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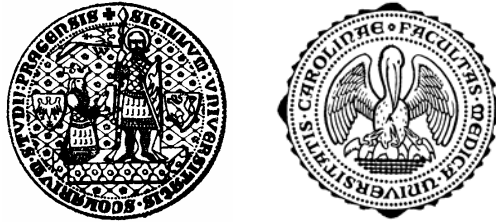# First Faculty of Medicine

Academic program: biochemistry and pathobiochemistry

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Studium poruch cytochrom c oxidasy a ATP synthasy na biochemické a molekulární úrovni

Biochemical and molecular studies of cytochrome c oxidase and ATP synthase deficiencies

PhD Thesis

Supervisor: Prof. MUDr. Jiří Zeman, DrSc.

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ABSTRAKT

Savčí organismus je plně závislý na systému oxidativní fosforylace (OXPHOS) jako hlavním zdroji produkce energie (ATP) v buňce. Poruchy OXPHOS mohou být způsobeny mutacemi v genech kódovaných mitochondriální DNA nebo jadernou DNA.

Část výzkumné práce je zaměřena na roli raně a pozdně se asemblujících, jaderně kódovaných, strukturních podjednotek cytochrom c oxidasy (CcO) a Oxa1l, lidského homologu kvasinkové mitochondriální Oxa1 translokasy, v biogenezi cytochrom c oxidasy a její funkci s využitím stabilní RNA interference COX4, COX5A, COX6A1 a OXA1L a ektopické exprese epitopově značených podjednotek Cox6a, Cox7a a Cox7b v buněčné linii HEK (lidské embryonální ledviny)-293. Naše výsledky ukazují, že zatímco podjednotky Cox4 a Cox5a jsou nezbytné pro asemblaci funkčního komplexu CcO, Cox6a podjednotka je důležitá pro její stabilitu. V buňkách se sníženou expresí OXA1L byla překvapivě zjištěna normální aktivita i hladina holoenzymu CcO, přestože inaktivace OXA1 u kvasinek vyvolá kompletní ztrátu aktivity CcO.

Při studiu poruch OXPHOS v izolovaných mitochondriích kosterního svalu, srdce, jater a frontálního kortexu získaných od pacientů s Leigh syndromem (mtDNA mutace 8363G>A), MERRF syndromem (mtDNA mutace 8344A>G) a MELAS syndromem (mtDNA mutace 3243A>G) jsme nalezli tkáňově specifické rozdíly v dopadu mt-tRNA mutací na OXPHOS mozku, které se významně lišily od dopadu těchto mutací v ostatních tkáních. Navíc jsme ukázali, že v případě mtDNA mikrodelece 9205ΔTA v ATP6 genu je výrazně omezena syntéza podjednotky a komplexu ATPasy a postižena biogeneze CcO.

Klíčová slova: mitochondrie, cytochrome c oxidasa, ATP synthasa (ATPasa), tkáňová specifita, asemblace proteinů, biogeneze, RNA interference (RNAi)

ABSTRACT

The mammalian organism fully depends on the oxidative phosphorylation system (OXPHOS) as the major energy (ATP) producer of the cell. Disturbances of OXPHOS may be caused by mutations in either mitochondrial DNA (mtDNA) or nuclear DNA.

One part of the thesis is focused on the role of early and late assembled nuclear-encoded structural subunits of cytochrome c oxidase (CcO) as well as Oxa1l, the human homologue of the yeast mitochondrial Oxa1 translocase, in the biogenesis and function of the human CcO complex using stable RNA interference of COX4, COX5A, COX6A1 and OXA1L, as well as expression of epitope-tagged Cox6a, Cox7a and Cox7b, in HEK (human embryonic kidney)-293 cells. Our results indicate that, whereas nuclear- encoded CcO subunits Cox4 and Cox5a are required for the assembly of the functional CcO complex, the Cox6a subunit is required for the overall stability of the holoenzyme. In OXA1L knockdown HEK-293 cells, intriguingly, CcO activity and holoenzyme content were unaffected, although the inactivation of OXA1 in yeast was shown to cause complete absence of CcO activity.

In addition, we compared OXPHOS protein deficiency patterns in mitochondria from skeletal muscle, heart, liver and frontal cortex of patients with Leigh (mtDNA mutation 8363G>A), MERRF (mtDNA mutation 8344A>G), and MELAS (mtDNA mutation 3243A>G) syndromes. Our data show new effects of mt-tRNA mutations on the brain which differ substantially from those described for other tissues. Furthermore, we found that mtDNA 9205ΔTA microdeletion in the ATP6 gene prevents the synthesis of ATPase subunit a and also affects the biogenesis of CcO.

Key words: mitochondria, cytochrome c oxidase, ATP synthase (ATPase), tissue specificity, protein assembly, biogenesis, RNA interference (RNAi)

ACKNOWLEDGEMENTS

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ABBREVIATIONS

2D	two-dimensional
Δ	deletion
ΔΨ	potential difference
$\Delta\Psi_m$	mitochondrial membrane potential
ΔpH	proton gradient
AAC	ADP/ATP carrier
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BN	blue native
bp	base pair
BSA	bovine serum albumin
CcO	cytochrome c oxidase, complex IV
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
CMV	cytomegalovirus (RNA polymerase II) promoter
CN	colorless native
CNS	central nervous system
CoQ	coenzyme Q
COX	cytochrome c oxidase, complex IV
Cox#	cytochrome c oxidase structural subunit number #
CS	citrate synthase
CV	complex V
CytC	cytochrome c
dADP	deoxy-adenosine diphosphate
dATP	deoxy- adenosine triphosphate
dCTP	deoxy-cytosine triphosphate
DDM	n-dodecyl-β-D-maltoside
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FACS	fluorescent-activated cell sorting
FAD	flavin adenine dinucleotide
$FADH_2$	flavin adenine dinucleotide reduced
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
FLAG	octapeptide tag sequence (N-DYKDDDDK-C, 1012 Da)
GFP	green fluorescent protein
HEK-293	human embryonic kidney - 293
IMAGE	integrated molecular analysis of genomes and their expression
IMM	inner mitochondrial membrane
IMS	intermembrane space
IRES-Neo	internal ribosome entry site - neomycin phosphotransferase
ISP	iron-sulfur protein
KD	knockdown
kDa	kilodalton

KO	knockout
MCA	metabolic flux control analysis
MDa	megadalton
MELAS	mitochondrial encephalopathy, lactic acidosis, and stroke like episodes
MERRF	myoclonic epilepsy and ragged-red fibers
mRNA	messenger ribonucleic acid
mt	mitochondrial
mt-tRNA	mitochondrial transfer ribonucleic acid
MW	molecular weight
n	nuclear
N side	negatively charged side
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced
NARP	neurogenic muscle weakness, ataxia, and retinitis pigmentosa
NS	non-silencing
nt	nucleotide(s)
OMM	outer mitochondrial membrane
ORF	open reading frame
OXA1	oxidase assembly 1
OXA1L	oxidase assembly 1-like
OXPHOS	oxidative phosphorylation system
P side	positively charged side
P_{50}	partial pressure of oxygen at half-maximal respiration rate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEO	progressive external ophthalmoplegia
Pi	inorganic phosphate
PIC	mitochondrial phosphate carrier
PMF	proton motive force
PVDF	polyvinylidene difluoride
QCR	ubiquinol:cytochrome c oxidoreductase, complex III
qRT-PCR	quantitative real-time PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
shRNAmir	microRNA-adapted shRNA
STE	sucrose-Tris-EDTA medium
TBS	Tris-buffered saline
TMPD	*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine
TMRM	tetramethylrhodamine methyl ester
tRNA	transfer ribonucleic acid
UTR	untranslated region

1   INTRODUCTION

Mitochondria hold a central position in cellular bioenergetics. The most important mitochondrial energy-yielding reaction is performed by the oxidative phosphorylation system (OXPHOS) which is mainly composed of five large multi-subunit complexes (CI-CV) embedded in the inner mitochondrial membrane. The importance of mitochondria for cell viability is most dramatically apparent from diseases resulting from its malfunction. OXPHOS disorders, first recognized 50 years ago (Luft *et al.* 1962), have been found to be the most frequent cause of metabolic abnormality in pediatric neurology (DiMauro and Schon 2008; Zeviani *et al.* 1996) but often present with nonneurological symptoms (e. g. hepatic, cardiac, renal, gastrointestinal, endocrine, hematological symptoms, failure to thrive) (Di Donato 2009; Munnich *et al.* 1996) or can contribute to severe diseases of adulthood (e. g. Parkinson's disease, Alzheimer's disease, Type 2 diabetes mellitus) (Civitarese and Ravussin 2008; Lin and Beal 2006; Zeviani and Carelli 2007). The clinical and genetic variability of OXPHOS disorders makes it extremely difficult to estimate prevalence accurately; however, the general incidence of mitochondrial disease is approximately 1:5000 (Schaefer *et al.* 2004; Thorburn 2004). Since the OXPHOS is composed of 13 structural subunits encoded by mitochondrial DNA (mtDNA) and more than 70 structural polypeptides encoded by nuclear DNA (nDNA), primary OXPHOS disorders can be classified genetically according to whether the primary defect is in the nuclear or mitochondrial genome. Furthermore, 24 additional mtDNA-encoded genes are involved in mitochondrial translational apparatus and numerous nDNA-encoded genes are necessary for synthesis of prosthetic groups and assembly of OXPHOS complexes, stability of mtDNA and mitochondrial biogenesis (Fig. 1). Therefore, inheritance of these mutations may be autosomal recessive or dominant, X-linked or maternal (Smeitink *et al.* 2001b). MtDNA mutations, as it currently is believed, account for mitochondrial encephalopathy in 20% to 30% of pediatric patients and a higher proportion of adult patients with mitochondrial disorders (Lebon *et al.* 2003; Thorburn 2004).

At the metabolic level, the impact of OXPHOS disease can be classified as systemic consequences (e. g. pathological increase in concentration of the lactic acid, alanin or higher ketone body ratio) or consequences at the tissue and cellular level (bio-energy deficiency, redox or metabolite imbalance or elevated or disturbed production of reactive oxygen species (ROS)) (Smeitink *et al.* 2006). Although often assumed to be the primary effect of mitochondrial disease, sufficient supply of ATP to meet cellular needs is not necessarily the only factor or in some cases even the main factor in OXPHOS disease (Smeitink *et al.* 2006).

Biochemically, OXPHOS defects are classified based on the affected enzyme as isolated (only one OXPHOS enzyme is deficient) or combined (more than one complex deficiency is observed). Due to high complexity of genes which are involved in optional functioning of OXPHOS, some of which very likely remain to be identified, most patients are still classified clinically or biochemically, simply because the genetic defect has not yet been established. Moreover, very common and severe pathogenic manifestation of OXPHOS dysfunction - Leigh syndrome (see Chapter 3.3.1.1 for clinical details), for example, may be caused by mutations in more than 25 different genes (Kirby and Thorburn 2008), thus in many cases there is no clear candidate gene to investigate and many potential candidates may need to be analyzed (Tucker *et al.* 2010). Therefore, there is a great need to better understand the genetics of mitochondrial disease as well as biogenesis of OXPHOS, which will enable prenatal diagnoses and will deliver deeper understanding of mitochondrial function necessary for development of effective therapies (Koene and Smeitink 2009).
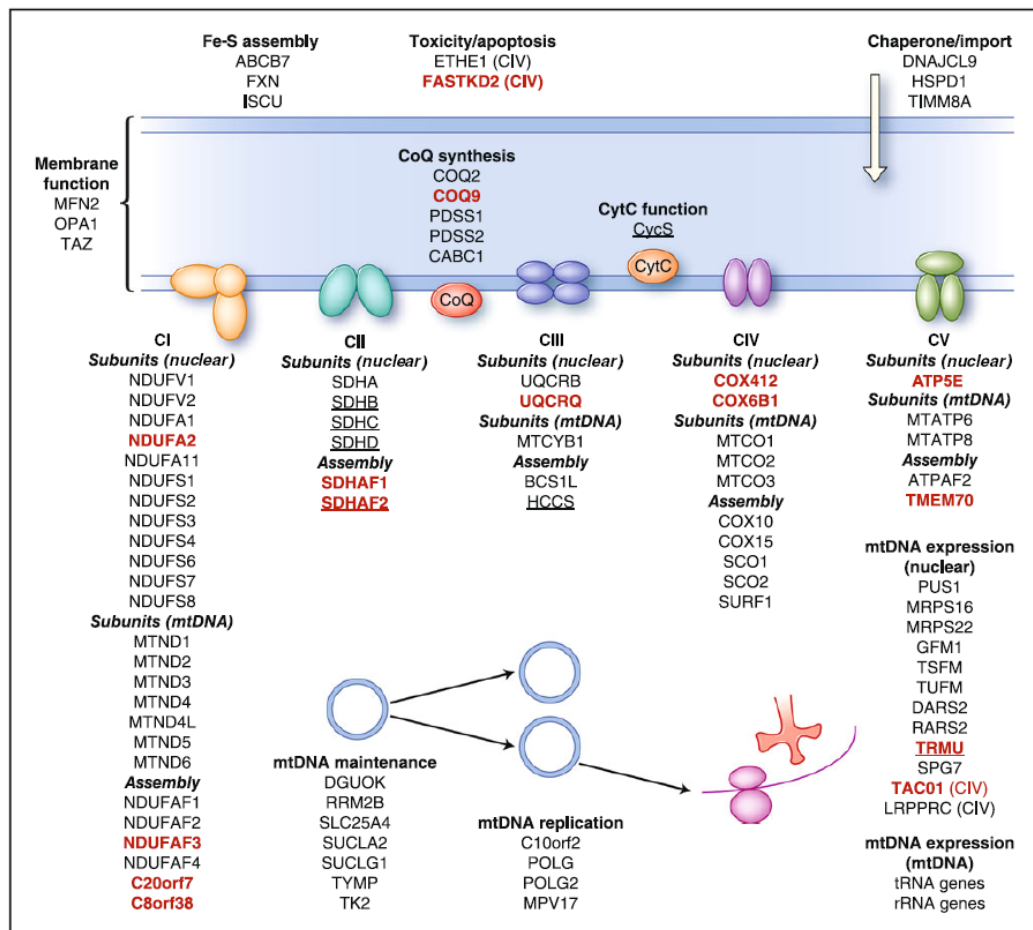
Figure 1 Genes associated with mitochondrial disease (Tucker *et al.* 2010). Mutations have been identified in genes encoding CI, CII, CIII, CIV, and CV subunits and assembly factors; genes involved in mitochondrial DNA (mtDNA) maintenance (via nucleotide metabolism), mtDNA replication, and mtDNA expression; genes affecting the electron carriers coenzyme Q (CoQ) and cytochrome *c* (CytC); genes affecting Fe-S assembly; and genes involved in protein import, toxicity/apoptosis, and membrane function. Recently identified genes are highlighted in red. Genes affecting oxidative phosphorylation for which mutations are not reported to cause neuropathology are underlined.

2   AIMS OF THE STUDY

Our laboratory for study of mitochondrial disorders specializes in biochemical and molecular diagnostics of OXPHOS deficiencies in the patients predominantly coming from Czech and Slovak Republics. One part of this thesis is based on the study of selected deleterious mutations of mtDNA in terms of their impact on OXPHOS steady-state levels and functioning in various patients´ tissues. All analyzed mutations, especially those in genes coding for mt-tRNAs, usually affects more than one OXPHOS complex, thus they manifest as combined OXPHOS defects. We were interested in differences in OXPHOS deficiency patterns among various tissues.

Second part of this thesis is aimed at biogenesis of cytochrome c oxidase (CcO), the complex IV of OXPHOS, which plays central role in oxidative metabolism. Defective CcO functionality results in heterogeneous group of diseases predominantly affecting tissues with high-energy demands. For its medical relevance, CcO biogenesis has received significant attention and has been intensively studied by biochemical, genetic, spectroscopic and crystallographic means. Data from medical records of 180 CcO-deficient children identified in Poland, Czech and Slovak Republics, that means from area accounting for more than 50 million inhabitants, revealed fairly high mortality of CcO deficiency, relatively early onset of the diseases resulting from CcO deficiency and pretty severe clinical symptoms, encephalopathy to be the most common (Bohm *et al.* 2006). In this group, molecular basis of CcO deficiency, which is fundamental e. g. for prenatal diagnostics in affected families, still remains to be elucidated approximately in the half of cases. Therefore, the study of CcO assembly also became an important interest in our laboratory and of my thesis.

The specific aims of this thesis have been:

1) to study new aspects of the CcO assembly pathway

   a) Characterization of the assembly and function of human nuclear-encoded CcO subunits 4, 5a, 6a, 7a and 7b.

   b) Characterization of the biochemical properties of OXA1L, the human homologue of the yeast mitochondrial Oxa1 translocase, and study of its role for CcO biogenesis.

2) to study the molecular basis of selected mtDNA-encoded mutations and their impact on OXPHOS function

a) Analysis of the tissue-specific effects of mt-tRNA point mutations in patients affected by Leigh syndrome (8363G>A), MERRF syndrome (8344A>G), and MELAS syndrome (3243A>G) on the steady-state levels and activity of OXPHOS complexes.

b) Study of molecular and biochemical impact of mtDNA mutation in the ATP6 gene (9205ΔTA) on the biosynthesis of ATPase subunit a and its structural and functional consequences.

3   REVIEW OF THE LITERATURE

3.1. Evolutionary trends in mitochondria

Mitochondria are the endosymbiotic semiautonomous ATP-generating organelles of eukaryotes. The divergence of mitochondria from bacteria and aerobic respiration in eukaryotes are conventionally associated with a global disaster about 2.0 billion years ago (Sicheritz-Ponten *et al.* 1998), when atmospheric oxygen levels increased rapidly, presumably from the accumulated activity of oceanic photosynthetic cyanobacteria (Andersson *et al.* 2003). A continuous presence of oxygen during the entire history of the biosphere, albeit primary at very low levels (Holland 1990), matches the ancient origins of the terminal oxidases characteristic of mitochondria as well as represented in the archaea and bacteria. Thus, the oxidative respiratory system was at the time of mitochondrial origin an ancient enzymatic system (Kurland and Andersson 2000). Phylogenetic reconstructions have by now provided convincing evidence that the various mitochondrial genomes have a single monophyletic origin in α-proteobacterial subdivision (Gray *et al.* 1999). A studying of mitochondrially encoded, chaperone and ribosomal proteins points specifically towards the Rickettsiaceaea family (Andersson *et al.* 2003; Andersson *et al.* 1998; Viale and Arakaki 1994). A weakness of all current theories of mitochondrial origins is the uncertain identity of the host of the ancestral endosymbiont (Andersson *et al.* 2003; Lang *et al.* 1999). Phylogenomic reconstructions show that the characteristic eukaryotic complexity arose without any intermediate grades seen between the prokaryotic and eukaryotic levels of organization (Koonin 2010).

ATP production in eukaryotes from glucose and oxygen normally consists of two catabolic processes (Voet *et al.* 2006). First, the glycolytic system of the eukaryotes, which seems not to trace its origin back to the α-proteobacteria (Andersson *et al.* 2003), supply mitochondria (as well as the obligate parasites of the genus *Rickettsia*) with pyruvate. Second, pyruvate dehydrogenase complex and Krebs cycle catalyze oxidative conversion of pyruvate to H_2O and CO_2. Substrates of Krebs cycle do not come only from glycolysis, but also from beta-oxidation of fatty acids and proteolysis. Concomitantly to these catabolic processes, electrons are transferred via coenzymes NAD^+ and FAD to pass into the mitochondrial electron-transport chain which produces a proton gradient across the mitochondrial membrane. The free energy stored in this electrochemical gradient drives the synthesis of ATP from ADP and phosphate through oxidative phosphorylation (Voet *et al.* 2006). The mechanistic principle of oxidative phosphorylation was first proposed by Peter Mitchell (Mitchell 1961) who received for chemiosmotic hypothesis Nobel prize in 1978.

