitochondrial transfer ribonucleic acid
MW	molecular weight
ND	NADH dehydrogenase
OM	outer membrane
ORF	open reading frame
OXA1	oxidase assembly 1
OXA1L	oxidase assembly 1-like
OXPHOS	oxidative phosphorylation system
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SCO	synthesis of cytochrome oxidase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
tRNA	transfer ribonucleic acid
VDAC	voltage-dependent anion-selective channel

SUMMARY

The thesis was worked out in The laboratory for study of mitochondrial disorders at the Department of Pediatrics, First Faculty of Medicine, Charles University in Prague.

The study of human mitochondrial biogenesis, particularly of the oxidative phosphorylation system (OXPHOS), and its pathologies has seen remarkable progress in past decade. The knowledge of the complete sequence of the human genome, together with the results of elegant yeast studies aimed at the identification of respiratory important gene products, enable us to identify and study the molecular and biochemical bases of numerous human mitochondrial pathologies. These studies not only continue to reveal the underlying molecular mechanisms of the disease etiopathogenesis but also shed completely new light on the various processes of mitochondrial assembly and function in humans.

The work presented in this thesis was aimed mainly at the understanding of the various roles of mitochondrial assembly factors Sco1, Sco2, Surf1 and Oxa1l in the assembly and/or maintenance of the five multimeric complexes of the OXPHOS system.

The presented data indicate that human Sco1 and Sco2 are involved, in a highly tissue-specific manner, in distinct posttranslational steps of maturation of the cytochrome c oxidase (CcO) subunit Cox2. Furthermore, both SCO proteins, together with the assembly factor Surf1, are implicated herein in the regulation of cellular copper homeostasis. The human homologue of the yeast Oxa1 translocase, OXA1L is shown to be required for the assembly/stability of the respiratory complex I and the F_1F_O-ATP synthase. Finally, the dissection of the assembly patterns of CcO in the various CcO deficient backgrounds suggests new important addition to the current model of human CcO assembly.

SOUHRN

Disertační práce byla vypracována v Laboratoři pro studium mitochondriálních poruch, Kliniky dětského a dorostového lékařství a 1.LF UK v Praze.

Studium mitochondriální biogeneze a jejích poruch, a to především s ohledem na biogenezi systému oxidativní fosforylace (OXPHOS), zaznamenalo v uplynulém desetiletí mimořádný pokrok. Znalost kompletní sekvence lidského genomu, spolu s výsledky elegantních kvasinkových studií zaměřených na identifikaci respiračně významných genových produktů, dovolila molekulární identifikaci a následné studium biochemické podstaty celé řady lidských mitochondriálních patologií. Tyto studie odhalují molekulární a biochemické mechanismy etiopatogeneze těchto chorob a současně přinášejí nové unikátní informace o lidské mitochondriální biogenezi jako takové.

Výsledky a původní publikace prezentované v této disertační práci se týkají snahy o pochopení molekulárních rolí lidských asemblačních faktorů Sco1, Sco2, Surf1 a Oxa1l v biogenezi multimerních membránových komplexů lidského systému OXPHOS.

Prezentovaná data ukazují, že lidské asemblační faktory Sco1 a Sco2 fungují vysoce tkáňově specifickým způsobem na úrovni odlišných posttranslačních kroků maturace podjednotky Cox2 cytochrom *c* oxidázy (CcO). Prezentovaná data dále naznačují, že oba výše zmíněné lidské SCO proteiny, a asemblační faktor Surf1 mohou hrát významnou roli v udržování buněčné homeostázy mědi. Studie zaměřená na biochemickou a funkční charakterizaci lidského homologu, OXA1L, kvasinkové translokázy Oxa1 ukázala, že tento integrální protein vnitřní mitochondriální membrány je u člověka nezbytný pro správnou asemblaci/stabilitu respiračního komplexu I a F_1F_O-ATP syntázy. A konečně, biochemické studie asemblačních defektů CcO na podkladě deficitu asemblačních faktorů Sco1, Sco2 a Surf1 přinesla významné upřesnění současného modelu asemblace lidské CcO.

INTRODUCTION

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 and synthesized on cytoplasmic ribosomes (1, 2). 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 (2).

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. 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 (1, 3, 4).

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. 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 (5, 6). However, mainly the hydrophobic nature of most mitochondrially-encoded proteins has hindered this process (7). 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 (8, 9). Hence, mitochondrial translation is thought to occur exclusively at the matrix face of the inner membrane bilayer (10). In contrast, the recognition and membrane-recruitment of translating ribosomes in the cytoplasm of eukaryotic and prokaryotic cells is mediated by signal recognition particles (11) that appear to be absent from mitochondria (12).

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 (13, 14). Members of this family possess a hydrophobic core domain containing five transmembrane helices that facilitate the membrane export of their protein substrates (15, 16). 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 (17, 18). 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 (17) represented by approx. 200 kDa homooligomeric assembly of four Oxa1 subunits (19). In a crosslinking experiment, mitochondrially-encoded cytochrome c oxidase subunits Cox1, Cox2 and Cox3 were shown to transiently interact with Oxa1 as nascent chains (20). 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 (21). 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 (22, 23). *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 (24). 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 (25).

The human Oxa1 orthologue, referred to as Oxa1l, shares 33% sequence identity with the corresponding yeast polypeptide (26). 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 (22). 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. 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 (27). 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 (28). 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 (28). 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 (28). 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.

2. Cytochrome c oxidase (CcO)

Eukaryotic cytochrome *c* oxidase (CcO) is the terminal multicomponent enzyme of the energy-transducing mitochondrial electron transport chain (29, 30). 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 (31). 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 (32, 33). 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 (30, 34). 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 (35, 36). 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 (37, 38). 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 (30, 37, 39). 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 (40, 41). However, the effects on CcO biogenesis of mutations in both of the gene products are thought to be essentially secondary.

2.1. Copper trafficking and homeostasis - Sco1, Sco2

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 (42). 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 (43). 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) (44). 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 (42, 45). Recently, it was shown that yeast mitochondria contain a significant pool of copper bound neither to proteins nor mitochondrial DNA (46). 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 (46), a compound with the same fluorescent and chromatographic properties was found to be conserved in mouse liver (45). 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 (42). 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 (30, 39).

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 (47). In addition, they have been

shown to be involved in the maintenance of cellular copper homeostasis, presumably by controlling cellular copper export (48, 49). Very recently, human SCO proteins were reported to carry out distinct, stage-specific roles during Cox2 synthesis and Cu_A site maturation (50). Interestingly, the tumor suppressor p53 was shown to directly regulate mitochondrial respiration through transactivation of human *SCO2* transcription (51). Mutations in both *SCO1* and *SCO2* cause severe tissue-specific CcO assembly impairment accompanied by marked copper deficiency (49, 52, 53). 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 (54). 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 (53). 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 (55), whereas the G132S allele appears to lead to a simple, yet almost complete loss of protein and function (53). In contrast to *SCO1*, mutations of *SCO2* are more common, with all reported patients carrying at least one E140K missense allele (56). 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 (56).

SCO proteins are integral inner membrane components consisting of a globular copper-binding domain that protrudes into the IMS (57). This domain exhibits a thioredoxin fold composed of a central four-stranded β sheet surrounded by four α helices (58). 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 (59). 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 (60). The human Sco2 conformer resembles human Sco1 with the exception of greater conformational dynamics (61). 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 (62). It was suggested that SCO proteins might form a complex in order to deliver two copper ions to Cox2 simultaneously (47). Recently, however, a sequential delivery scenario is favored (50, 63). 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 (54, 63). 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 (47, 64). 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 (62).

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 (45), this has no apparent function in CcO assembly (65). Both yeast Sco1 and Sco2 were shown to physically interact with Cox2, albeit only upon overexpression (66). Recently, we have reported that human Sco1 physically interacts with the fully assembled CcO complex in both skeletal muscle and HEK293 cell mitochondria (53). 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 (67). Furthermore, on the basis of high-resolution structural data, human Sco1 has been implicated to function as a redox switch in the IMS (58).