The processes transforming the ancestral α-proteobacterium into the modern mitochondrion were obviously incremental and presumably spread over millions of years. Many ancestral mitochondrial genes have been lost, a smaller number of mitochondrial proteins have been transferred from the mitochondrial to the nuclear genome and the minimum of mitochondrial genes remained to be encoded by mitochondrial DNA (mtDNA). The tendency to transfer the vast majority of residual ancestral mitochondrial genes to the nuclear genome might have been favored for its well-developed sexual mechanisms compared to the asexual character of mitochondrial lineages which suggests that they might be particularly vulnerable to Muller's ratchet[1] (Kurland and Andersson 2000). Like a typical bacterial genome, many mtDNAs map as circular molecules, although linear mtDNA exist as well (Nosek *et al.* 1998). Although the genetic role of mtDNA appears to be universally conserved, this genome exhibits remarkable variation in conformation and size, which ranges from <6kbp in *Plasmodium falciparum* (the human malaria parasite) to >200kbp in land plants, as well as in actual gene content, arrangement and expression. The contrast between the expansive plant and condensed animal mitochondrial genomes in virtually every parameter (e. g. proportion of coding to noncoding sequence, rate of primary sequence divergence, conservation of gene order) suggests that these mtDNAs exhibit entirely opposite evolutionary trends (Gray *et al.* 1999).

The largest number of mitochondrial proteins, which have no bacterial or archaeal orthologues, constituted a novel "eukaryotic" group of mitochondrial proteins, which evidently evolved from the nuclear genome by duplication and functional divergence of sequences that predate the arrival of the endosymbiont ancestor of mitochondria (Karlberg *et al.* 2000). The phylogenetic clustering of the mitochondrial proteins into α-proteobacterial and eukaryotic homologues goes hand in hand with the functional profiles of the clusters (Kurland and Andersson 2000). Thus, the bacterial homologues seem to be mainly involved in translation and energy metabolism. In contrast, the eukaryotic proteins are typically associated with transport and regulatory functions. An important group of eukaryotic proteins, which originated subsequent to the integration of the endosymbiont into the eukaryotic cell, involves protein and ATP transport machinery (Andersson *et al.* 2003). Transport of mitochondrial precursor proteins to mitochondria is posttranslational process subsequent to their synthesis by cytosolic ribosomes. A recognition, import and sorting of mitochondrial precursor proteins based on their targeting sequence signals is mediated by translocases in the outer and inner membrane of mitochondria (Neupert and Herrmann 2007). The evolution of mitochondrial targeting system gave a possibility to the host cell to decorate the

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[1] **Muller´s ratchet** is the process by which the genomes of an asexual population accumulate deleterious mutations in an irreversible manner.

mitochondrial core complexes with a variety of new proteins, e. g. participating in the assembly of complexes or functioning in regulation. It is assumed, that the coevolution of the core α-proteobacterial components and the complementary eukaryotic nuclear components transformed the endosymbiont into an organelle (Kurland and Andersson 2000). The presence of ATP/ADP transporter systems made it possible for the mitochondriate cell to fully exploit the benefits of the high ATP yields obtained by aerobic respiration and may even have been critical for the transition from single cells to early, undifferentiated forms of multicellular eukaryotes (Pfeiffer *et al.* 2001).

3.2. The main characteristic features of mitochondria

Mitochondria display striking interspecies and inter-tissue variations in shape, connectivity, and inner membrane morphology (Mannella 2008). The general structure of mitochondrion is defined by its two membranes (Frey and Mannella 2000; Logan 2006). A topologically simple and limiting outer membrane enwraps the energy-transducing inner membrane whose surface area is considerably larger and which in turn encloses a protein-rich matrix. To accommodate the volume constraint imposed by the outer membrane, the inner membrane has numerous invaginations, the cristae, each of which can have one or more tubular connections to the membrane periphery (Mannella 2006b). In addition to the scaffold function of the inner membrane for the assembly and operation of the respiratory chain complexes, this highly pleomorphic structure provides the permeability barrier across which the respiratory machinery generates its chemiosmotic gradient. Electron tomographic analyses of various mitochondria provided overwhelming evidence that cristae are not simply random folds in the inner membrane but rather internal compartments formed by invaginations of the membrane, which originates at narrow neck-like segments referred as crista junctions. In other words, crista junctions delimit an intercristal space, thereby presumably functionally restricting diffusion between intercristal compartments and the peripheral intermembrane space (Mannella 2006a; Mannella 2006b; Mannella *et al.* 1997). The number of crista junctions and the morphology of the intercristal space have been shown to change with the metabolic state of the mitochondria (Hackenbrock 1968; Mannella *et al.* 1997; Mannella *et al.* 1994) and can only be achieved by the inner membrane undergoing fusion and fission (Mannella *et al.* 2001). The relative activities of proteins involved in mitochondrial fusion and fission determine an overall morphology, integrity and turnover of the mitochondrial population as well as segregation and protection of mitochondrial DNA (Berman *et al.* 2008; Cerveny *et al.* 2007; Chen and Chan 2005; Scott *et al.* 2003). In many cases, mitochondria form a complex reticulum that interacts with other cellular components, in particular the cytoskeleton and endoplasmic reticulum (Mannella 2000; Rutter and Rizzuto 2000). Interaction of mitochondrial and endoplasmic reticulum networks and the connectivity state of mitochondria controls metabolic flow, production of ROS, protein transport, intracellular Ca^{2+} signaling and cell death (Csordas *et al.* 2006; de Brito and Scorrano 2008; Giorgi *et al.* 2009; Kakkar and Singh 2007; Szabadkai and Rizzuto 2007).

3.3. System of oxidative phosphorylation

Mitochondria comprise the site of most ATP production in eukaryotic cells by a process known as oxidative phosphorylation (Saraste 1999). This remarkably complex process, which is performed by system of oxidative phosphorylation (OXPHOS) (Fig. 2), requires four major events, electron transport (CI-CIV) and generation of a proton gradient (CI, CIII and CIV), transport of Pi and ADP and finally coupling the proton gradient to ATP synthesis (CV).
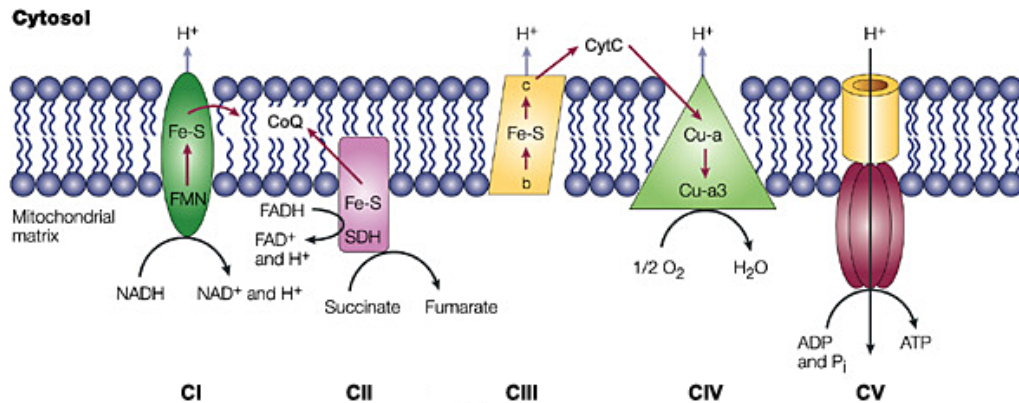
Figure 2 The complexes of OXPHOS (Eng *et al.* 2003). The diagram shows the five complexes of OXPHOS (CI – CV). Complexes I–IV are the electron-transport complexes, whereas complex V synthesizes ATP. Electrons are passed down the four complexes (black and red arrows) to molecular oxygen and then complex V generates ATP from ADP and Pi. The blue arrows show where the protons are pumped to the cytosolic side of the inter-membrane to generate an electrochemical gradient, and where proton movement back across complex V (the ATP synthase) is used to drive ATP synthesis.

3.3.1. Electron-transport chain

Oxidation of NADH and $FADH_2$ and transfer of electrons to O_2 is carried out by the electron transport chain also known as respiratory chain. Electrons travel through this chain from lower to higher standard reduction potentials through series of redox centers, so that the overall process is exergonic. Some of these redox centers are mobile, and others are components of integral membrane protein complexes (Fig. 2). The released energy is used at three locations within the chain (CI, CIII and CIV) to expel protons from the matrix into the IMS, resulting in a potential difference ($\Delta\Psi$) and proton gradient (ΔpH) across the IMM (Voet *et al.* 2006). The energy stored in this electrochemical proton gradient, generally referred to as proton motive force (PMF) can be used for chemical, osmotic and mechanical work. The PMF is dominated by $\Delta\Psi$, with ΔpH contributing ~15% to its total magnitude (Koopman *et al.* 2008; Nicholls and Ferguson 2002). In addition to crucial role of PMF in ATP synthesis, it is also used for a variety of other energy-dependent processes, which are (indirectly) driven by either $\Delta\Psi$ or ΔpH (Fig. 3).
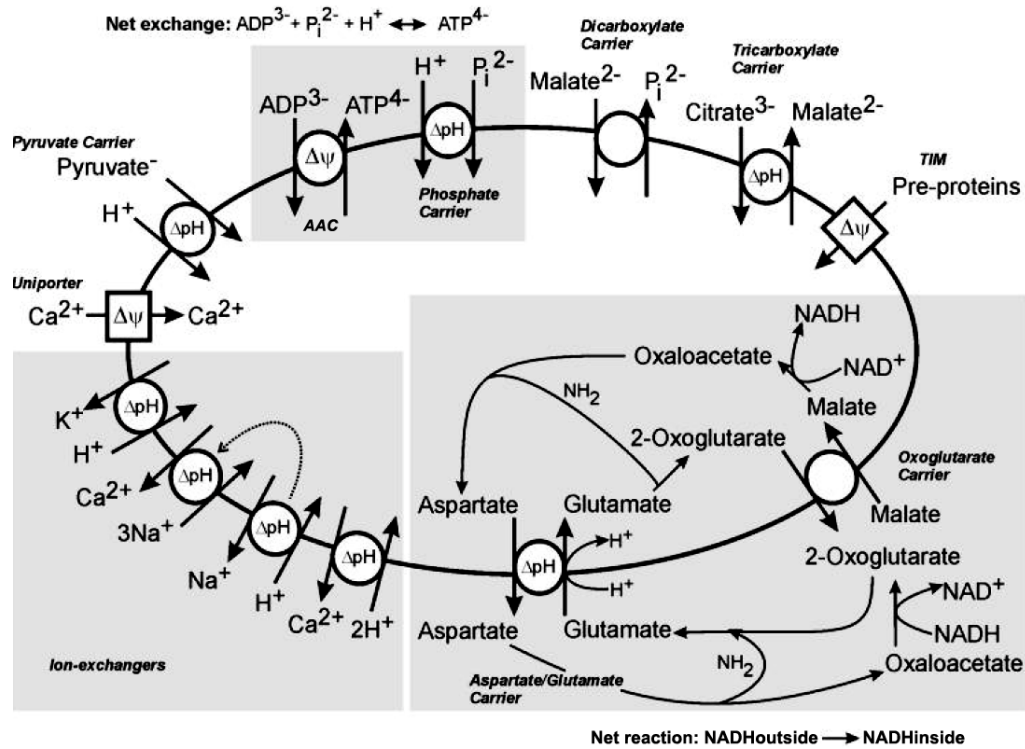
Figure 3 Processes driven by proton motive force (PMF) (Koopman *et al.* 2010). In addition to CV-mediated ATP generation, the PMF also is required for transport processes across the IMM. These processes are (indirectly) driven by either $\Delta\Psi$ or ΔpH. For instance, mitochondrial Ca^{2+} uptake, ATP/ADP exchange by the adenine-nucleotide translocator (AAC), trans-IMM Na^+ and K^+ fluxes (important in mitochondria volume regulation), and import of nDNA-encoded preproteins are $\Delta\Psi$ dependent, whereas several mitochondrial antiporters (aspartate/glutamate, H^+/K^+, H^+/Na^+, $2H^+/Ca^{2+}$) and symporters (H+/pyruvate and Pi/ADP) are ΔpH dependent. In this panel, indirect dependencies on ΔpH are marked by dotted lines.

3.3.1.1. Complex I

Complex I, NADH:ubiquinone oxidoreductase, is a very large integral membrane protein complex which oxidizes NADH, produced predominantly by the tricarboxylic acid cycle (Krebs cycle) and the β-oxidation of fatty acids, to regenerate the NAD^+ pool in the mitochondrial matrix. The transfer of two electrons from NADH which reduce ubiquinone (oxidized CoQ) to ubiquinol (reduced CoQ) in the membrane is coupled to the vectorial translocation of four protons across both the inner mitochondrial membrane and the plasma membrane of many bacteria thus contributing to generation of the proton motive force across the membrane. The ubiquinol is subsequently reoxidized by complex III (Hirst 2010).

Complex I has an unusual L-shaped structure, with one arm in the plane of the mitochondrial inner membrane, and one arm protruding into the matrix, both arms being of approximately equal length (Zickermann *et al.* 2009). The L-shaped structure of complex I, observed by electron microscopy, is conserved among species (Guenebaut *et al.* 1998; Radermacher *et al.* 2006), although the protein composition of the eukaryotic

and prokaryotic enzymes varies significantly. The 14 central subunits are conserved in prokaryotes and eukaryotes and harbor all bioenergetic core functions sufficient for energy transduction (Weidner *et al.* 1993). These core subunits consist of seven highly hydrophobic (in eukaryotes mtDNA-encoded) subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6; human nomenclature) and seven hydrophilic (in eukaryotes nDNA-encoded) subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7 and NDUFS8; human nomenclature). The eukaryotic enzyme, which accounts for a total mass in the range of 1MDa, contains numerous accessory subunits which vary between species and augment both enzyme domains. The function of these subunits is largely unknown, but they are very unlikely to participate directly in energy transduction (Koopman *et al.* 2010). In man and cow, there is 31 accessory subunits (Carroll *et al.* 2006). The conserved cofactors of complex I are a non-covalently bound FMN (flavin mononucleotide) and eight iron-sulfur clusters (Fe-S) with the iron coordinated with two to four cysteine side chains from the polypeptide. FMN accepts electrons from NADH (Gostimskaya *et al.* 2007; Grivennikova and Vinogradov 2006), Fe-S clusters transport electrons through the enzyme to ubiquinone. All Fe-S clusters are bound by the hydrophilic domain. Evidence was provided that mitochondrial complex I can undergo a so called active/deactive transition (Vinogradov 1998). To facilitate the proper buildup and stability of complex I protein, several assembly chaperones are required (Vogel *et al.* 2007).

The prokaryotic minimal form of complex I has been characterized e. g. from *Escherichia coli* (Weidner *et al.* 1993) or *Paracoccus denitrificans* (Usui *et al.* 1990). To establish a yeast genetic approach to complex I, obligate aerobic yeast *Yarrowia lipolytica* has been extensively used (Kerscher *et al.* 2002), since this enzyme is absent in fermentative yeasts like *Saccharomyces cerevisiae* (Buschges *et al.* 1994). Further eukaryotic model organisms are e. g. *Bos taurus* (Carroll *et al.* 2003), fungi *Neurospora crassa* (Marques *et al.* 2005) or plant *Arabidopsis thaliana* (Perales *et al.* 2005). Nevertheless, no high resolution structure of the whole complex I holoenzyme is available to date.

Isolated complex I deficiency is the most common cause of OXPHOS dysfunction (Smeitink *et al.* 2001a; Smeitink *et al.* 2001b). Clinical phenotypes of complex I deficiency can result from mutations in nuclear-encoded subunits, generally presenting in infancy and early childhood. Mutations in mtDNA-encoded subunits are more common in the late childhood or adolescence. Disease causing mutations have been described in all seven mtDNA-encoded structural subunits, twelve of the nDNA-encoded structural subunits and nDNA-encoded assembly factors NDUFAF2, NDUFAF1, C6orf66, C8orf38 and C20orf7 (Distelmaier *et al.* 2009; Janssen *et al.* 2006). MtDNA mutation-based diseases are inherited maternally, while nDNA

mutation-based diseases are mostly inherited in an autosomal recessive manner, although also X-linked inheritance has been described (Fernandez-Moreira *et al.* 2007). The most common and devastating clinical phenotype associated with early-onset complex I deficiency is Leigh syndrome, first described as subacute necrotizing encephalomyelopathy (Leigh 1951). Leigh syndrome is an early-onset, progressive neurological disorder characterized by motor and intellectual developmental delay, signs of brainstem and basal ganglia involvement, and increased lactate levels in blood and/or cerebrospinal fluid (Rahman *et al.* 1996).

3.3.1.2. Complex II

Complex II, succinate dehydrogenase (SDH) or succinate:ubiquinone oxidoreductase is a functional member of both the Krebs cycle (tricarboxylic acid cycle) and respiratory electron transfer chain. Within the Krebs cycle, SDH oxidizes succinate to fumarate in the mitochondrial matrix (or cytoplasm in bacteria) which is coupled to the reduction of ubiquinone in the membrane. SDH is the only OXPHOS complex to lack subunits encoded by the mitochondrial genome and the only respiratory complex not to pump protons across the membrane during its catalytic cycle. The physiological form of the mitochondrial SDH is most likely to be a monomer (Sun *et al.* 2005), unlike that for *Escherichia coli*, which is believed to be a trimer (Yankovskaya *et al.* 2003). Eukaryotic holoenzyme of SDH consists of equimolar amounts of four subunits, two of which, subunit A with a covalently bound FAD cofactor and subunit B containing three iron-sulfur clusters, form a hydrophilic catalytic head. This heterodimer is anchored in the inner membrane by hydrophobic tail composed of subunits C and D, which contain one heme b at the subunit interface each providing one of the two axial His ligands. The heme moiety is present in mammalian, yeast and *Escherichia coli* SDHs, but diverse SDH species vary in the number of heme groups as well as in the number of their hydrophobic subunits (Lemos *et al.* 2002; Sun *et al.* 2005). The electron transfer pathway in the oxidation of succinate by SDH involves initial reduction of a FAD cofactor followed by electron transfer through three Fe-S centers to ubiquinone (Hagerhall 1997). Two ubiquinone binding sites have been identified in SDH complexes in mammals and *Escherichia coli* (Sun *et al.* 2005; Yankovskaya *et al.* 2003). The high affinity ubiquinone site lies on the matrix side of the inner membrane and is formed by residues in subunits B, C and D. The second, low affinity ubiquinone site resides in the membrane closer to the intermembrane space. SDH is closely related to fumarate reductase (manaquinol:fumarate oxidoreductase), which catalyzes the opposite reaction to that of SDH during anaerobic respiration in bacteria (Kroger *et al.* 1992). Both enzymes are suggested to have evolved from a common ancestor.

Mutations of SDH genes can manifest themselves with a wide variety of clinical phenotypes in humans (Rustin and Rotig 2002). A mutation in SDHA gene for subunit A was described, first by Bourgeron et al (1995), to contribute to complex II deficiency and Leigh syndrome (Bourgeron *et al.* 1995; Parfait *et al.* 2000) or late-onset optic atrophy, ataxia and myopathy (Birch-Machin *et al.* 2000). Mutations in genes coding for subunits B, C and D (SDHB, SDHC and SDHD respectively) have never been described in any of these progressive neurodegenerative syndromes related to mitochondrial complex II deficiencies, however, they cause the tumors observed in hereditary paraganglioma and/or pheochromocytoma (Baysal *et al.* 2000; Niemann and Muller 2000; Pasini and Stratakis 2009). Very recently, mutations in SDHAF1, encoding a new LYR-motif protein, were described to manifest as defective SDH and infantile leukoencephalopathy in patients (Ghezzi *et al.* 2009). Being a soluble protein, the authors concluded that SDHAF1 is not a stable component of the SDH complex and, therefore, must be an assembly factor.