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 (52). 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 (68). 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 (53). 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 (50). 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 (50).

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 (49). 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 (50). 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 (53).

2.2. Assembly of the mammalian CcO

The spatiotemporal assembly of mammalian CcO within the inner mitochondrial membrane is a sequential, tissue-specific and relatively slow process (69, 70). The half-life of the holoenzyme is thought to be about three days (71). 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 (30). In contrast, yeast CcO subcomplexes are difficult to detect as they likely undergo rapid proteolytic degradation (72). The nuclear encoded CcO subunits are imported into mitochondria upon synthesis on free cytoplasmic polysomes (73). 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 (73). 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 (52). 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 (52). This subcomplex readily accumulates under conditions of blocked/retarded assembly, pointing to its high intrinsic stability (52, 70, 74).

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 (75). Second, human cells deficient in heme *a* synthesis do not accumulate Cox1·Cox4·Cox5a subassembly (74, 76). 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 (77). 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 (78). 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 (79). The intrinsic inner-membrane protein Cox11 might be responsible for the formation of Cu_B site (79). 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 (52, 74), 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 (80). 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 (45). 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 (70). 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 (81). 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 (82). Finally, the mature holoenzyme complex associates with complex I and dimeric complex III, to form the 1.7-MDa respiratory supercomplex (83, 84). The role of cardiolipin in final maturation of CcO and the function of cytochrome *c* during CcO assembly remains elusive.

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 (85). 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 (86), separately expressed N- and C-terminal transmembrane domains of human Surf1 are not able to form a functional protein (85). 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 (53, 74). 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 (3, 87). 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 (78, 88).

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 (89). 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 (90). 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 (90).

Two Surf1 knockout mouse models were generated so far by using either replacement of exons 5-7 by neomycin-resistance (NEO) cassette (91) or insertion of a *loxP* sequence in exon 7 of the gene (92). 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 (92). 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 (3). Consistent with these findings, CcO deficiency was associated with reduced oxidative stress in CNS of *COX10* knockout mice (93).

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 (94). 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 (85). 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 (86). 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 (95). 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 (96).

The severe CcO deficiency of *SURF1* fibroblasts is accompanied by barely detectable changes in cellular respiratory rates under normoxic conditions (97). 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 (98).

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 (52). 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 (53). Interestingly, yeast cells lacking Shy1 are deficient in mitochondrial copper, whereas the total cellular copper content remains normal (99). Supplementation of *shy1*Δ cultures with exogenous copper partially rescues the respiratory capacity of these cells (96).

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. IF 4.371

Stiburek, L., Fornuskova, D., Wenchich, L., Pejznochova, M., Hansikova, H., Zeman, J. (2007). Knockdown of human Oxa1l impairs the biogenesis of F_1F_O-ATP synthase and NADH:ubiquinone oxidoreductase. *J Mol Biol,* 374: 506-16. IF 4.146

Stiburek, L., Hansikova, H., Tesarova, M., Cerna, L., Zeman, J. (2006) Biogenesis of eukaryotic cytochrome *c* oxidase. *Physiol Res,* 55 Suppl 2, S27-41. IF 1.653

Stiburek, L., Vesela, K., Hansikova, H., Hulkova, H., Zeman, J. (2009) Loss of function of Sco1 and its interaction with cytochrome *c* oxidase. *Am J Physiol Cell Physiol*, 296(5):C1218-26. IF 4.230

Fornuskova, D., Brantova, O., Tesarova, M., Stiburek, L., Honzik, T., Wenchich, L., Tietzeova, E., Hansikova, H., Zeman, J. (2008) The impact of mitochondrial tRNA mutations on the amount of ATP synthase differs in the brain compared to other tissues. *Biochim Biophys Acta* 1782: 317-25. IF 4.579

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

Biochemical Journal *2005*; *392*, 625-32.

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.

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.