3.3.1.3. Complex III

Complex III, ubiquinol:cytochrome c oxidoreductase (QCR) or bc_1 complex, is a component of the eukaryotic or bacterial respiratory chain and of the photosynthetic apparatus in purple bacteria. Every bc_1 complex (bacterial as well as mitochondrial) contains three common subunits with active redox centers. These catalytic core components are cytochrome b with two noncovalently attached b-type hemes b_L (also called b_{565}) and b_H (b_{562}), cytochrome c_1 with covalently attached heme c group and the "Rieske" iron-sulfur protein (ISP) with an [2Fe-2S] iron-sulfur cluster. The mitochondrial system contains additional nonredox subunits absent in the bacterial complexes (Hunte *et al.* 2000; Iwata *et al.* 1998; Schagger *et al.* 1986; Zhang *et al.* 1998), seven in yeast and eight in mammalian enzymes (Zara *et al.* 2009). The redox subunit b is encoded by mtDNA in these systems. In mitochondria, more than half of the total molecular mass of this structurally dimeric transmembrane protein complex protrudes into the matrix region, including two core proteins (Leonard *et al.* 1981; Xia *et al.* 1997). Both these subunits, Core1 and Core2, have been shown to be members of the mitochondrial processing peptidase family of proteins, suggesting that the QCR may be a bifunctional protein also involved in mitochondrial import protein processing (Braun *et al.* 1992; Braun and Schmitz 1995).

In respiratory chain, QCR functions to enable one molecule of ubiquinol, a two-electron carrier, to reduce two molecules of cytochrome c, a one-electron carrier (Voet *et al.* 2006). This occurs by a modified version of the "proton-motive Q cycle" of Mitchell (Mitchell 1976), that permits QCR to pump protons from the matrix to the intermembrane space and explains the main features of the enzyme activity (Brandt and

Trumpower 1994; Crofts *et al.* 1999a; Crofts *et al.* 1999b). The key trait of the model is that there are two separate binding sites for ubiquinone and ubiquinol. Ubiquinol is oxidized at the Q_o site of the complex in a bifurcated reaction, in which one electron is transferred to a high-potential chain and the other to a low-potential chain. The high-potential chain, consisting of the ISP, cytochrome c_1 and CytC, transfers one electron from ubiquinol to an acceptor (cytochrome c oxidase, Compolex IV), what leaves a ubisemiquinone at the Q_o site. The low potential chain consists of two cytochrome b hemes (b_L and b_H, for low- and high-potential hemes), which serve as a pathway through which electrons are transferred across the coupling membrane from ubisemiquinone at the Q_o site to the Q_i site, at which ubiquinone binds and is reduced to ubiquinol. The two electrons at the Q_i site required for reduction of ubiquinone are provided in successive turnovers. The first electron at the Qi site generates a relatively stable ubisemiquinone that is reduced to ubiquinol by the second electron. Thus, for every two ubiquinols that enter the Q cycle, one ubiquinol is regenerated. Proton translocation is the result of deprotonation of ubiquinol at the Q_o site (near the P side of the IMM) and protonation of the reduced ubiquinone at the Q_i site (near the N side of the IMM). The circuitous route of electron transfer in complex III is tied to the ability of CoQ to diffuse within the hydrophobic core of the membrane in order to bind to both the Q_o and Q_i sites. Binding of mobile CytC to subunit cytochrome c_1 of the enzyme is mainly mediated by nonpolar interactions (Lange and Hunte 2002). The exclusive reduction of heme b_L from Q_o-bound ubisemiquinone rather than the Rieske Fe-S cluster of the ISP protein, despite the greater reduction potential difference favoring the latter reaction, is secured by mechanic movement of the extrinsic domain of the Fe-S component after its reduction by ubiquinol from Q_o site to the proximity of heme c_1 group. Merely after oxidation of the Fe-S protein by the cytochrome, the subunit can move back to the docking interface on cytochrome b, close to the Q_o site to undergo further reduction by the ubiquinol (Zhang *et al.* 1998).

Complex III deficiency was first described by Spiro *et al.* (1970) in a 46-year-old man and his 16-year-old son with progressive ataxia, predominantly proximal muscle weakness, areflexia, extensor plantar responses and dementia. Studies of muscle mitochondria showed very loose coupling of oxidative phosphorylation and marked reduction in cytochrome b content, representing a defect in complex III (Spiro *et al.* 1970). Disorders related to complex III deficiency are clinically heterogeneous and relatively rare. Interestingly, absence of complex III was described to be associated with combined I-III deficiency, possibly due to structural importance of complex III for stability of complex I in the form of supercomplexes (see Chapter 3.4.1). Most of the cases of complex III deficiency are caused by mutations in the mtDNA-encoded cytochrome b subunit. Patients with these mutations usually present with an isolated

myopathy with ragged red fibers, characterized by exercise intolerance, weakness and myoglobinuria (Fernandez-Vizarra *et al.* 2009). Only one mutation in nDNA-encoded structural subunit has been described so far (Haut *et al.* 2003), a homozygous 4-bp deletion in the UQCRB gene, encoding the ubuquinone-binding protein presented with hypoglycemia and liver dysfunction. More than 20 pathogenic mutations have been reported in the complex III assembly factor BCS1L, dysfunction of which is associated with a wide variety of phenotypic manifestations, e. g. GRACILE syndrome (growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death), encephalopathy or Björnstad syndrome (sensorineural hearing loss and pili torti) (Fernandez-Vizarra *et al.* 2009).

3.3.1.4. Complex IV

Complex IV, cytochrome c oxidase (CcO), is the terminal enzyme complex of the mitochondrial electron-transport chain, which couples the electron transfer from reduced cytochrome c to molecular oxygen with vectorial proton translocation across the inner membrane. The mammalian CcO complex is composed of 13 different polypeptide subunits, encoded by both the nuclear and mitochondrial genomes. Mitochondrially encoded Cox1 and Cox2 form the redox site involved in electron transfer. Electrons enter the CcO complex at the binuclear copper site (Cu_A) in the Cox2 subunit, which also mediates electrostatic binding of cytochrome c (Zhen *et al.* 1999). From Cu_A, electrons pass to other metal centers in the Cox1 subunit, first to heme a and then to a heterobimetallic heme a_3/Cu_B center (Stiburek *et al.* 2006). Together with Cox3, mitochondrially encoded subunits constitute the evolutionarily conserved structural core of the enzyme. The remaining ten subunits (Cox4, Cox5a, Cox5b, Cox6a, Cox6b, Cox6c, Cox7a, Cox7b, Cox7c and Cox8), which are encoded by nuclear DNA, are associated with the surface of the complex core. These small polypeptides are required for the assembly and stability of the holoenzyme and are thought to function in the regulation of its activity (Fontanesi *et al.* 2008; Helling *et al.* 2008; Huttemann *et al.* 2008). Tissue-specific isoforms of subunits Cox4, Cox6a, Cox6b, Cox7a and Cox8 have been identified in mammals (Huttemann *et al.* 2001; Kadenbach *et al.* 2000). Most CcO subunits have one or more transmembrane domains, with the exception of Cox5a and Cox5b, which are located at the matrix side, and Cox6b, which is associated with the surface of the complex in the intermembrane space (Tsukihara *et al.* 1996).

Subunit Cox4 is the largest nuclear-encoded subunit of the complex. It was shown to be involved in the allosteric inhibition of CcO activity by ATP, which binds to the matrix portion of the subunit (Arnold and Kadenbach 1997). Isoforms 1 and 2 of Cox4 are encoded by two separate genes and are likely to differ with respect to ATP-induced inhibition of CcO activity (Horvat *et al.* 2006). In mammalian cells, the first step of

CcO assembly is the membrane integration of Cox1, followed by the association of the Cox4–Cox5a heterodimer (Stiburek *et al.* 2005) (Fig. 4B, C). Subunit Cox5a binds indirectly to subunit Cox1 via the matrix domain of subunit Cox4 and the extramembrane segment of Cox6c. Knockdown (KD) of both early-assembled subunits Cox4 and Cox5a, which we performed, resulted in accumulation of four subcomplexes consisting merely of subunit Cox1. The absence of Cox1–Cox4/Cox5a heterodimers confirmed further the interdependence of the assembly of Cox4 and Cox5a (Barrientos *et al.* 2009; Stiburek *et al.* 2005). Together with the lack of accumulation of higher-molecular-mass intermediates, our findings suggest that the assembly of the Cox4–Cox5a heterodimer with Cox1 is necessary for the subsequent association of Cox2, and thus for the rest of the assembly to proceed (Fornuskova *et al.* 2010).

Either under mild detergent conditions or when crystallized or reconstituted in phospholipids vesicles, the majority of mammalian CcO exists as a homodimer. Subunits involved in the stabilization of the dimeric state of CcO are Cox6a and Cox6b. Subunit Cox6a may also contribute to the formation of an interaction site for cytochrome c (Tsukihara *et al.* 1996). Subunit Cox6a (Nijtmans *et al.* 1998) and very likely also subunit Cox6b (Massa *et al.* 2008) were shown to associate with the complex, together with subunits Cox7a or Cox7b, at a very late stage of CcO assembly (Nijtmans *et al.* 1998) (Fig. 4A). The expression of FLAG-tagged versions of Cox7a2, Cox7b and Cox6a2 in both wild-type and Cox6a1-deficient backgrounds allowed us to elucidate for the first time the very late events in human CcO assembly (Fornuskova *et al.* 2010). We identified the particular entry points of these three subunits and demonstrated the significance of the CcO holoenzyme bands a_1 and a_2; detected e. g. by (Massa *et al.* 2008). According to our results, band a_1 probably represents the 13-subunit CcO holoenzyme (S4). In contrast, band a_2 lacks subunit Cox6a, which appears to be added as the last assembled structural subunit. Band a_2 (S4*) is formed by the addition of Cox7a2 and probably Cox6b1 (Massa *et al.* 2008) to the assembly intermediate S3. Finally, subunit Cox7b was found to join the assembling complex during or at the end of the formation of the assembly intermediate S3 (Fig. 4C). Furthermore, unlike COX4- and COX5A-KD mitochondria, in which the reduction in the holoenzyme band a_1 was accompanied by a more pronounced decrease in band a_2, Cox6a-deficient mitochondria retained significantly higher levels of the latter species. These results indicate that band a_2 represents another rate limiting step in human CcO assembly (Fornuskova *et al.* 2010).

The biogenesis of CcO is a complicated, sequential process that necessitates sophisticated coordination. Studies in yeast revealed that the CcO assembly requires more than 30 auxiliary proteins, encoded in the nuclear genome, that are essential for proper biogenesis of the CcO complex but are not part of the mature enzyme

(Barrientos *et al.* 2002; Winge and Tzagoloff 2009). These nonstructural factors (assembly factors), accomplish diverse functions at all the levels of the assembly process which include transcription and mRNA maturation, translation of CcO mitochondrial genes, as well as import into mitochondria of nuclear nDNA-encoded subunits and insertion of transmembrane subunits into the inner mitochondrial membrane. Essential additional roles involve heme a biosynthesis, copper homeostasis and insertion into the apoenzyme and formation of assembly intermediates (Fontanesi *et al.* 2008). A number of these factors were shown to have human homologues some of which were studied at the protein level (Stiburek *et al.* 2006). These include copper metallochaperones Sco1, Sco2, Cox11 and Cox17, which are involved in trafficking/insertion of copper ions into Cu_A and Cu_B centers in Cox2 and Cox1 subunits, respectively; inner membrane proteins Cox10, Cox15 and Surf1, which are required for synthesis and incorporation of heme a moieties into Cox1; and Oxa1 and Cox18 translocases, which are involved in the export/translocation of transmembrane segments of integral inner membrane proteins, including CcO subunits (Stiburek and Zeman 2010). In our laboratory, we addressed the role of human Oxa1l, the human orthologue of the yeast Oxa1, in the biogenesis of OXPHOS. Oxa1 protein is a member of the evolutionarily conserved Oxa1/Alb3/YidC protein family, which is involved in the biogenesis of membrane proteins in mitochondria, chloroplasts and bacteria (Herrmann and Neupert 2003; Yi and Dalbey 2005). The predicted human Oxa1l shares 33% sequence identity with the corresponding yeast polypeptide. The inactivation of OXA1 in yeast was shown to cause pleiotropic OXPHOS defects, characterized mainly by the complete absence of CcO activity (Altamura *et al.* 1996), pointing to Oxa1 as essential factor for CcO biogenesis. Oxa1l-depleted cells showed markedly decreased protein levels and ATP hydrolytic activity of the complex V and moderately reduced levels and activity of complex I. Intriguingly, the cytochrome oxidase activity and content of CcO, as well as the ascorbate/TMPD fuelled, sodium azide-sensitive oxygen uptake, were either unaffected or even increased when compared to negative controls (Stiburek *et al.* 2007). Since the yeast Oxa1 is strictly required for the N-terminal export of Cox2 precursor, it is possible that the complete absence of the N-terminal leader peptide in nascent human Cox2 might be responsible for the observed Oxa1-independent assembly of human CcO. The fact that human Oxa1l is able to partially restore the respiratory growth of yeast *oxa1* cells suggests that both proteins share at least basic functional features.

CcO deficiency, in addition to complex I deficiency, is frequent cause of mitochondrial encephalomyopathies. Although several pathogenic mutations in each of the three mitochondrially encoded CcO subunits (Shoubridge 2001) and two mutations in nDNA-encoded genes for structural subunits Cox4i2 (Shteyer *et al.* 2009) and Cox6b

(Massa *et al.* 2008) have been reported, the majority of fatal infantile CcO deficiencies identified so far result from autosomal recessive mutations in genes encoding CcO assembly factors (e. g. (Barrientos *et al.* 2009; Diaz 2010)). Indeed, two of the human CcO assembly factors, LRPPRC (leucine-rich pentatricopeptide repeat cassette) and TACO1 (translational activator of Cox1), both of which are involved in mitochondrial CcO mRNA translation, were identified primarily on the basis of their mutant phenotypes in human patients (Mootha *et al.* 2003; Weraarpachai *et al.* 2009).
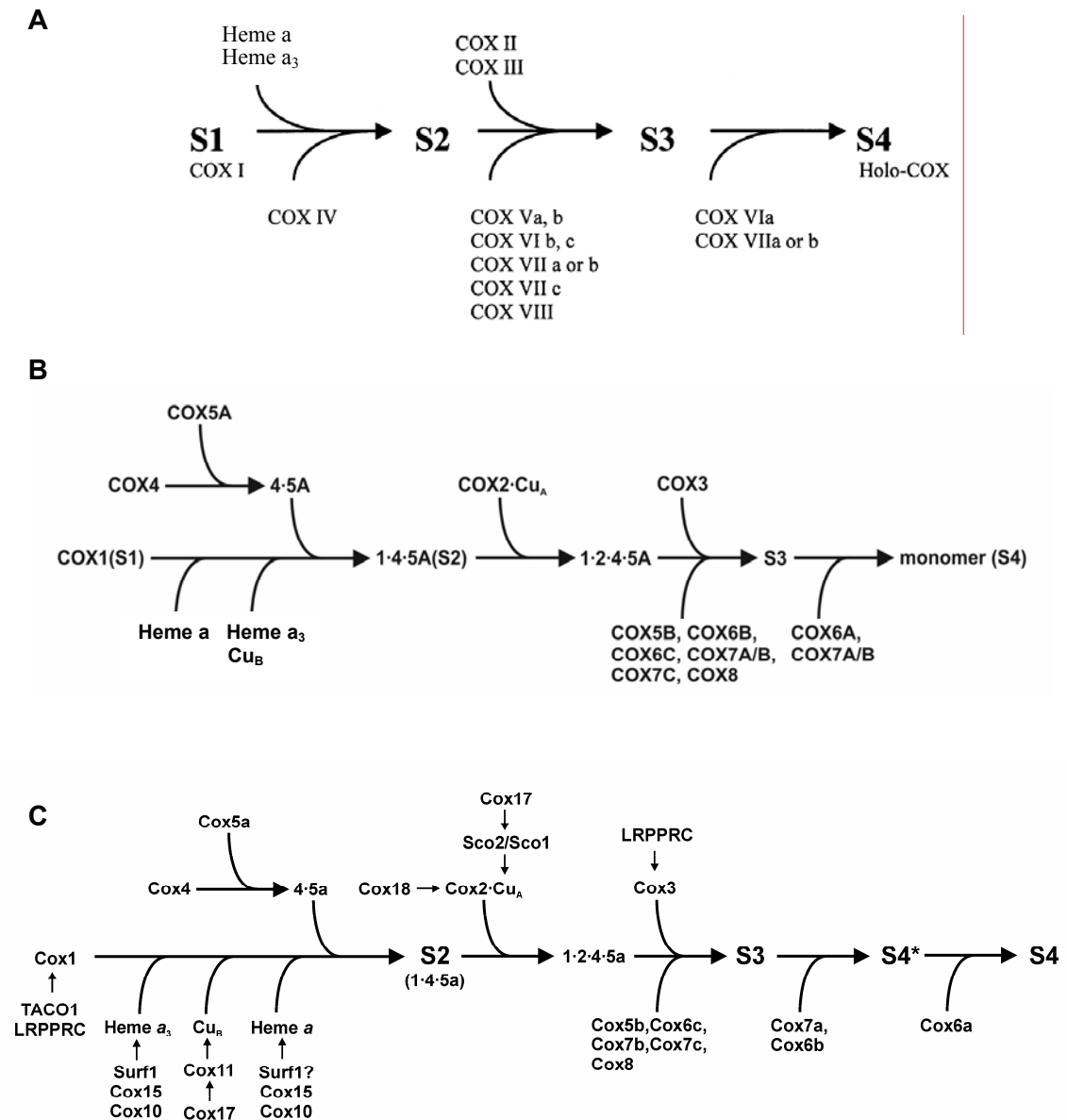
Figure 4 Models of the mammalian CcO assembly pathway according to Nijtmans *et al.* (1998) (A), Stiburek *et al.* (2005) (B) and Fornuskova *et al.* (2010) (C). Prosthetic groups and assembly factors are indicated. Roman (A) or Arabic (B, C) numerals denote CcO subunits within subcomplexes. S2 and S3 indicate assembly intermediates. S4 represents the 13-subunit CcO holoenzyme, and S4* indicates the late assembly intermediate that immediately precedes the formation of the holoenzyme.

3.3.2. Complex V

Complex V, F_1F_o-ATP synthase or F-type ATPase is multisubunit enzyme nearly ubiquitous in the cell membranes of eubacteria, inner membranes of mitochondria and tylakoid membranes of chloroplasts (Boyer 1998). Under oxidative conditions, ATP synthase is a critically important for synthesis of ATP from ADP and P_i driven by a proton or sodium motive force (Meier *et al.* 2009; Meier *et al.* 2005; Pogoryelov *et al.* 2009; Preiss *et al.* 2010), thereby providing the vast majority of ATP in the cell. Under anaerobic conditions, eubacterial enzymes catalyze the reverse reaction and generate the proton motive force using energy liberated by hydrolysis of ATP produced by fermentation, whereas specific inhibitory mechanisms prevent the chloroplast and mitochondrial enzymes from carrying out the reverse reaction. Inhibition of the ATP hydrolysis activity of F_1 under certain conditions was shown in context of IF1 regulatory polypeptide (ATPase inhibitory factor 1) in mammals and Inh1p, Stf1p and Stf2p in yeast (Hong and Pedersen 2002; Walker 1994).

F_1F_o-ATP synthase contains an extramembranous cytoplasmic F_1 part ($\alpha_3\beta_3\gamma\delta\epsilon$ in bacteria) and a membrane-embedded F_o part (ab_2c_{10-12} in bacterial F_1F_o) (Fig. 5A). Both parts are linked by a peripheral and a central stalk. Overall, this large complex has eight different subunits in prokaryotes and 16-18 in mammals (Meyer *et al.* 2007), where the additional subunits are mostly in the stalk region. The central stalk, containing F_1-subunits γ and ϵ, is associated with a ring of F_o-subunits c. This central stalk/subunit c assembly constitutes the rotor in the fully assembled ATP synthase. In mitochondria (Fig. 5B), there is an additional subunit in the rotor, which is inconveniently called „ϵ" and has no equivalent in bacteria or chloroplasts. The mitochondrial „δ" subunit is the equivalent of the bacterial „ϵ" (Nakamoto *et al.* 2008). Peripheral stalk extends from the top (membrane distal) region of F_1, along the external surface of the $\alpha_3\beta_3$ domain, and then reaches down into and across the membrane, where it is associated with subunit a (Walker and Dickson 2006). Its role is to act as stator which connects catalytic $\alpha_3\beta_3$ complex to the transport mediating subunit a. In most bacteria is made up of two copies of the helical b subunits (b_2) and the single δ subunit (the homologue of the bovine OSCP subunit). In some photosynthetic bacteria and chloroplasts, the two b subunits are similar but are the products of two genes (bb´) (Nakamoto *et al.* 2008). In the mitochondrial complex, there is only one subunit b with two additional subunits in the peripheral stalk, d and F6 (known as subunit h in *Saccharomyces cerevisiae* (Rubinstein *et al.* 2005)) and four additional membranous subunits e, f, g and A6L are associated with subunit a (Dickson *et al.* 2006). Unlike the situation in bovine heart where are subunits e and g tightly bound as the part of monomeric ATP synthase, yeast subunits e and g as well as k were proposed to be a dimer-specific (Arnold *et al.* 1998). Subunit a

(ATP6 gene) and A6L (ATP8) are encoded by mammalian mtDNA, whereas yeast mitochondrial genome codes also for the ATP synthase subunit c, (ATP9).
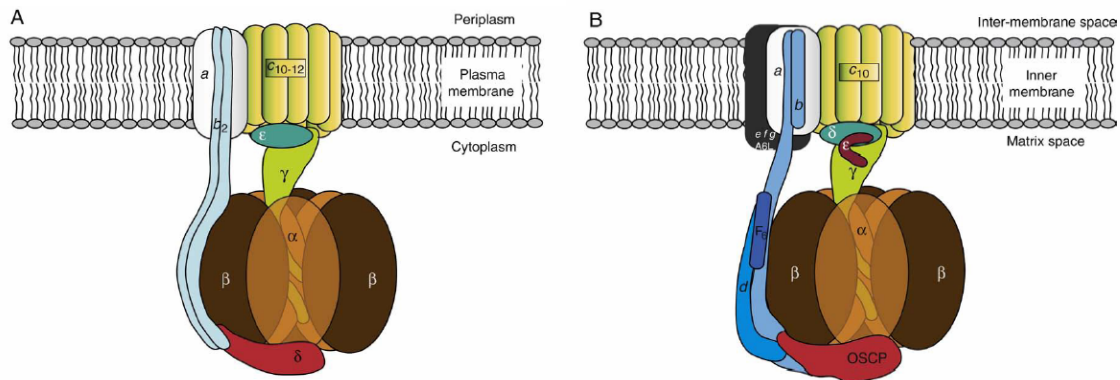
Figure 5 Schematic representation of the bacterial and mitochondrial F_1F_O-ATP synthases (Kucharczyk *et al.* 2009). The enzyme of *Escherichia coli* (A), with a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon ab_2c_{10-12}$, is organized in four subdomains, i.e. the catalytic headpiece ($\alpha_3\beta_3$), the central rotor stalk ($\gamma\epsilon$), the stator stalk ($b_2\delta$), and the proton channel (ac_{10-12}). The human mitochondrial enzyme (B) contains the additional subunit ϵ in the central stalk ($\gamma\delta\epsilon$); the stator stalk is composed of one subunit b, subunits d, F6, f and OSCP; subunit c is present in 10 copies and subunits e and g mediate enzyme dimerization.

The F_1 part contains three catalytic sites, which lie mainly in β-subunits at interfaces with α-subunits. The study of these catalytic sites (Weber and Senior 2000) indicated that F_1 part is effectively a trimeric complex of three α and three β subunits that displays string negative cooperativity of substrate binding and, at the same time, string positive cooperativity of enzymatic activity. To explain these unusual properties, Paul Boyer proposed mechanism named as binding change hypothesis (or also alternating site hypothesis) (Boyer 1993). The key feature of this hypothesis is that three interacting catalytic αβ-protomers are each in a different conformational state at any one time. One binds substrates and products loosely, one binds them tightly and one does not bind them at all. The cyclic nature of the binding change mechanism led Boyer to propose that the binding changes are driven by the rotation of the catalytic assembly ($\alpha_3\beta_3$) with respect to other portions of the F_1F_o ATPase (Boyer 1993; Boyer 2000). We know now that C-terminal region of the γ-subunit penetrates $\alpha_3\beta_3$ domain in asymmetric manner and as it rotates, it brings about a series of conformational changes given by this asymmetry of the central stalk (Walker and Dickson 2006). Each 360° rotation of the central stalk in a clockwise manner (as viewed from the membrane) inside the F_1 domain generates three ATP molecules, and the rotation of the central stalk proceeds, to a first approximation, in 120° steps. If each c subunit translocates one proton, and if it takes the energy for translocation of three or four protons to synthesize one ATP molecule, then three or four small rotations (each through an angle that depends on the number of c subunits in the ring) are required to drive a 120° rotation of γε relative to the $\alpha_3\beta_3$ domain. During the rotation of the central stalk, energy is stored transiently,

presumably in an elastic element, and then released in a quantum to drive each 120° step (Beke-Somfai *et al.* 2010; Capaldi and Aggeler 2002; Nakamoto *et al.* 2008; Tsunoda *et al.* 2001). The two most obvious candidates for such elastic element are the α-helical region of the γ-subunit in the central stalk and the peripheral stalk (Walker and Dickson 2006).

In humans, the most common cause of ATPase defects are missense heteroplasmic mtDNA mutations in the ATP6 gene for subunit a, which affect the protonophoric function of ATPase (Hutcheon *et al.* 2001; Stock *et al.* 1999). Higher prevalence show 8993T>G(C) mutations (Ciafaloni *et al.* 1993; Holt *et al.* 1990; Puddu *et al.* 1993; Shoffner *et al.* 1992; Tatuch *et al.* 1994) which change Leu[156] to Arg or Pro. At a lower mutation load, they manifest as neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP syndrome); at heteroplasmy exceeding 90% they present as maternally inherited Leigh syndrome (severe and fatal encephalopathy). Less common are 9176T>G(C) mutations, which change Leu[217] (Carrozzo *et al.* 2001; Dionisi-Vici *et al.* 1998; Thyagarajan *et al.* 1995), or a 8851T>G mutation (De Meirleir *et al.* 1995) that changes Trp[109], all manifesting also as striatal necrosis syndromes (Schon *et al.* 2001). Impairment of the ATPase H^+ channel results often, but not always, in decreased ATP production, whereas the ATPase hydrolytic activity remains largely unchanged (Houstek *et al.* 1995b; Tatuch and Robinson 1993). Otherwise, a mutation in which A8527G changes the ATPase 6 initiation codon AUG into GUG (Met > Val), has been found in an adult patient with neuropathy, mental retardation, myopathy and retinopathy (Dubot *et al.* 2004). More rarely, ATP synthase dysfunction can be caused by a mutation in the ATP8 gene (Jonckheere *et al.* 2008). A homoplasmic nonsense mutation in overlapping region of ATP6 and ATP8 gene, which results in truncated A6L protein without the last 14 amino acids, has been detected in a 16-year-old patient with hypertrophic cardiomyopathy and neuropathy. A different type of pathogenic mechanism represents mtDNA 2bp microdeletion 9205delTA, which was found in a newborn with transient lactic acidosis (Seneca *et al.* 1996) and also in our patient, a child with encephalopathy and severe psychomotor retardation (Jesina *et al.* 2004). A 9205delTA mutation removes the stop codon of the ATP6 gene and affects the cleavage site between the ATP6 and COX3 transcripts.

Our study on fibroblasts with primary defects in mitochondrial ATP synthase due to heteroplasmic mtDNA mutations in the ATP6 gene, affecting protonophoric function or synthesis of subunit a, show that at high mutation loads, mitochondrial membrane potential $\Delta\Psi_m$ at state 4 is normal, but ADP-induced discharge of $\Delta\Psi_m$ is impaired and ATP synthesis at state 3-ADP is decreased. Increased $\Delta\Psi_m$ and low ATP synthesis is also found when the ATPase content is diminished by altered biogenesis of the enzyme complex. Irrespective of the different pathogenic mechanisms, elevated $\Delta\Psi_m$ in primary

ATPase disorders could increase mitochondrial production of ROS and decrease an energy provision (Vojtiskova *et al.* 2004).

A distinct group of inborn defects of ATP synthase is represented by the enzyme deficiency due to nuclear genome mutations which are very rare and characterized by a selective inhibition of ATP synthase biogenesis. ATPase deficiency of possible nonmitochondrial origin was first described in a child with 3-methylglutaconic aciduria and severe lactic acidosis (Holme *et al.* 1992). Extremely low ATPase activity and low, tightly coupled respiration rates were observed in muscle mitochondria, but no mutation was found in mtDNA genes encoding ATPase subunits. A nuclear origin of ATP synthase deficiency was demonstrated for the first time in 1999 (Houstek *et al.* 1999) in a new type of fatal mitochondrial disorder: a child with severe lactic acidosis, cardiomegaly, and hepatomegaly died 2 days after birth. 70 to 80% decrease in ATPase activity and ATP production was associated with a corresponding selective decrease in the ATPase complex, which had normal size and subunit composition. Increased biosynthesis of the β subunit with a very short half-life contrasted with decreased biosynthesis of the assembled ATPase and indicated assembly defect at the level of F_1-ATPase. Cybrid cells made of patient fibroblasts fully complemented the ATPase defect and confirmed the nuclear origin of impaired biogenesis of the enzyme complex (Houstek *et al.* 1999). In 2004, the search for the diseased-causing gene resulted in the identification of a homozygous missense mutation in the third exon of the ATP12 (ATPAF2) gene which replaced tryptophan 94 with arginine. The mutation was found in a patient with severe encephalopathy and it was confirmed that the primary defect was in the assembly of the F_1 catalytic part of ATP synthase (De Meirleir *et al.* 2004). In 2008, the expression profiling and homozygosity mapping identified a mutation in the second intron of TMEM70 encoding a 30kDa mitochondrial protein of unknown function. The mutation leading to aberrant splicing and loss of *TMEM70* transcript was found in 24 patients (Cizkova *et al.* 2008). This protein turned out to be a novel ancillary factor of ATP synthase biogenesis, interestingly, the first one specific for higher eukaryotes. The first case of a mitochondrial disease due to a mutation in a nuclear encoded structural subunit of ATP synthase was described most recently. Missense mutation in ATP5E gene coding for ε subunit replaced tyrosine 12 with cysteine in patient with neonatal onset, lactic acidosis, 3-methylglutaconic aciduria, mild mental retardation and developed peripheral neuropathy (Mayr *et al.* 2010).

3.3.3. Transporters of ATP synthase substrates

The net efflux of phosphate across the inner mitochondrial membrane occurs to a great extent as γ-phosphate of ATP via the adenine nucleotide carrier, which catalyses efflux of ATP in exchange for ADP. Moreover, ADP as well as inorganic phosphate

(Pi) must be transported into the mitochondrial matrix to be utilized for oxidative phosphorylation.

3.3.3.1. Transport of ADP

The protein, which mediates link between the mitochondrial and cytosolic compartments of all aerobic eukaryotic cells by catalysis of the transmembrane exchange between ATP from oxidative phosphorylation generated inside mitochondria and cytosolic ADP, is ADP/ATP carrier (AAC) or translocase. The popular term adenine nucleotide transporter (ANT) is misleading since this term also implies transport of AMP which is explicitly excluded by the AAC (Klingenberg 2008). The transported substrates are large, hydrophilic, highly charged molecules, averse to low dielectric membrane environment, thus their transport through biomembranes requires a carrier with highly efficient catalytic qualities. Paradoxically to this point, transport by AAC requires adenine nucleotides with their full charge as ATP^{4-} and ADP^{3-} and the less charged AMP is excluded from both uptake and efflux. dADP and dATP are transported at 10-15% the rates of their ribose homologues. Concerning the base moiety, a stringent specificity for adenine is maintained, excluding most other bases (Duee and Vignais 1969; Pfaff and Klingenberg 1968). With AAC reconstituted vesicles, also a 1:1 exchange of pyrophosphate (PPi) against ADP was characterized (Kramer and Klingenberg 1985). Although a fractional charge, corresponding to a partial ADP^{3-} - $ATPH^{3-}$ transport cannot be ruled out and may serve to adjust the energy share used in the exchange in dependence on $\Delta\psi$ and ΔpH, various studies substantiate a fully electric ATP^{4-} - ADP^{3-} exchange (LaNoue *et al.* 1978; Villiers *et al.* 1979; Wulf *et al.* 1978). Under *in vivo* conditions, ADP and ATP are offered simultaneously at varying proportions. In the energized state, ATP is released preferably to ADP but in the uncoupled state ADP leaves the mitochondria more rapidly than ATP (Klingenberg 1980), although equal rates should be expected, just as observed for uptake (Klingenberg 2008). This can be explained by considering that in the mitochondria the nucleotides are present mostly as the Mg^{2+} complexes which are not transported and thus the concentration of free ATP which binds Mg^{2+} about 5-times tighter than ADP (Heldt *et al.* 1972) is decreased more than of ADP (Klingenberg 2008). Without membrane potential the AAC can catalyze the four exchange combinations of ADP and ATP, two homoexchange modes (ATP-ATP, ADP-ADP) and two heteroexchange modes (ATP-ADP, ADP-ATP) at nearly equal rates. Only the ADP-ATP mode (ADP uptake, ATP release) is productive for the ATP delivery whereas the ATP-ADP mode is counter-productive. The homo-modes are diverting exchange capacity. The membrane potential skews this distribution in favor of the ADP-ATP mode and suppresses the ATP-ADP mode (Klingenberg 2008). The central role of the AAC in oxidative

metabolism requires high expression levels in most eukaryotic cells exceeding other transporters.

The amino acid sequence of the AAC was first established in the bovine heart (Aquila *et al.* 1982). The sequence comprising 297 amino acids is likely to be segmented into three similar domains, each featuring two helices with a total of six transmembrane helices (Saraste and Walker 1982). It closely resembles the typical structure of members of the mitochondrial carrier protein family. The striking sequence RRRMMM regarded as an AAC signature was present in all forthcoming AAC sequences, such as of *Neurospora crassa* (Arends and Sebald 1984) and *Saccharomyces cerevisiae* (Adrian *et al.* 1986). By using cross-linking and analytical ultracentrifuge techniques it was shown that the AAC as well as the mitochondrial uncoupling protein, at least in the solubilized state, forms a homodimer (Hackenberg and Klingenberg 1980; Lin *et al.* 1980). A dimer state of yeast yAAC2 was demonstrated using native gel electrophoresis of mitochondria (Ryan *et al.* 1999).

First in yeast and then in other organisms, particularly in mammals, genes of AAC isoform were identified. *Saccharomyces cerevisiae* contains three genes (AAC1, AAC2 also known as PET9, AAC3) for AAC isoforms. yAAC2 is the principal AAC in aerobic yeast. The study of involved promoters and transcription factors showed the N-terminal extension of yAAC2 which is instrumental for the high expression level (Betina *et al.* 1995). yAAC1 is barely expressed (Lawson *et al.* 1990). yAAC3 is obligatory for anaerobic growth (Drgon *et al.* 1991; Kolarov *et al.* 1990) and is suggested to act in reversed order, supplying ATP from glycolysis to the mitochondria. Notably, in the obligate aerob *Neurospora crassa* only one ncAAC gene has been found (Arends and Sebald 1984). In man as well as in cow, four tissue-specific isoforms were described. h- and bAAC1 is predominantly expressed in skeletal and cardiac muscles (Stepien *et al.* 1992). hAAC2 expression is strongly stimulated by growth factor in fibroblasts (Ku *et al.* 1990) and also increased in neoplastic cells (Torroni *et al.* 1990) as well in early myoblasts (Stepien *et al.* 1992) indicating that this isoform is related to enhanced glycolysis in the proliferating cells and has the role of supplying glycolytic ATP into the mitochondria, similarly as suggested for yAAC3 in yeast. At very low level, hAAC3 is expressed in brain, liver, kidney, heart and skeletal muscle (Stepien *et al.* 1992) and hAAC4 in liver and testis, marginally in brain (Dolce *et al.* 2005). Overall, the sequence differences in AAC isoforms are small, as compared to those in the yeast, retaining about 85% identity, with the exception of h- and bAAC4 with only 66-68% identity to the other isoforms (Dolce *et al.* 2005).

Mutations in hAAC1 gene (SLC25A4) have been shown predominantly to result in autosomal dominant progressive external ophthalmoplegia and familial hypertrophic cardiomyopathy. Kaukonen *et al.* (2000) identified a missense mutation in the AAC1

gene (A114P) in 5 families with autosomal dominant progressive external ophthalmoplegia (PEOA2). The analogous mutation in yeast caused a respiratory defect. The authors also identified mutations in the AAC1 gene (V289M) in a sporadic case of PEO (Kaukonen *et al.* 2000). Chen (2002) determined that the A128P mutation of the *Saccharomyces cerevisiae* yAAC2 protein, equivalent to A114P in human AAC1, does not always affect respiratory growth. Rather, expression of A128P resulted in depolarization, structural swelling, disintegration of mitochondria and ultimately an arrest of cell growth in a dominant-negative manner. The author proposed that the A128P mutation may induce an unregulated channel, allowing free passage of solutes across the inner membrane, rather than interfere specifically with ATP/ADP exchange (Chen 2002). Fontanesi *et al.* (2004) introduced dominant-acting missense mutations associated with PEO into yAAC2, the yeast ortholog of human AAC1. Expression of the equivalent mutations in aac2-defective haploid strains of *Saccharomyces cerevisiae* resulted in a marked growth defect on nonfermentable carbon sources and a concurrent reduction of the amount of mitochondrial cytochromes, cytochrome c oxidase activity and cellular respiration. The AAC2 pathogenic mutants showed a significant defect in ADP versus ATP transport compared with wildtype AAC2. The aac2 mutant alleles were also inserted in combination with the endogenous wildtype AAC2 gene. The heteroallelic strains behaved as recessive for oxidative growth and petite-negative phenotype. In contrast, reduction in cytochrome content and increased mtDNA instability appeared to behave as dominant traits in heteroallelic strains (Fontanesi *et al.* 2004). Palmieri *et al.* (2005) first reported a recessive mutation in the AAC1 gene in a sporadic patient who presented with hypertrophic cardiomyopathy, exercise intolerance and lactic acidosis but without ophthalmoplegia. A muscle biopsy showed numerous ragged-red fibers and Southern blot analysis disclosed multiple mtDNA deletions. Homozygous mutation 368C>A in AAC1 gene converting a highly conserved alanine into an aspartic acid at codon 123 was found. The equivalent mutation in AAC2, the yeast ortholog of human AAC1, resulted in a complete loss of transport activity and in the inability to rescue the severe OXPHOS phenotype displayed by WB-12, an AAC1/AAC2 defective strain. Interestingly, exposure to ROS scavengers dramatically increased the viability of the WB-12 transformant, suggesting that increased redox stress is involved in the pathogenesis of the disease (Palmieri *et al.* 2005).

In mouse, two AAC isoforms, mAAC1 and mAAC2, were described (Levy *et al.* 2000). A knockout of heart/muscle specific isoform (mAAC1) (Graham *et al.* 1997) revealed ragged-red fibers in skeletal muscle and cardiac hypertrophy accompanied by dramatic proliferation of mitochondria. In mutant adults, a lactic acidosis and severe exercise intolerance were observed. Muscle specific AAC1 isoform with low expression in myoblasts, is settled to be predominantly expressed by muscle development (Stepien

et al. 1992). Surprisingly, the absence of mAAC1 in knockout mouse was not compensated by an increase in the other AAC isoform. Interestingly, comparable observations were characterized in context of cytochrome c oxidase isoforms of structural subunit Cox6a. Liver-type subunit Cox6a (Cox6a1/Cox6aL) is found in all non-muscle tissues, whereas heart/muscle-type subunit Cox6a (Cox6a2/Cox6aH), encoded by a different gene, is expressed only in striated muscles (Schlerf *et al.* 1988). Mature Cox6a1 and Cox6a2 subunits share lower intra-species (approx. 60%) than inter-species (80–88%) amino acid sequence identity in humans, rats and cows (Fabrizi *et al.* 1992). The tissue-specific pattern of these isoforms is also (similarly to AAC isoforms) established during tissue differentiation (Bonne *et al.* 1993; Kim *et al.* 1995; Taanman *et al.* 1992). A switch from COX6A1 to COX6A2 isoforms was described to occur during mammalian postnatal development in skeletal muscle and heart as well as during differentiation of myogenic cells *in vitro*, and is assumed to be essential for normal function of tissues with high aerobic metabolic demands (Kim *et al.* 1995; Taanman *et al.* 1992). In the heart of mice lacking the COX6A2 gene, the content of CcO comprising Cox6a1 appeared to be unchanged; in other words, without apparent compensation by induction of the expression of subunit Cox6a1 in the knockout heart (Radford *et al.* 2002). In our study (Fornuskova *et al.* 2010), used HEK-293 cells are derived from embryonic kidney tissue, which is specific by almost exclusive (both prenatal and postnatal) expression of the COX6A1 isoform (Bonne *et al.* 1993; Kim *et al.* 1995; Taanman *et al.* 1992). Indeed, the vast majority of the *COX6A* transcripts in HEK-293 cells is represented by the *COX6A1* isoform. Similar to the results from mCOX6A2-KO and mAAC1-KO, the decrease in the major isoform in our COX6A1-KD cells was not accompanied by up-regulation of the minor isoform (COX6A2). However, ectopic expression of Cox6a2 in COX6A1-KD cells tends to complement the CcO defect (Fornuskova *et al.* 2010). It appears that the mechanisms responsible for maintenance of tissue-specific patterns of COX6A as well as AAC isoforms cannot react in response to actual cellular state. In contrast, it is also possible that the pronounced biochemical defect still did not provoke sufficient physiological impairment, which would otherwise trigger the expression of the minor subunit.

3.3.3.2. Transport of Pi

A smaller fraction of phosphate may be transported by the dicarboxylate carrier, which catalyzes a Pi/dicarboxylate or Pi/Pi exchange (Wohlrab 1986). Furthermore, the protein, which is mainly responsible for phosphate transport, is the mitochondrial phosphate carrier (PIC) also named as phosphate transport protein (PTP) (Fig. 3). Three different modes of PIC action were elucidated. Homologous phosphate/phosphate (Pi^-/Pi^-) antiport, heterologous phosphate/hydroxyl (Pi^-/OH^-) antiport or phosphate/proton

(Pi^-/H^+) symport represents the physiological transport mode of unidirectional phosphate uptake. In the functionally active catalytic complex, two binding sites have to be simultaneously occupied in the transport cycle thus forming a ternary complex. The lack of any dependence on the electrical potential confirmed the electroneutrality of the transport reaction (Wohlrab 1986), which is, however, dependent on the membrane pH gradient as driving force of the mitochondrial phosphate carrier (Wohlrab and Flowers 1982). Kinetic modeling of the pH dependence of the unidirectional phosphate transport reaction favors the Pi^-/OH^- antiport rather than Pi^-/H^+ symport (Stappen and Kramer 1994). In addition to the physiological transport mode, PIC can undergo a functional shift from coupled antiport to uncoupled uniport after modification of cysteine residues by mercurial reagents (Stappen and Kramer 1993). A similar shift has been observed for the ATP/ADP and the carnitine carrier from mitochondria (Kramer 1998).

The amino acid sequence of the phosphate carrier from several species including bovine (Runswick *et al.* 1987) or human (Dolce *et al.* 1991) heart and yeast (Phelps *et al.* 1991) has been determined. It closely resembles the typical structure of members of the mitochondrial carrier protein family with subunits of six transmembrane segments and a molecular mass of ~32 kDa (Aquila *et al.* 1985; Kuan *et al.* 1995). Similarly to many mitochondrial transport proteins (Wohlrab 2009; Wohlrab 2010), PIC appears to function as homodimer (Phelps and Wohlrab 2004; Schroers *et al.* 1998; Wohlrab 2004). Among the family members, the mammalian, but not the yeast, PIC is exceptional in having a processed N-terminal sequence that helps to target the protein into mitochondria (Zara *et al.* 1992). Only one gene for the PIC has been detected in human (SLC25A3) and cow (Dolce *et al.* 1994), in both, with two closely related exons named 3A and 3B which appear to be alternatively spliced (Dolce *et al.* 1994). At transcript and protein levels, two PIC isoforms (A and B) has been demonstrated. PIC-A is present only in heart and skeletal muscles, whereas PIC-B is ubiquitous (Fiermonte *et al.* 1998). The recombinant, reconstituted isoforms exhibit similar substrate specificity and inhibitor sensitivity, but they differ in kinetic parameters.

Mayr *et al.* (2007) described the first patients with mitochondrial phosphate carrier deficiency caused by a homozygous mutation in the SLC25A3 gene, i. e. 215G>A transition in alternatively spliced exon 3A, resulting in an amino acid substitution of Gly[72] to Glu. Two siblings showed lactic acidosis, hypertrophic cardiomyopathy, and muscular hypotonia and died within the first year of life. Functional investigation of intact mitochondria showed a deficiency of ATP syntase in muscle but not in fibroblasts, which correlated with the tissue-specific expression of exon 3A in muscle versus exon 3B in fibroblasts (Mayr *et al.* 2007).

3.4. Organization of OXPHOS, functional and structural relevance

The molar ratios of the respiratory components of mitochondria from different species as well as from different organs of the same species vary significantly (Lenaz and Genova 2007). These different stoichiometries could reflect the adaptability of the tissues to a variable energy demand. In bovine heart, the main components of OXPHOS best fit unit stoichiometry of 1 complex I : 1.3 complex II : 3 complex III : 6.7 complex IV (Schagger and Pfeiffer 2001). For each complex IV are 0.5 unit of ATP synthase and 3-5 units of the ADP/ATP translocase (Capaldi 1982). The amount of CoQ is in considerable molar excess to complex III and CytC is usually in slight molar excess to complex IV (Lenaz and Genova 2009).

3.4.1. Random diffusion vs. solid-state assembly of electron transfer enzymes

In the prevalent view, the large enzymatic complexes are randomly distributed in the lipid bilayer of the inner membrane and functionally connected by lateral diffusion of small redox molecules, i. e., coenzyme Q and cytochrome c. All these components undergo independent lateral diffusion in the plane of the membrane, however, diffusion rates of mobile electron carriers are faster than those of the bulkier macromolecular complexes. The diffusion-coupled collision frequencies may be either higher or lower than any given reaction step within the complexes, and consequently electron transfer would be either reaction limited or diffusion limited. Such a random liquid-state organization is usually referred as fluid mosaic model, random diffusion model or random collision model of electron transfer (Lenaz and Genova 2007; Lenaz and Genova 2009) and was essentially based on the isolation of functional individual complexes (Hatefi 1985), on the failure of electron microscopic and liposomal fusion studies to identify associations of complexes (Capaldi 1982), and on the pool behavior of CoQ and CytC in bovine mitochondria (Gupte and Hackenbrock 1988; Kroger and Klingenberg 1973).

Alternatively, the components of the chain form aggregates ranging from small clusters of few complexes to the extreme of a solid-state assembly. The aggregates may be either permanent or transient, but their duration in time must be larger than any electron transfer turnover to show kinetic differences from the previous model (Lenaz and Genova 2007; Lenaz and Genova 2009). The evidence that the respiratory chain complexes are associated to form specific stoichiometric supramolecular units with well defined structure provided the electrophoretic analyses by both BN-PAGE and colorless native PAGE (CN-PAGE) supporting the idea of highly ordered associations of the respiratory supercomplexes and discarding most doubts on artificial interactions. The interactions may be species- or kingdom- specific (Schagger and Pfeiffer 2000; Vonck

and Schafer 2009) presumably within the plane of the membrane, whereas the matrix-exposed protein domains are in close vicinity but probably do not substantially interact (Dudkina *et al.* 2005).

In bovine heart mitochondria, complexes I-III interactions were apparent from the presence of about 17% of total complex I in the form of I_1III_2 supercomplex that was found further assembled into two major supercomplexes (respirasomes) comprising different copy numbers of complex IV ($I_1III_2IV_1$ and $I_1III_2IV_2$ contain 54% and 9% of total complex I, respectively). Only 14-16% of total complex I, 40-42% of complex III and majority of complex IV (>85%) was found in free form in the presence of digitonin (Schagger and Pfeiffer 2001). Associations of complex II with other complexes of the OXPHOS system could not be identified under the conditions of BN-PAGE (Lenaz and Genova 2009). However, Acin-Perez and colleagues demonstrated recently that some of the previously identified mammalian supercomplexes contain also complex II, and complex V, as well as electron carriers CoQ and CytC (Acin-Perez *et al.* 2008).

In *Saccharomyces cerevisiae*, complex I is absent and replaced by three NADH dehydrogenases. Compared to bovine heart mitochondria, yeast complex IV does not occur significantly in free form. It is quantitatively associated with complex III, and the amount of the supercomplexes formed depends on the amount of complex IV available at different growth conditions. The amount of supercomplexes seems to reflect the demand of the cell for energy supply via the OXPHOS system (Schagger and Pfeiffer 2000). Stable supercomplexes of complexes III and IV were isolated also from several bacteria, e. g. from *Paracoccus denitrificans* (Berry and Trumpower 1985), the thermophilic *Bacillus* PS3 (Sone *et al.* 1987), the thermoacidophilic archaeon *Sulfolobus* sp. strain 7 (Iwasaki *et al.* 1995a; Iwasaki *et al.* 1995b) and *Corynebacterium glutamicum* (Niebisch and Bott 2003).

Supercomplexes are stabilized by cardiolipin (Claypool *et al.* 2008; McKenzie *et al.* 2006; Pfeiffer *et al.* 2003), an anionic phospholipid consisting of two phosphatidyl residues that are linked by a glycerol moiety (Lecocq and Ballou 1964; Macfarlane 1964). Cardiolipin is exclusively found in bacterial and mitochondrial membranes, which are designed to generate an electrochemical potential for substrate transport and ATP synthesis (Schlame *et al.* 2000). It is buried within the protein complexes having important structural and catalytic role (Lange *et al.* 2001; Sedlak and Robinson 1999).

Although, from a structural point of view, little doubt exists that respiratory chain complexes are organized, at least in part, as supramolecular aggregates, characterization of the supercomplexes by biochemical functional analysis is still poor. The close proximity of the enzymes catalyzing consecutive reactions enables increased metabolic flow in the pathway by ensuring the substrate channeling of quinones and/or CytC and sequestration of reactive intermediates like ubisemiquinone, which can react with

oxygen to generate superoxide anion radical (Grivennikova and Vinogradov 2006; Schagger and Pfeiffer 2000). Kinetic testing using metabolic flux control analysis (see Chapter 3.4.3) can discriminate whether the enzyme complexes are independently embedded in the lipid bilayer of the inner membrane and connected by randomly diffusing mobile electron carriers or functions as the part of multicomplex units with direct electron channeling between complexes. The former model implies that each enzyme may be rate-controlling to a different extent, whereas in the latter, the whole metabolic pathway would behave as a single supercomplex and inhibition of any one of its components would elicit the same flux control. In bovine heart mitochondria particles and submitochondrial particles, both complexes I and III were found to be highly rate-controlling over NADH oxidation, a strong kinetic evidence suggesting the existence of functionally relevant association between the two complexes, whereas complex IV appears randomly distributed, indicating that electron transfer occurs via a random pool of CytC molecules. Moreover, complex II is fully rate-limiting for succinate oxidation, clearly indicating the absence of substrate channeling toward complexes III and IV (Bianchi *et al.* 2004). The presence of a large excess of free complex IV (Schagger and Pfeiffer 2001) is in line with this conclusion, however, this fact also prevents, using this approach, to exclude substrate channeling within those complex IV units that are bound in a respirasome. Interestingly, in yeast, where the majority of complexes III and IV is associated in form of III-IV supercomplex, overall quinol oxidase rate (rate of complexes III-IV) was significantly decreased after DDM treatment of digitonin-solubilized yeast mitochondria. Indeed, the decrease in quinol oxidase activity was observed in the same range of DDM as dissociation of supercomplexes III-IV was detected by BN-PAGE (Schagger and Pfeiffer 2000). Taken together, substrate channeling of electrons in bovine heart mitochondria, which was confirmed between complexes I and III, may occur also between complexes III and IV but is not required for electron transfer (Genova *et al.* 2008).

In addition, the supercomplex assembly appears to play role in structural stabilization of individual complexes. Analysis of the structural integrity of human respirasomes in patients with defined defects of individual respiratory chain complexes (Schagger *et al.* 2004) provided evidence that a loss of complex III prevented respirasome formation and led to secondary loss of complex I, therefore primary complex III assembly deficiencies presented as combined complex III/I defects. Conversely, absence of complex I did not influenced stability of complex III. Also several other studies in mutants and patients suggested that complex III is involved in the assembly and stabilization of complex I (Acin-Perez *et al.* 2004; Blakely *et al.* 2005; Stroh *et al.* 2004). Moreover, Marques *et al.* (2007) studied biogenesis of complex I in *Neurospora crassa* using a collection of deletion mutants that can survive for the

presence of alternative NADH dehydrogenases. They found that the biogenesis of complex I is obligatorily linked with its assembly in supercomplexes. Furthermore, complex I of nuo51 mutant, that lacks the NADH-binding 51kDa subunit and is enzymatically inactive, is assembled into supercomplexes like those found in wild-type mitochondria, showing that a major function of supercomplex organization might be non-catalytic, but rather to direct assembly of the respiratory complexes (Marques *et al.* 2007).

Regarding structural relevance of complex IV for stability of complex I, the recent evidence is not so straightforward. In the study of Schagger *et al.* (2004), complex IV deficiency led to normal steady-state levels of complexes I and III (Schagger *et al.* 2004). However, similar authors observed reduced stability of complex I under the conditions of BN-PAGE and substantial decrease in NADH-ubiquinone oxidoreductase activity in the mutant strain of *Paracoccus denitrificans* lacking complex IV (Stroh *et al.* 2004). Similarly, the total loss of CcO in a mouse COX10-knockout model (Diaz *et al.* 2006) as well as substantial decrease of CcO due to RNAi KD of Cox4 in murine cultured cells (Li *et al.* 2007) resulted in decreased stability and activity of complex I. Nevertheless, the knockdown of Cox4 and Cox5a in *Caenorhabditis elegans* led to enzymatic defect of complex I with otherwise normal amount of the assembled complex (Suthammarak *et al.* 2009). In contrast, the CcO deficiency in our COX4-, COX5A- and COX6A-KD cells did not lead to any significant changes in the amount and/or activity of other OXPHOS complexes, including complex I (Fornuskova *et al.* 2010). Similarly, normal levels and activity of complex I were also found in murine COX5BKD cells with severe CcO deficiency (Galati *et al.* 2009).

Another aspect of supercomplex assembly concerns at what stage of biogenesis of respiratory complexes are the supercomplexes organized. In four *Neurospora crassa* deletion mutants of complex I membrane arm subunits nuo9.8, nuo11.5 and nuo20.8, 2D BN-SDS immunoblots of digitonin-solubilized mitochondria detected that subcomplexes of peripheral arm containing at least the 30.4kDa subunit and accumulating in these samples comigrate with III/IV supercomplexes and/or the prohibitin complex, thus pointing to them as putative candidates for interaction partners (Marques *et al.* 2007). In another study, Mick *et al.* (2007) investigated the role of Shy1, the yeast homolog of human Surf1, in the assembly of complex IV from *Saccharomyces cerevisiae* by analyzing the nature of intermediate subcomplexes by gel electrophoresis. They found that complex IV associates with complex III already in the form of incomplete subcomplexes, and that some of the late assembled subunits are probably added directly to the III/IV supercomplex (Mick *et al.* 2007). An example of such a subunit is Cox13, the yeast homologue of human Cox6a (Brandner *et al.* 2005). We performed BN/BN-PAGE immunoblots in our CcO deficient samples with

knockdown of selected structural CcO subunits, including subunit Cox6a. In all samples, including controls, additional spots that migrated at the level of supercomplexes in the first dimension, but with apparent molecular masses lower than that of the CcO holoenzyme were detected. These spots were most apparent in COX5A-KD mitochondria, which exhibited the most severe CcO holoenzyme defect and the highest accumulation of incomplete CcO assemblies (Fornuskova *et al.* 2010). Although we cannot exclude the removal of some of the peripheral subunits because of detergent treatment, these spots might represent incomplete CcO assemblies that are already present in supercomplexes. Collectively, subcomplexes resulting from pathological conditions as well as those accumulating in the mitochondria under physiological conditions seem to be capable, at least to some extent, to assemble into supercomplexes.

3.4.2. Supramolecular organization of ATP synthase and pleiotropic effects of its dysfunction

While prokaryotic ATP synthase is thought to occur exclusively as a monomer, mitochondrial ATP synthase adopts supramolecular structures that have been found in a large range of organisms. Originally, ATP synthases arrangements were observed by electron microscopy of deep-etched *Paramecium* mitochondria as a double row of particles, which form a zipper-like array along the outer curved margin of the helically shaped tubular cristae (Allen *et al.* 1989), however, the same approach failed to reveal a similar arrangement in mammalian specimens (Allen *et al.* 1989). Therefore, it was unclear whether or not the dimer ribbons were a special feature of ciliate mitochondria. Ten years later, rows of F_1 parts were also seen by cryo-electron tomography of *Neurospora crassa* mitochondria (Nicastro *et al.* 2000) and then by atomic force microscopy in yeast (Buzhynskyy *et al.* 2007) and by cryo-tomography in rat and bovine mitochondria (Strauss *et al.* 2008) indicating that the ribbons of ATP synthase dimers are rather common to all eukaryotes. Furthermore, dimeric and occasionally higher oligomeric forms of ATP synthase were isolated with high yield by native gel electrophoresis after mild detergent treatment of mitochondrial membranes, first in yeast (Arnold *et al.* 1998) and later in mammalian (Schagger and Pfeiffer 2000) and plant (Eubel *et al.* 2003) mitochondria. A gentle separation by CN-PAGE, without the use of Coomassie stain, promotes the retention of even-numbered oligomers like tetramers, hexamers and octamers suggesting that oligomeric ATP synthase is formed from dimeric building blocks (Wittig and Schagger 2005). A higher digitonin/protein ratio, used for membrane solubilization, induces breakdown of the oligomers into dimers and monomers.

Association between the two ATP synthases in a dimer occurs via the F_o parts which are linked together so that the dimer adopts a V-like structure. The dimer ribbons

seem to assemble from two dimer types assigned as "true dimer" and "pseudo dimer" (Dudkina *et al.* 2006). The former appears to be characterized by large angle (70-90°) between the ATP synthase monomers across both rows and the latter is characteristic by rather small angle (20-35°) made up of neighbors within a row (Vonck and Schafer 2009; Wittig and Schagger 2009). In *Saccharomyces cerevisiae*, it has been shown that subunits e and g that are not involved in the ATPase or ATP synthase activity are essential actors for dimerization (Arnold *et al.* 1998) and oligomerization (Fronzes *et al.* 2006). Both subunits are F_o components with a single transmembrane span (Belogrudov *et al.* 1996). Other components such as subunit 4, the homologous subunit to the b-subunit of beef ATP synthase, and other proteins of the peripheral stalk are involved *in vivo* in the interactions between monomers (Fronzes *et al.* 2006; Gavin *et al.* 2005; Wittig and Schagger 2008).

Deletion mutants of e and g not only lacked ATP synthase dimers but also had altered inner membrane morphology with the inner membranes forming onion-like structures. Thus, the association of ATP synthase dimers was strongly suggested to be involved in the control of the biogenesis of the inner mitochondrial membrane (Giraud *et al.* 2002; Paumard *et al.* 2002). Strauss et al (2008) further characterized in bovine and rat mitochondria, that the dimer ribbons are associated with a sharp bend (~ 60°) in the lipid bilayer implying that the ATP synthase arrays are a key factor in shaping cristae morphology (Strauss *et al.* 2008). Cristae are perhaps the most notable ultrastructural feature of active mitochondria. Numerical simulation indicates that, although the electrochemical potential must be the same along the entire inner membrane surface under equilibrium conditions, the local pH gradient, and hence the ΔpH contribution to the proton motive force is significantly higher in regions of high membrane curvature (Strauss *et al.* 2008). As the ΔpH contribution seems to be more effective than $\Delta\Psi$ in driving ATP synthesis (Kaim and Dimroth 1999), this would suggest that a position of the ATP synthase at the apex of mitochondrial cristae is optimal for ATP production.

ATP synthase is fundamentally dependent on the phosphate (PIC) and adenine nucleotide (AAC) carriers (Kaplan 2001), which are responsible for transport of Pi and ADP, the substrates for ATP synthesis, to the mitochondrial matrix (see Chapter 3.3.3). Each carrier consists of a single polypeptide chain in dimeric form and both were found to sub-fractionate under the mild conditions in complex with ATP synthase as ATP synthase/PIC/AAC supercomplex of 1:1:1 stoichiometry named also as "ATP synthasome" (Ko *et al.* 2003). A likely location of PIC and AAC is adjacent to one another and although they are near the more centrally located subunit c ring of ATP synthase F_o part, do not overlap with it (Chen *et al.* 2004).

In some yeast mutants of complex V subunits, combined deficiencies in complexes IV and V were found. The complex IV was affected more than the other respiratory enzymes in strains with mutation in ATP4 (Paul *et al.* 1989), ATP6 (Choo *et al.* 1985), ATP8 (Marzuki *et al.* 1989), ATP9 (Hadikusumo *et al.* 1988) or ATP17 (Spannagel *et al.* 1997), however, high level of petite production, i. e. cells bearing large deletions in the mtDNA or totally lacking mtDNA, rendered the interpretation difficult. More recently, Δatp6 (Rak *et al.* 2007), Δatp1 and Δatp2 (Marsy *et al.* 2008) mutant strains with minimal petite production also showed substantial decrease in the accumulation of complex IV. Interestingly, no decrease in complex IV levels was observed in uncoupled complex V mutants of ATP3, ATP16 or ATP15 genes (coding for subunits γ, δ and ε, respectively) where maintenance of a mitochondrial potential was found to be severely compromised by massive proton leaks through the F_o (Duvezin-Caubet *et al.* 2003; Guelin *et al.* 1993; Xiao *et al.* 2000). Therefore, it was hypothesized that reduction of the amount of complex IV would be observed only in complex V mutants unable to dissipate the proton gradient. Indeed, blocking proton translocation in wild-type yeast cells with use of oligomycin also induced a strong quantitative deficit in complex IV, which was reversible when the cells were returned to an oligomycin-free medium (Marsy *et al.* 2008).

In tightly coupled mitochondria, the oxygen consumption rate and ATP synthesis activity depend on each other (Kadenbach 2003). The first mechanism of respiratory control suggest that an uptake of ADP into mitochondria stimulates the ATP synthase, thereby decreasing proton motive force, which in consequence stimulates the activity of the three proton pumps and thus mitochondrial respiration (Nicholls and Ferguson 2002); the proton pumps are inhibited at high membrane potential (150-200 mV). The second mechanism of respiratory control, however, involves allosteric inhibition of cytochrome c oxidase by ATP at high intramitochondrial ATP/ADP ratios (Arnold and Kadenbach 1997; Kadenbach and Arnold 1999) which is suggested to keep *in vivo* the mitochondrial membrane potential at low values (Lee *et al.* 2001), close to optimal values for the synthesis of ATP by the ATP synthase (100-140mV) (Kaim and Dimroth 1999). Thus in the system with minimal or no activity of ATP synthase, low energy provision (low ATP) can presumably stimulate cytochrome c oxidase activity, which in turn increases membrane potential without its dissipation by ATP synthase. At high mitochondrial membrane potentials (>140 mV), an exponential increase of mitochondrial ROS production occurs (Korshunov *et al.* 1997; Liu 1999), which could be prevented by an uncoupler treatment (Houstek *et al.* 2004). High membrane potential across the inner membrane would also hamper import of glycolytic ATP into mitochondria unable to perform oxidative phosphorylation (Brustovetsky *et al.* 1996), since this is an electrogenic reaction exchanging four against three negative charges by

reversal of the ADP/ATP translocase (see Chapter 3.3.3.1). Defective OXPHOS presumably tends to lower the respiratory chain in attempt to prevent either a too high membrane potential or the accumulation of reduced intermediary components of the respiratory chain. In accordance with the second mechanism of respiratory control, the pleiotropic effects of severe complex V deficiency are mainly striking for complex IV.

In human skin fibroblasts from Leigh patients with ATP synthase deficiency due to mtDNA 8993T>C heteroplasmic mutation (>95%) in ATP6 gene, the pleiotropic effects similar to those observed in yeast mutants with ATP synthase deficiency (Rak *et al.* 2007) were absent (Marsy *et al.* 2008). In our study, we investigated impact of mitochondrial tRNA mutations on the amount and stability of OXPHOS in various tissues including skeletal muscle, heart, frontal cortex and liver (Fornuskova *et al.* 2008). In skeletal muscle and heart, the patterns of OXPHOS deficiencies were typical for mutations affecting mt-tRNA$^{\text{Leu(UUR)}}$ and mt-tRNA$^{\text{Lys}}$ with respect to abundance of respective codons in mitochondrially-translated subunits of OXPHOS, i. e. isolated defect of complex I in the patient with a mutation in mt-tRNA$^{\text{Leu(UUR)}}$ and combined severe deficiency of complexes I and IV in patients with mutations in mt-tRNA$^{\text{Lys}}$ (complex V was also significantly decreased, albeit less than complexes I and IV). Accordingly, immunoblotting of BN/SDS/PAGE in skeletal muscle of patient with dysfunctional mt-tRNA$^{\text{Lys}}$ detected the level of free S1 subcomplex (related to free mtDNA-encoded subunit Cox1 of complex IV with most abundant AAR codons specific for Lys) to be below the detection limit of the method what very likely reflected the limiting character of this subunit in the holoenzyme assembly and clearly demonstrated aberrant CcO assembly due to dysfunction of mt-tRNA$^{\text{Lys}}$. However, in frontal cortex mitochondria of both types (with affected mt-tRNA$^{\text{Leu(UUR)}}$ as well as mt-tRNA$^{\text{Lys}}$), the patterns of OXPHOS deficiencies differed substantially from those observed in other tissues, what was particularly striking for ATP synthase. Surprisingly, in the frontal cortex of the patient with mutation in mt-tRNA$^{\text{Leu(UUR)}}$, whose ATP synthase level was below the detection limit, the assembly of complex IV appeared to be hindered by some factor other than the availability of mtDNA-encoded subunits, based on the massive accumulation of all three mitochondrially encoded subunits, either free or partially assembled. Although the Cox1 subunit was shown to be a key regulatory target for CcO reduction in yeast cells (Rak *et al.* 2007), hindered CcO assembly in frontal cortex of our patient (Fornuskova *et al.* 2008) employing other mechanism might be comparable to pleiotropic (presumably protective) effects of very severe ATP synthase deficiencies observed in yeast mutants (Marsy *et al.* 2008; Rak *et al.* 2007).

3.4.3. Tissue-specific physiological diversity of OXPHOS

Mitochondria of various tissues exhibit large diversity in mitochondrial shape and organization with an important variability of organellar section profile, intracellular localization and heterogeneity (Benard *et al.* 2006). Accordingly, the different tissues present with large differences in the composition of the OXPHOS machinery and the organization of mitochondria, which could reflect their variable physiological activity. The tissue specific nature of mitochondria was proposed to contribute to the observed tissue variation in the control of oxidative phosphorylation, which was studied by metabolic flux control analysis (MCA). MCA is usually performed by titrating the whole respiratory chain activity (global flux) and its single steps with inhibitors of the individual complexes thus quantitatively expressing the effect of a pertubation of a step on a flux. It allows determination of control coefficients or threshold curves. The control coefficients are calculated from the measured per cent changes in the enzyme activities upon the addition of a small concentration of specific inhibitors (Groen *et al.* 1982; Rossignol *et al.* 2000). In the case of the threshold curves, each point represents the respiratory rate inhibition percentage as a function of inhibition percentage of the isolated step activity for the same inhibitor concentration. The threshold curves exhibit two types according to their profile (Mazat *et al.* 2000). Type I threshold curves present a large plateau phase followed by a steep breakage allowing a precise determination of the threshold value, whereas in type II, a plateau is no longer apparent and the threshold value is hardly defined. Threshold curve profiles can be explained by an excess of enzyme activity that accounts for the plateau phase of type I threshold curves. Conversely, in the case of no excess of enzyme activity, the isolated step inhibition will have a direct effect on the flux (type II profile), so the threshold curve will no longer have a plateau phase. Type I threshold curves for complexes I and III and type II threshold curves for oxidative phosphorylation network (ATP synthase, AAC, PIC) were observed in mitochondria originated from various rat tissues (muscle, heart, brain, liver and kidney). Complex IV presented the two profiles according to the tissue with a type I in the liver, the brain and the kidney and a type II in the muscle and the heart (Rossignol *et al.* 1999). The authors proposed two tissue groups, each characterized by similar threshold values (determined from threshold curves). The first group included the muscle and the heart and the second group encompassed the kidney and the brain. The liver could be associated to either one or the other of these two groups according to the complex considered. Based on the control coefficient distribution, two slightly different tissue groups were obtained by the same authors (Rossignol *et al.* 2000). The muscle and the heart on the one hand, controlled essentially at the level of the respiratory chain and the liver, the kidney and the brain on the other hand, controlled mainly at the phosphorylation level by ATP synthase and the phosphate carrier. Indeed,

when we studied tissue-specific impact of mt-tRNA mutations with comparable tissue heteroplasmy on OXPHOS deficiency patterns using Western immunoblotting, the observed steady-state levels of OXPHOS complexes suggested that the brain ATP synthase is most sensitive to disturbances of the mitochondrial translational system, however, in the skeletal muscle and the heart, steady-state levels of respiratory chain complexes I (and IV) were most severely affected. In skeletal muscle and heart, the preferential deficiency of complex I and also IV is in line with the particular abundance and distribution of codons for affected mt-tRNAs in mitochondrially-translated subunits of these complexes (Fornuskova *et al.* 2008).

Finally, when the tissue diversity of OXPHOS was considered also based on mitochondrial morphology, overall mitochondrial content, relative and absolute content of respiratory chain complexes II, III an IV, their kinetics and stoichiometry with respective substrate as well as global functioning of the respiratory chain, three groups of tissues were obtained. The first group, skeletal muscle and heart, presented with the highest OXPHOS capacity and a low resistance against the occurrence of respiratory chain perturbation. The second group, liver and kidney, was characterized by a lower OXPHOS capacity and a lower sensitivity to OXPHOS defects. The third group, which contained solely the brain, was between the first and the second group regarding the OXPHOS capacity and flux response (Benard *et al.* 2006).

Taken together, oxidative phosphorylation capacity is highly variable and diverse in tissues. It appaers to be determined by different combinations of the mitochondrial content, the amount of respiratory chain complexes and their intrinsic activity. Tissue specificity of these parameters can partly explain the tissue specificity of mitochondrial deficiencies.

4 MATERIAL AND METHODS

4.1. Tissues and cell lines

Human samples for analyses were obtained from patients with mtDNA microdeletion 9205ΔTA, from three patients harboring two mutations in mt-tRNA^{Lys} (8363G>A and 8344A>G) or one mutation in mt-tRNA$^{Leu(UUR)}$ (3243A>G), as well as from age-related controls. Open muscle biopsies from the tibialis anterior muscle were frozen at -80°C. Post-mortem tissue specimens obtained at autopsy of patients with mtDNA mutations 8363G>A and 3243A>G, and controls were frozen less than 2 h after death. The studies were performed in available stored material from the muscle (tibialis anterior), heart, liver, and brain (frontal cortex). Fibroblast cultures were established from skin biopsies. HEK-293 cells (CRL-1573) were obtained from A.T.C.C. (Manassas VA, U.S.A.).

4.2. Ethics

All studies were carried out in accordance with the Declaration of Helsinki of the World Medical Association and were approved by the Committees of Medical Ethics at all collaborating institutions. Informed consent was obtained from investigated individuals or their parents.

4.3. Plasmid construction

The nucleotide sequences of 33 different candidate miR-30-based shRNAs [shRNAmirs (microRNA-adapted shRNAs)] targeted to COX4I1, COX5A and COX6A1 mRNAs were designed with shRNA Retriever (http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA). The nucleotide sequences of eight different shRNAmirs targeted to the coding sequence of human OXA1L were downloaded from the publicly accessible RNAi Codex database (Olson *et al.* 2006). The corresponding 97-mer oligonucleotides were synthesized (Invitrogen) and used as template sequences for PCR amplification to produce clonable double-stranded products. The corresponding XhoI/EcoRI restriction fragments were inserted downstream of the CMV-GFP-IRESNeo expression cassette of the lentiviral pCMV-GIN-Zeo vector (Open Biosystems). The negative control (non-silencing) shRNAmir pCMV-GIN-Zeo derivative was obtained from Open Biosystems. The coding sequences of COX7A2 (IMAGE ID: 40002220) and COX7B (IMAGE ID: 3861730) were amplified from the respective full-length cDNA clones (ImaGenes), fused to the C-terminal FLAG epitope and cloned (EcoRI/NotI) into the modified pmaxFP-Red-N plasmid (Amaxa). The full length human OXA1L coding sequence (IMAGE ID: 40017377) was amplified and inserted into the C-FLAG fusion mammalian expression vector pCMV-Tag4a (Stratagene). The fidelity of all constructs was confirmed by automated DNA

sequencing. Plasmids pReceiver-M02 (EXC0224) and pReceiver-M13 (EX-C0224) (GeneCopoeia) were used to express Cox6a2 and Cox6a2–FLAG respectively.

4.4. Cell culture, transfections and flow cytometry

Fibroblast cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (Sigma) at 37°C in 5% CO_2. Cells were grown to ~90% confluence and harvested using 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. Detached cells were diluted with an ice-cold culture medium, sedimented by centrifugation and washed twice in PBS (Sigma).

HEK-293 cells were grown at 37°C in a 5% (v/v) CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium (PAA) supplemented with 10% (v/v) fetal bovine serum Gold (PAA). Cell lines stably expressing shRNAmir were prepared using the Nucleofector™ device (Amaxa) and the HEK-293 cell-specific transfection kit. At 48 h after the transfection the cells were split into culture medium containing 720 μg/ml of G418 sulfate (Clontech) and antibiotic-resistant colonies were selected over a period of three weeks. The cells were further maintained in the presence of 720 μg/ml G418.

The GFP fluorescence of stable G418-resistant HEK-293 cells was measured with a FACSCalibur flow cytometer and analyzed using the Cell Quest 3.3 application (Becton Dickinson).

The transient expression of Oxa1l or selected CcO subunits in HEK-293 cells was accomplished using the Lipofectamine 2000 reagent (Invitrogen) or the Express-In Transfection Reagent (Open Biosystems). To obtain optimal accumulation of the CcO subunit polypeptides, the cells were transfected twice consecutively, leading to transgene expression for a total of 4 days.

To assess the efficiency of the shRNAmir constructs interfering with *OXA1* transcripts, HEK-293 cells were co-transfected using a Nucleofector™ device with either one of the eight OXA1L-targeted shRNA constructs or the negative control (non-silencing) shRNA construct and with the OXA1L-FLAG expression construct. At 48 h after the transfection, the cells were lysed and the expression level of the Oxa1l-FLAG fusion protein was examined by immunoblot analysis. The three OXA1L-targeted constructs that contained shRNA 1 (mp_id 433861, 5′-CAAGTTAGCAGGAGACCAT-3′), shRNA 2 (mp_id 19616, 5′-CCTACAACCTGGAAAGGAT-3′) and shRNA 3 (mp_id 19615, 5′- GAGACCATATTGAGTATTA-3′) (RNAi Codex) showed the highest potential to interfere with the expression of Oxa1l-FLAG protein in the transient assay. For the generation of stable transfectants, HEK-293 cells (10^6) were transfected using a Nucleofector™ device with the three functionally validated OXA1L-targeted shRNA constructs, the negative control (non-silencing) shRNA construct and with the empty vector.

4.5. Isolation of mitochondria

Mitochondria were isolated from fibroblasts by a hypo-osmotic shock method (Bentlage *et al.* 1996). The freshly harvested cells were disrupted in 10 mM Tris buffer (pH 7.4) and quickly homogenized in a Teflon/glass homogenizer at 4°C. Sucrose was added to a final concentration of 0.25 M immediately after homogenization. The nuclei were removed by centrifugation for 10 min at 4°C and 600 g and the mitochondrial fraction was isolated from the postnuclear supernatant by centrifugation for 10 min at 4°C and 10 000 g. The mitochondrial pellet was washed and finally resuspended in 0.25 M sucrose, 2 mM EGTA, 40 mM KCl and 20 mM Tris, pH 7.4, and stored at -70°C. Mitoplasts were prepared from fibroblasts as described previously (Klement *et al.* 1995). In brief, trypsinized cells suspended in isotonic STE medium [0.25 M sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1% (w/v) Protease inhibitor cocktail (Sigma)] were treated with digitonin (0.4 mg/mg of protein; Fluka) on ice for 15 min. The suspension was diluted 10-fold with STE and centrifuged for 10 min at 4°C and 12 000 g. The pellet was washed by centrifugation and resuspended in STE to a final concentration of 1–2 mg of protein/ml. Based on immunodetection and enzyme activity measurements, >95% of the mitochondrial inner membrane proteins were recovered in this fraction.

To isolate mitochondria from HEK-293 cells, collected and thawed cells were resuspended in STE buffer, homogenized in a glass/glass homogenizer at 4°C. Unbroken cells and nuclei were removed by centrifugation for 15 min at 4°C and 600 g and the mitochondrial fraction was isolated from the postnuclear supernatant by centrifugation for 25 min at 4°C and 10 000 g. The mitochondrial pellet was washed, resuspended in STE buffer (2.5 volume of the mitochondrial pellet) and stored at -80°C.

Muscle mitochondria were isolated according to (Schagger and von Jagow 1991), but without use of protease. Tissue samples were homogenized at 4°C in a KCl medium (100 mM KCl, 50 mM Tris, 2 mM EDTA, 10 mg/ml aprotinin, pH 7.5). The homogenate was centrifuged for 10 min at 4°C and 600 g, the supernatant was filtered through a 100 μm nylon screen, and mitochondria were sedimented by centrifugation for 10 min at 4°C and 10 000 g. The mitochondrial pellet was washed by centrifugation and resuspended to a final protein concentration of 20-25 mg/ml.

4.6. Immunofluorescence

HEK-293 cells (1.5×10^5) grown on 70 mm^2 glass chamber slides (BD Falcon) were transfected with an OXA1L–FLAG expression construct using the Lipofectamine 2000 reagent. At 24 h after the transfection, the cells were incubated with 200 nM MitoTracker Red CMX Ros (Molecular Probes) for 15 min, and then fixed and permeabilized with PBS, 4% (v/v) paraformaldehyde and 0.1% (v/v) Triton X-100 solutions, respectively. Subsequently, the cells were incubated in PBS containing

10% (w/v) BSA for 1 h at 37°C to block non-specific binding. Immunocytochemical detection was then performed with a monoclonal M2 anti-FLAG antibody (1:1000) and with an anti-mouse IgG$_1$ Alexa Fluor® 488 antibody (1:500).

4.7. Laser scanning confocal microscopy

xyz images sampled according to the Nyquist criterion were acquired using a Nikon Eclipse TE2000 microscope equipped with a C1*si* confocal scanning head and an Apo TIRF 60× (N.A. 1.49) objective. The 488 nm and 543 nm laser lines and appropriate 515±15 and 590±15 nm band pass filter sets were used for excitation and fluorescence detection, respectively. Individual channel images were acquired separately. Images were restored using the measured point spread function (PSF) and the classic maximum likelihood deconvolution algorithm in Huygens Professional Software (SVI).

4.8. Sub-cellular and submitochondrial fractionation

The postnuclear supernatant from 10^7 HEK-293 cells (see Chapter 4.5) was centrifuged at 10 000 g for 25 min to pellet the mitochondria. The resulting supernatant corresponding to the cytoplasmic fraction was collected, and the mitochondrial pellet was washed once with STE buffer. For sonical disruption, isolated mitochondria were adjusted to a protein concentration of 2.5 mg/ml, sonicated and centrifuged at 100 000 g for 30 min. Alkaline sodium carbonate extraction of mitochondrial membranes was performed essentially as described (Fujiki *et al.* 1982).

4.9. Preparation of a polyclonal antibody to human Oxa1l

An Oxa1l-specific antibody was prepared by Open Biosystems. An Oxa1l-specific antiserum was generated by immunizing chicken with a synthetic peptide (KLH-coupled) corresponding to the C-terminal part of human Oxa1l (CKPKSKYPWHDT). The polyclonal antibody to human Oxa1l was affinity-purified from the total IgY with the respective peptide-packed column. The specificity of the produced antibody was tested by immunodetection of the Oxa1l-FLAG fusion protein.

4.10. Electrophoresis and Western blot analysis

In cultured skin fibroblasts, BN-PAGE (Schagger and von Jagow 1991) was used for the separation of native mitochondrial OXPHOS complexes on 6–15% (w/v) polyacrylamide gradient minigels (Mini Protean system; Bio-Rad) as described previously (Klement *et al.* 1995). Mitoplasts were pelleted by centrifugation for 10 min at 4°C and 10 000 g, and solubilized using 1 g of DDM/g of protein for 20 min on ice in a buffer containing 1.75 M aminocaproic acid, 2 mM EDTA and 75 mM Bis-Tris (pH 7.0). Samples were centrifuged for 20 min at 20 000 g and Serva Blue G dye was added

to collected supernatants at a concentration of 0.1 g/g of detergent. Electrophoresis was performed at 50 V for 30 min and then at 90 V. SDS/PAGE (Schagger and von Jagow 1987) was performed on 10% (w/v) polyacrylamide slab minigels, and analysis of [^{35}S] methionine-labelled proteins was performed on a 16 cm long 15-20% (w/v) gradient polyacrylamide slab gels (Protean system; Bio-Rad). The samples were boiled for 3 min in sample lysis buffer [2% (v/v) mercaptoethanol, 4% (w/v) SDS, 10 mM Tris/HCl, 10% (v/v) glycerol]. For 2D analysis, strips of the first-dimension BN-PAGE gel were incubated for 1 h in 1% (w/v) SDS and 1% (v/v) mercaptoethanol and then subjected to SDS/PAGE (10% polyacrylamide) for separation in the second dimension (Schagger and von Jagow 1987). Gels were blotted onto Hybond C-extra nitrocellulose membranes (Amersham) by semi-dry electrotransfer for 1 h at 0.8 mA/cm^2 and the membrane was blocked in PBS containing 0.2% (v/v) Tween 20. The membranes were used whole or were cut according to molecular mass markers into portions containing individual OXPHOS complexes or their subunits. Membranes were incubated for 3 h with primary antibodies diluted in PBS containing 2% (w/v) BSA. Previously characterized polyclonal antibodies were used at the indicated titres: those against the F_oc subunit of ATPase (1:900) (Houstek *et al.* 1995a) and those against the F_oa (ATP6) subunit of ATPase (1:500) (Dubot *et al.* 2004). In addition, we used monoclonal antibodies against complex IV subunits Cox1 (1:330), Cox4 (1:670) and Cox6c (1:200), complex I subunit NADH39 (1:250) and the 70 kDa flavoprotein subunit of SDH (SDHA) (1:2000), all obtained from Molecular Probes, as well as subunit F_1-α of ATPase (1:200 000 (Moradi-Ameli and Godinot 1983). Incubation with peroxidase-labelled secondary antibodies in PBS with 2% (w/v) BSA was performed for 1 h using either goat anti-mouse IgG (1:1000; Sigma A8924) or goat anti-rabbit IgG (1:1000; Sigma F0382). The chemiluminescence reaction with an ECL® kit (Amersham) was detected on an LAS 1000 instrument (Fuji) and the signal was quantified using Aida 2.11 Image Analyzer software.

In bioptic or autoptic tissues, proteins were electroblotted from the gels onto Immobilon™-P PVDF membranes (Millipore) using semi-dry transfer for 60-90 min at a constant current of 0.6-0.8 mA/cm^2. Membranes were air-dried overnight, rinsed twice with 100% (v/v) methanol, and blocked in TBS and 10% (w/v) non-fat dried milk for 1-2 h. Primary detection of BN/PAGE-blots was performed with mouse monoclonal antibodies raised against the complex I subunit NDUFA9 (2 μg/ml), ATP synthase subunit F_1-α (2-3 μg/ml), complex III subunit Core2 (0.5 μg/ml), complex IV subunit Cox2 (0.5–1 μg/ml), and complex II subunit 70 kDa protein (1 μg/ ml) (Mitosciences), at indicated dilutions. Primary detection of two-dimensional BN/SDS/PAGE-blots for the CcO assembly was performed as described previously (Stiburek *et al.* 2005). Blots were incubated with primary antibodies in TBS, 0.3% (v/v) Tween 20 and 2% non-fat

dried milk for 2 h. Secondary detection was carried out with a goat anti-mouse IgG-horseradish peroxidase conjugate (1:1000–1:4000; Sigma A8924) in TBS, 0.1% Tween 20 and 2% non-fat dried milk, for 1 h. The immunoblots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Signal acquisition was performed using either a VersaDoc 4000 imaging system (Bio-Rad Laboratories) or Kodak BioMax Light films (Eastman Kodak Co.). Digital images were analyzed using the Quantity One application (Bio-Rad Laboratories). All blotting experiments were repeated with independently isolated mitochondrial samples. Duplicate experiments yielded consistent results.

In HEK-293 cells, protein sample preparations, electrophoresis, immunoblot analysis and signal acquisition for SDS, BN (blue native) and BN/SDS/PAGE immunoblot analysis were performed essentially as described above for bioptic and autoptic tissues. Monoclonal antibodies against the mitochondrial outer membrane protein porin, CcO subunits Cox1, Cox2, Cox4, Cox5a, Cox6a1 and Cox6c, the 70 kDa flavoprotein subunit of SDH (SDHA), complex III subunit Core2, complex I subunits NDUFA9 and NDUFB6, complex V subunits F_1-α, F_1-β, subunit d and OSCP, and pyruvate dehydrogenase (PDH) subunit E2 were obtained from Mitosciences. The mouse monoclonal antibody to mtHSP70 was from Alexis Biochemicals. Polyclonal antibodies to α-tubulin and eIF2α were from Cell Signaling Technology, and the monoclonal anti-FLAG antibody was from Sigma. For analysis of respiratory supercomplexes by BN/SDS/PAGE and BN/BN/PAGE, isolated mitochondria were extracted using digitonin (detergent/protein ratio of 6). Primary detection of BN/SDS/PAGE and BN/BN/PAGE immunoblots was performed with mouse monoclonal antibodies (Mitosciences) raised against the complex I subunit NDUFB6, complex III subunit Core1 and complex IV subunit Cox1. The first dimension gel strips for BN/BN/PAGE immunodetection were soaked in cathode buffer containing 0.1% DDM for 15 min and then in cathode buffer containing 0.02% DDM for another 15 min. The second dimension of BN-PAGE separation was performed in the presence of 0.02% DDM as described in (Wittig *et al.* 2006).

4.11. Immunoprecipitation of Oxa1l–FLAG protein

HEK-293 cells (~10^6) were transiently (48 h) transfected with the Oxa1l–FLAG expression construct, lysed with a buffer containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA and 50 mM Tris/HCl (pH 7.4), and the lysate was incubated for 6 h at 4°C with previously washed 50 μl of an ANTI-FLAG® M2 affinity agarose resin (flagipt-1; Sigma). Subsequently, the resin was washed five times with a buffer containing 150 mM NaCl, 50 mM Tris/HCl (pH 7.4), and the bound protein was eluted by competition with 3× FLAG peptide. Finally, the eluted immunoprecipitate was combined with SDS sample buffer and resolved using SDS/PAGE.

4.12. Spectrophotometric assays

The activities of respiratory chain complexes were measured spectrophotometrically with a UV-2401PC instrument (Shimadzu). The activities of the mitochondrial enzymes NADH:coenzyme Q_{10} reductase (NQR, complex I), succinate:coenzyme Q_{10} reductase (SQR, complex II), succinate:cytochrome c reductase (SCCR, complex II+III), NADH:cytochrome c reductase (NCCR, complex I+III), coenzyme Q_{10}:cytochrome c reductase (QCCR, complex III), and cytochrome c oxidase (COX, CcO, complex IV) were measured spectrophotometrically by standard methods at 37°C in muscle homogenate and isolated muscle mitochondria (Fischer *et al.* 1986; Makinen and Lee 1968) or in cultured fibroblasts (Chowdhury *et al.* 2000). In HEK-293 cells, rotenone-sensitive complex I activity (NADH:ubiquinone oxidoreductase) was measured by incubating 45 μg of mitochondrial protein in 1 ml of assay medium (50 mMTris/HCl, pH 8.1, 2.5 mg/ml BSA, 50 μM decylubiquinone, 0.3 mM KCN and 0.1 mM NADH without or with 3 μM rotenone). The decrease in absorbance at 340 nm due to the NADH oxidation was followed. Complex II activity (succinate:2,6-dichloroindophenol oxidoreductase) was measured by incubating 20 μg of mitochondrial extract in 1 ml of assay medium (10 mM potassium phosphate, pH 7.8, 2 mM EDTA, 1 mg/ml BSA, 0.3 mM KCN, 10 mM succinate, 3 μM rotenone, 0.2 mM ATP, 80 μM 2,6-dichloroindophenol, 1 μM antimycin and 50 μM decylubiquinone). The decrease in absorbance at 600 nm due to the reduction of 2,6-dichloroindophenol was monitored. CcO activity was measured by incubating 15–18 μg of mitochondrial protein in 1 ml of assay medium (40 mM potassium phosphate, pH 7.0, 1 mg/ml BSA, 25 μM reduced cytochrome c and 2.5 mM DDM) and the oxidation of cytochrome c (II) at 550 nm was monitored. All assays were performed at 37°C. The total protein was determined using the method of Lowry (Lowry *et al.* 1951).

Aurovertin-sensitive ATP hydrolytic activity was measured in a ATP-regenerating system as described in (Baracca *et al.* 1989). Mitochondria (8-22 μg of protein/ml) were incubated in a medium containing 40 mM Tris (pH 7.4), 5 mM $MgCl_2$, 10 mM KCl, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 1 μg/ml rotenone, 0.1% (w/v) BSA, 5 units of pyruvate kinase and 5 units of lactate dehydrogenase for 2 min. The reaction was started by the addition of 1 mM ATP and the rate of NADH oxidation, equimolar to ATP hydrolysis, was monitored as the decrease in absorbance at 340 nm. Sensitivity to aurovertin was determined by parallel measurements in the presence of 2 μM inhibitor.

4.13. High-resolution oxygraphy

Oxygen consumption in cultured fibroblasts was determined at 30°C as described in (Chowdhury *et al.* 2000; Pecina *et al.* 2003) using an Oxygraph-2k (Oroboros). Freshly harvested fibroblasts were suspended in a KCl medium (80 mM KCl, 10 mM Tris/HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.4) and cells

were permeabilized by digitonin (0.1 mg of detergent/mg of protein). Respiratory substrates and inhibitors were used at the concentrations indicated. Oxygen consumption was expressed in pmol of oxygen/s per mg of protein. CcO activity was measured with 5 mM ascorbate and 1 mM TMPD, and was corrected for substrate autooxidation (determined as oxygen uptake insensitive to 0.33 mM KCN).

Muscle fibers were separated mechanically according to (Kunz *et al.* 1993), and oxygen consumption by saponin-skinned muscle fibers was determined using multiple substrate inhibitor titrations as described (Wenchich *et al.* 2003).

The oxygen consumption of digitonin-permeabilized HEK-293 cells was measured at 30°C using an OROBOROS Oxygraph (Anton Paar) in a medium containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 110 mM sucrose, 1 g/l BSA, 20 mM Hepes (pH 7.1). The measurements were carried out in the presence of 30-75 μg/ml of digitonin, 2.5 μM antimycin A, 2 mM ascorbate, 500 μM TMPD and 0.5-1.5 μM FCCP. Respiration was inhibited by the addition of sodium azide to a final concentration of 10 mM. The P_{50} (partial pressure of oxygen at the half-maximal respiration rate) value was measured in the presence of 0.5 μM FCCP and 10 mM succinate essentially as described in (Pecina *et al.* 2004). All measurements were performed independently three to six times for each cell line.

4.14. Cytofluorimetric analysis of mitochondrial membrane potential

Freshly harvested fibroblasts were resuspended in 80 mM KCl, 10 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate and 10 mM succinate at a protein concentration of 1 mg/ml. Cells (~10^4 per each measurement) were permeabilized by 0.1 mg digitonin per mg protein (Sigma) and stained with 20 nM TMRM (Molecular Probes) for 15 min at room temperature. ADP, inhibitors, or both were added at indicated concentrations 1 min before cytofluorometric measurements, which were performed as described elsewhere (Floryk and Houstek 1999) on a FACSort flow cytometer (Becton Dickinson) equipped with an argon laser, 488 nm. The TMRM signal was analyzed in the FL2 channel, equipped with a band pass filter 585±21 nm; the photomultiplier value of the detector was at 631 V in FL2. Data were acquired in log scale using CellQuest (Becton Dickinson) and analyzed with WinMDI 2.8 software (TSRI). Arithmetic mean values of the fluorescence signal in arbitrary units were determined for each sample for the purpose of subsequent graphic representation.

4.15. ATP synthesis

The rate of ATP synthesis was measured at 37°C in 150 mM KCl, 25 mM Tris/HCl, 10 mM potassium phosphate, 2 mM EDTA and 1% (w/v) BSA, pH 7.2, using 0.5 mM ADP and 10 mM succinate or 10 mM pyruvate + 10 mM malate (or

ketoglutarate + malate) as substrate, as described previously (Wanders *et al.* 1996). Protein concentration was 1 mg/ml. For permeabilization of fibroblasts and cybrids, 0.1 and 0.03 mg of digitonin/mg of protein, respectively, was used. The reaction was started by addition of the cells and performed for the indicated time intervals. Reaction mixture aliquots of 200 μl were added to 200 μl of DMSO, and ATP content was determined in DMSO-quenched samples by a luciferase assay according to (Ouhabi *et al.* 1998). ATP production was expressed in nmol of ATP/min per mg of protein.

4.16. Biosynthesis of mitochondrial proteins

Growth medium was removed from cultured fibroblasts, and the cells were rinsed with methionine-free medium without serum (Gibco medium 21013; 1 mM pyruvate, 2 mM glutamine and 30 mg/l cysteine) and incubated in the same medium containing 10% (v/v) dialysed fetal calf serum and 100 μg/ml emetine for 10 min. The cells were labelled for 3 h with 300 μCi/ml L-[^{35}S] methionine, as described in (Chomyn 1996). The products were separated by 15-20% (w/v) polyacrylamide gradient SDS-PAGE. A small aliquot of the samples prepared for electrophoresis was used to measure the total incorporation of radioactivity in the mitochondrial fraction as trichloroacetic acid-precipitable counts. The radioactivity of proteins was quantified in dried gels using a BAS-5000 system (Fuji). Labelled proteins were identified according to their molecular mass as reported previously in *ex vivo* translation assays (Chomyn 1996).

4.17. Protein determination

The protein content was measured by the Bradford or Micro BCA protein kit assays (Bio-Rad Laboratories), using BSA as a standard. Samples were sonicated for 20 s prior to protein determination.

4.18. DNA analysis and sequencing

Total genomic DNA from muscle and cultured fibroblasts was isolated by phenol extraction. The entire mtDNA was amplified in six overlapping fragments by PCR (7-3148, 2073-5719, 5645-8815, 8403-11 132, 11 005-14 684 and 13 863-136). Purified fragments were sequenced on the automatic sequencer ALF Express II (Amersham Biosciences) using cycle sequencing with 41 Cy5-labelled internal sequencing primers or on an AbiPrism 3100 Avant Genetic Analyzer (Applied Biosystems).

4.19. Restriction analysis

To determine the amount of mtDNA mutations, PCR/RFLP (restriction fragment length polymorphism) analysis method was performed. 9205delTA microdeletion was quentified according to (Chrzanowska-Lightowlers *et al.* 2004) using the mismatched (bold) primers 5′-CCT CTA CCT GCA CGA CAA **TGC** A-3′ (forward) and 5′-CGT

TATG**CAT**TGGAAGTGAAATCA C-3′ (reverse), which introduce two novel NsiI restriction sites in the case of the wild-type mtDNA and one NsiI restriction site in the case of mutant mtDNA. PCR products were radioactively labelled with [α-^{32}P]dCTP in the final cycle of PCR and run on a non-denaturing 13% (w/v) polyacrylamide gel after complete digestion. To quantify 8363G>A mutation, PCR products (8279–8485) were radioactively labelled with [α-^{32}P]dCTP in the final cycle of PCR, and run on a non-denaturing 10% (w/v) polyacrylamide gel after complete digestion with TspRI (New England BioLabs). The mutation abolishes one of two TspRI restriction sites on the fragment. The levels of heteroplasmy of the prevalent 3243A>G and 8344A>G mutations were determined as described elsewhere (Brantova *et al.* 2006). The proportions of wild-type to mutant mtDNA were measured using PhosphorImager and ImageQuant software (Molecular Dynamics).

4.20. Northern blot analysis

Total RNA was isolated from cultured fibroblasts by phenol/guanidium thiocyanate/chloroform extraction (Sambrook and Russell 2001). ~20 μg of total RNA per lane was separated through a 1.2% (w/v) agarose/formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham) in 20×SSC (1×SSC is 0.15 M NaCl/ 0.015 M sodium citrate). The membrane was prehybridized for 2 h at 45°C in 5×SSC, Denhardt's solution, 0.5% SDS and 100 μg/ml sonicated herring sperm. The membranes were hybridized overnight at 45°C with [α-^{32}P]dCTP-labelled probes corresponding to regions of the genes ATP6 (8361–9060), COX3 (9269–9912), ND1 (3313–4252) and COX1 (6120–6960). The radioactivity was detected by PhosphorImager and ImageQuant software (Molecular Dynamics).

4.21. Quantitative RT-PCR (reverse transcription–PCR) analysis

Total RNA was isolated from cultured fibroblasts using RNA Blue reagent (Top-Bio, Prague, Czech Republic). Following DNase I treatment (Invitrogen), first-strand cDNA was synthesized from 1 μg RNA aliquots with 200 units SuperScript II reverse transcriptase using either 200 ng of random hexamer primers or 500 ng of oligo(dT)12−18 (all Invitrogen) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed on a LightCycler instrument (Roche Diagnostics) using a QuantiTect SYBR Green PCR kit (Qiagen). PCR reactions were performed on cDNAs using primer pairs specific for the ATP6 gene transcript (forward, 5′-CCT TAT GAG CGG GCA CAG T-3′; reverse, 5′- CAG GGC TAT TGG AA-3′; nt 8846–8994), for the COX3 gene transcript (forward, 5′-GCC CTC TCA GCC CTC CTA ATG-3′; reverse, 5′-GTG GCC TTG GTA TGT GCT TTC TCG-3′; nt 9267–9416), for the ATP6–COX3 gene transcript (forward, 5′- AAT CCA AGC CTA CGT TTT CAC ACT-3′; reverse, 5′-TAG GCC GGA GGT CAT TAG G-3′; nt 9150–9299), for the

CYTB gene transcript (forward, 5′-GACCTCCCCACCCCATCCA-3′; reverse, 5′-AAA GGC GGT TGA GGC GTC TG-3′; nt 14 804– 14 935) and for the ND1 gene transcript (forward, 5′-CAA CCT CAA CCT AGG CCT CCT-3′; reverse, 5′-ACG GCT AGG CTA GAG GTG GC-3′; nt 3595–3644). The primer pair for ATP6–COX3 was designed to flank the splice site of the ATP6–COX3 transcript. Amplified regions of the ATP6 or COX3 transcript were present in both processed and unprocessed RNAs. All reactions were run at an annealing temperature of 60°C. The PCR mixture contained 5 µl of 2×SYBR Green PCR Master Mix, 2 µl of 100×diluted reverse transcription product and 200 nM of each primer in a total volume of 10 µl. All reactions were performed in triplicate. For each primer pair, non-template controls were included to check for the absence of contaminants and primerdimers that would interfere with quantification when SYBR Green is used. The external standard curve was generated in parallel for all reactions using serial dilutions of cDNA synthesized from control RNA. For each sample, the relative amounts of ATP6, COX3, CYTB, ND1 and unprocessed ATP6–COX3 transcripts were determined from the standard curves. Each sample was analyzed in two separate experiments.

Total RNA from HEK-293 cells was isolated using TriReagent solution (MRC). First-strand cDNA was synthesized from 4 µg of total RNA with the use of Superscript III reverse transcriptase (Invitrogen) and Oligo-dT primers (Promega). Pre-amplification and relative quantification was performed according to the manufacturer's instructions (Applied Biosystems). Ten pre-amplification cycles were run with 12.5 µl of cDNA and a 0.05×pooled mixture of eight TaqMan Gene Expression Assays [Hs00971639_m1, COX4I1; Hs00261747_m1, COX4I2; Hs01924685_g1, COX6A1; Hs00193226_g1, COX6A2; Hs00427620_m1, TBP (TATA-box-binding protein); Hs00173304_m1, PPARGC1A (peroxisome-proliferator-activated receptor γ co-activator 1α); Hs00188166_m1, SDHA (succinate dehydrogenase complex subunit A; Hs01082775_m1, TFAM (transcription factor A, mitochondrial)]. Relative quantification was performed in duplicates twice on the 7300 Real-Time PCR System (Applied Biosystems). The transcript levels of all mRNAs were normalized to the level of TBP mRNA. Because the amplification efficiency of the reference and target genes was the same, the comparative $\Delta\Delta C^t$ method was used for relative quantification.

4.22. Statistical analysis

A Student's t test was performed using Microsoft Excel. Results are expressed as mean±S.D. A P value of less than 0.05 was considered as statistically significant, and asterisks are used to denote significance as follows: *$P<0.05$; ** $P<0.01$; *** $P<0.001$.

5 RESULTS AND DISCUSSION

5.1. Characterization of the assembly and function of human nDNA-encoded CcO subunits 4, 5a, 6a, 7a and 7b *(specific aim 1a)*

Fornuskova D, Stiburek L, Wenchich L, Vinsova K, Hansikova H and Zeman J

To study the role of nuclear-encoded cytochrome c oxidase (CcO) structural subunits Cox4, Cox5a and Cox6a as well as the sequence of late events in the CcO assembly pathway, we prepared stable HEK-293 knockdown lines using microRNA-adopted design evoking RNA interference of *COX4*, *COX5A* and *COX6A1*. Furthermore, we used ectopic expression of C-end epitope-tagged Cox6a, Cox7a and Cox7b in wild-type HEK-293 cells and COX6A knockdowns to elucidate entry points of these subunits into the CcO assembly pathway. To estimate functionality and integrity of OXPHOS in context of CcO deficiency, we employed quantitative real-time RT-PCR, isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, spectrophotometric enzyme activity assays, high-resolution respirometry and blue-native (BN), denaturing (SDS) and two-dimensional (BN/SDS or BN/BN) PAGE with downstream immunoblot detections using monoclonal antibodies against various mitochondrial proteins.

Knockdown of Cox4, Cox5a and Cox6a resulted in reduced CcO activity, diminished affinity of the residual enzyme for oxygen, decreased CcO holoenzyme and CcO dimer levels, increased accumulation of CcO subcomplexes and gave rise to an altered pattern of respiratory supercomplexes. An analysis of the patterns of CcO subcomplexes found in both knockdown and overexpressing cells identified a novel CcO assembly intermediate, identified the entry points of three late-assembled subunits and demonstrated directly the essential character as well as the interdependence of the assembly of Cox4 and Cox5a. The ectopic expression of the heart/muscle-specific isoform of the Cox6 subunit (COX6A2) resulted in restoration of both CcO holoenzyme and activity in COX6A1-knockdown cells. This was in sharp contrast with the unaltered levels of *COX6A2* mRNA in these cells suggesting the existence of a fixed expression

programme. The normal amount and function of respiratory complex I in all of our CcO-deficient knockdown cell lines suggest that, unlike non-human CcO-deficient models, even relatively small amounts of CcO can maintain the normal biogenesis of respiratory complex I in cultured human cells.

I contributed fundamentally to this study by designing the research, cloning of pCMV-GIN-ZEO plasmid derivatives coding for candidate shRNAs to achieve the most efficient knockdown, maintaining the HEK-293 cell culture, carrying out expression cloning, preparation of knockdown cell lines and transiently transfected cellular materials, performing mitochondrial isolations and all Western blot analyses and by writing the paper.

5.2. Characterization of the biochemical properties of OXA1L, the human homologue of the yeast mitochondrial Oxa1 translocase, and study of its role for CcO biogenesis *(specific aim 1b)*

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

Knockdown of human Oxa1l impairs the biogenesis of F_1F_o-ATP synthase and NADH:ubiquinone oxidoreductase.

We addressed the role of human Oxa1l in the biogenesis of oxidative phosphorylation system.

To study the molecular role and biochemical properties of human OXA1L gene product, we employed expression cloning and propagation of plasmid constructs in bacteria, rabbit OXA1L antibody design and preparation, immunocytochemistry, confocal microscopy, co-immunoprecipitation as well as subcellular and submitochondrial fractionation and localization. To estimate functionality and integrity of OXPHOS in context of Oxa1l deficiency, we prepared stable human HEK-293 knockdown lines using human microRNA-adopted design evoking RNA interference. The obtained material was analyzed with use of isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, FACS analysis, spectrophotometric measurements of specific enzyme activities, high-resolution respirometry and blue-native (BN), denaturing (SDS) and two-dimensional (BN/SDS) PAGE with downstream immunoblot detections using monoclonal or polyclonal antibodies against various mitochondrial proteins.

The Oxa1 protein is a 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. Here 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 HEK-293 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 HEK-293 cells resulted in markedly decreased steady-state levels and ATP hydrolytic activity of the 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 complex III. Taken together, our results indicate that human Oxa1l represents a mitochondrial integral membrane protein required for the correct biogenesis of ATP synthase and complex I.

I contributed to this study by assisting in research design, by cloning of pCMV-GIN-ZEO plasmid derivatives coding for candidate shRNAs to achieve the most efficient knockdown and by performing the part of electrophoretic and immunoblot analyses.

5.3. Analysis of the tissue-specific effects of mt-tRNA point mutations in patients affected by Leigh syndrome (8363G>A), MERRF syndrome (8344A>G), and MELAS syndrome (3243A>G) on the steady-state levels and activity of OXPHOS complexes *(specific aim 2a)*

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

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

Numerous studies have characterized the molecular mechanisms of point mutations in mitochondrial tRNA genes *in vitro*, but less was known how these mutations affect the amount and stability of OXPHOS complexes in human tissues. We have characterized the tissue- and gene-specific impact of 8363G>A, 8344A>G and 3243A>G mutations affecting mt-tRNA^{Lys} and mt-tRNA$^{Leu(UUR)}$ on OXPHOS complexes in various tissues of patients carrying these mutations with clinical phenotypes of Leigh, MERRF and MELAS syndromes.

We used spectrophotometric measurements of specific enzyme activities, high-resolution respirometry and isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation to perform blue-native (BN) and two-dimensional (BN/SDS) PAGE with downstream immunoblot detections using monoclonal antibodies against various mitochondrial proteins.

In skeletal muscle mitochondria, 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 designing the research, carrying out the part of one-dimensional and two-dimensional electrophoretic and immunoblot analyses, and by writing the paper.

5.4. Study of molecular and biochemical impact of mtDNA mutation in the ATP6 gene (9205ΔTA) on the biosynthesis of ATPase subunit a and its structural and functional consequences *(specific aim 2b)*

Jesina P, Tesarova M, **Fornuskova D**, Vojtiskova A, Pecina P, Kaplanova V, Hansikova H, Zeman J and Houstek J

Diminished synthesis of subunit a (ATP6) and altered function of ATP synthase and cytochrome c oxidase due to the mtDNA 2 bp microdeletion of TA at positions 9205 and 9206.

In patient with combined deficiency of complexes IV and V, we identified and studied rare mtDNA mutation in the ATP6 gene – a 2bp microdeletion at positions 9205 and 9206 (9205ΔTA). This mutation cancels the STOP codon of ATP6 gene and changes the cleavage site between the *ATP6* and *COX3* transcripts.

To unravel the molecular basis of combined cytochrome c oxidase and ATP synthase deficiency in patient with fatal infantile encephalopathy, severe psychomotor delay, frontal lobe atrophy and lactic acidosis, we used mtDNA sequencing. The amount of mtDNA containing the microdeletion was determined by the PCR/RFLP analysis in available tissues of the patient and his relatives. The functionality and integrity of OXPHOS, especially of complexes IV and V, was studied using isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, spectrophotometric measurements of specific enzyme activities, rate of ATP synthesis, quantitative real-time RT-PCR and Northern blot analyses, assay for biosynthesis of mitochondrial proteins, high-resolution respirometry, cytofluorimetric analysis of mitochondrial membrane potential, and blue-native (BN), denaturing (SDS) and two-dimensional (BN/SDS) PAGE with downstream immunoblot detections using monoclonal or polyclonal antibodies against various mitochondrial proteins.

The mutation was present at increasing load in a three-generation family (in blood: grandmother - 16%, mother - 82%, proband - >98%). In the affected boy with severe encephalopathy, a homoplasmic mutation was present in blood, fibroblasts and muscle. The fibroblasts from the patient showed normal aurovertin-sensitive ATPase hydrolytic activity, a 70 % decrease in ATP synthesis and an 85 % decrease in CcO activity.

ADP-stimulated respiration and the ADP-induced decrease in the mitochondrial membrane potential at state 4 were decreased by 50 %. The content of subunit a was decreased 10-fold compared with other ATP synthase subunits, and [^{35}S] methionine labelling showed a 9-fold decrease in subunit a biosynthesis. The content of CcO subunits 1, 4 and 6c was decreased by 30–60 %. Northern Blot and quantitative real-time reverse transcription–PCR analysis further demonstrated that the primary *ATP6-COX3* transcript is cleaved to the *ATP6* and *COX3* mRNAs 2-3-fold less efficiently. Structural studies by blue-native and two-dimensional electrophoresis revealed an altered pattern of CcO assembly and instability of the ATP synthase complex, which dissociated into subcomplexes. The results indicate that the 9205ΔTA mutation prevents the synthesis of ATP synthase subunit a, and causes the formation of incomplete ATP synthase complexes that are capable of ATP hydrolysis but not ATP synthesis. The mutation also affects the biogenesis of CcO, which is present in a decreased amount in cells from affected individuals.

I contributed to this study by identification of the 9205ΔTA mutation in mitochondrial genome of the patient and his relatives.

Vojtiskova A, Jesina P, Kalous M, Kaplanova V, Houstek J, Tesarova M, **Fornuskova D**, Zeman J, Dubot A and Godinot C

Mitochondrial membrane potential and ATP production in primary disorders of ATP synthase.

Toxicol Mech Methods 2004; 14: 7-11.

We summarize the functional consequences of primary ATP synthase defects resulting from 8993T>G, 9205ΔTA and 8527A>G mutations in the ATP6 gene at the level of mitochondrial ATP synthesis and maintenance as well as discharge of the mitochondrial membrane potential.

Studies of fibroblasts with primary defects in mitochondrial ATP synthase due to heteroplasmic mtDNA mutations in the *ATP6* gene, affecting protonophoric function or synthesis of subunit a, show that at high mutation loads, mitochondrial membrane potential $\Delta\Psi_m$ at state 4 is normal, but ADP-induced discharge of $\Delta\Psi_m$ is impaired and ATP synthesis at state 3-ADP is decreased. Increased $\Delta\Psi_m$ and low ATP synthesis is also found when the ATP synthase content is diminished by altered biogenesis of the enzyme complex. Irrespective of the different pathogenic mechanisms, elevated $\Delta\Psi_m$ in

primary ATP synthase disorders could increase mitochondrial production of reactive oxygen species and decrease energy provision.

I contributed to this study by molecular analysis and preparation of cultured skin fibroblasts derived from patients.

6   CONCLUSIONS

- Whereas nDNA-encoded CcO subunits Cox4 and Cox5a are required for the assembly of the functional CcO complex, the Cox6a subunit is required for the overall stability of the holoenzyme. Consequently, the heterogeneous CcO population of Cox6a-deficient cells exhibits higher residual respiration at low oxygen levels than the various CcO forms found in COX5A-KD cells. The description of a novel assembly intermediate at the very last step of CcO assembly suggests additional regulatory level of the process.

- The fact that the ectopic expression of heart/muscle-specific isoform of Cox6a can complement the CcO defect in COX6A1-KD cells is in sharp contrast with unaltered levels of this isoform in our CcO-deficient model, and suggests the existence of a fixed differentiation programme regarding human Cox6a isoforms. The normal amount and function of complex I in all of our CcO-deficient cell lines suggest that even relatively small residual amounts of CcO can maintain normal biogenesis of this respiratory complex in human cells.

- The RNAi knockdown of OXA1L in HEK-293 cells showed that the protein plays an important role in the biogenesis of 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 complex III, suggesting functional divergence during evolution.

- In skeletal muscle tissue, comparably high mutant loads (~ 90%) of 3243A>G affecting mt-tRNA$^{Leu(UUR)}$ and 8344A>G affecting mt-tRNA^{Lys} have been associated with severe defect of complex I, but only 8344A>G mutation also resulted in severe deficiency of complex IV. Similarly, 80% heteroplasmy of 8363G>A mutation affecting mt-tRNA^{Lys} resulted in combined severe deficiency of complexes I and IV. Virtually the same patterns of OXPHOS holoenzyme deficiencies were observed in heart mitochondrial samples. However, the patterns of OXPHOS deficiencies in frontal cortex mitochondria of 8363G>A and 3243A>G patients differed substantially from those of other tissues. This difference was particularly striking for ATP synthase. Although it is necessary to analyze considerably more samples with high levels of heteroplasmy (such samples are difficult to obtain), effects of mt-tRNA mutations on the brain OXPHOS are likely to be particularly different from those described for skeletal muscle, heart, and liver tissues.

- The mechanism by which the 9205ΔTA mutation affects mitochondrial function is associated with changes in the transcription of the ATP6 and COX3 genes and their translational competence and efficacy. The decrease in the amount of the mature *ATP6* transcript agreed well with the decreased synthesis and content of subunit a. Interestingly, the labelling of subunit 8 was increased, indicating up-regulated translation of the ATP8 gene, which precedes and partially overlaps the ATP6 gene. The translation of the *ATP8* and *ATP6* mRNAs is well described in yeast, but the structure of these genes and its regulation differs completely from that in mammalian mitochondria, where the mechanism of ATP8 and ATP6 biosynthesis is largely unknown. The question arises whether increased labelling of subunit 8 could be caused by translation of *ATP8* from an unspliced form of the *ATP8-ATP6-COX3* transcript, part of which is, according to our results, polyadenylated [cDNA synthesis with random primers and oligo(dT) primer] and could be therefore subjected to translation.

- Based on the cytofluorometric studies in cultured skin fibroblasts from patients with complex V defects, higher physiological levels of $\Delta\Psi_m$ can be expected because of low amount of enzyme or altered function of the F_o proton channel. Consequently, increased and unbalanced ROS production, rather than diminished energy provision, would be the key pathogenic process in primary ATP synthase diseases.

7 SUMMARY

In this PhD thesis, we utilized molecular approaches to manipulating the gene expression in human HEK-293 cell line and rare autoptic/bioptic tissue materials or cultured skin fibroblasts from patients with mitochondrial disorders to deal with molecular, biochemical and functional aspects of OXPHOS deficiencies.

We prepared stable HEK-293 cell lines with downregulated expression of selected structural CcO subunits (Cox4i1, Cox5a and Cox6a1) and OXA1L gene to study new aspects of the CcO assembly pathway. The obtained knockdown samples in combination with ectopic expression of C-end epitope-tagged Cox6a, Cox7a and Cox7b in wild-type HEK-293 cells and knockdown cell lines allowed us to elucidate early and very late events of CcO assembly and let us to propose new scheme of human CcO holoenzyme assembly pathway. Based on the study of OXA1L knockdown material, we showed that OXA1L is to a great extent expendable, unlike the loss of OXA1 in yeast, for CcO biogenesis.

Due to unique collection of tissues from patients with comparably high heteroplasmy of mt-tRNA mutations resulting in MELAS, MERRF and Leigh syndromes, we found that in skeletal muscle of the patients, the impact of mt-tRNA mutations seems to be gene-specific, whereas tissue-specificity of OXPHOS deficiency patterns was found among different tissues of the patients. Furthermore, we clarified at molecular and biochemical level a mechanism by which very rare mutation 9205ΔTA affects mitochondrial function. The data on complex V deficient cultured skin fibroblasts contributes to the growing idea that insufficient supply of ATP to meet cellular needs is not necessarily the only factor decisive for pathogenic processes in primary ATPase diseases but also increased and/or unbalanced ROS production might be underlying.

The data presented in this PhD thesis were published in 5 scientific journals and were reported at specialized national or international meetings.

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9.1. Journal articles

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9.2. Abstracts in journals

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