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**The role of nuclear-encoded subunits of cytochrome *c* oxidase in mitochondrial
metabolism**

Úloha jaderně kódovaných podjednotek cytochrom *c* oxidázy v mitochondriálním
metabolismu

Doctoral thesis

Supervisor: Mgr. Petr Pecina, Ph.D.

Prague, 2022

Prohlášení

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Abstract

Mitochondria, ‘the powerhouses of the cell’, house the integral metabolism pathway of oxidative phosphorylation to produce the majority of cellular energy. Mammalian cytochrome *c* oxidase, also called complex IV (cIV), is indispensable for the overall oxidative phosphorylation function as the terminal oxidase, and for its regulation to sustain energetic needs. Since cIV is a multimeric enzyme composed of subunits encoded by nuclear and mitochondrial genomes, its biogenesis is a complicated process, which needs to be coordinated to complete a fully functional complex. Further, the setup of individual nuclear-encoded subunits isoforms of cIV may fine-tune cIV function based on the tissue or the environment context. Despite the physiological and pathological relevance of cIV composition, biogenesis, and the secondary deficiencies triggered by cIV defects, nuclear-encoded subunits’ function remains poorly understood.

At first, mammalian COX4 subunit isoforms with tissue- and oxygen-dependent expression were studied in the HEK293 cellular model with an exclusive expression of COX4I1 or COX4I2 isoform. Remarkably, the COX4I2 isozyme showed lower affinity to oxygen, which may imply regulation of cIV activity under hypoxia, and is of physiological relevance for the oxygen-sensing mechanism.

Further, analysis of the cIV assembly process utilizing COX4 and COX6B lacking cells approved the indisputable role of the COX4 subunit in the cIV assembly initiation. Unexpectedly, COX6B subunit absence led to complete cIV deficiency with the MT-CO1 module being preserved. This reflects COX6B’s importance not only in the later stages of assembly but also in the stabilization of early assembly intermediates. Moreover, results obtained in this study supported the non-canonical assembly route of cIV subunits directly into the respirasome, which still represents a highly uncharacterized process.

Finally, COX4 and COX6B lacking cells served as ideal models to explore the interdependence of cIV and complex I maintenance, and the respective signaling. cIV deficiency triggered complex I content decrease, presumably through the mitochondrial protein synthesis attenuation. Functional replacement of cIV by alternative oxidase in cIV deficient cells improved its phenotype, indicating functional rather than a structural trigger

of cIV and cI interdependence. Mitochondrial integrated stress response was active in cIV deficient models, being a highly probable signaling pathway in interdependence.

To summarize, the present study explored the role of cIV subunits COX4 and COX6B on multiple levels of its impact on cIV itself, and the oxidative phosphorylation system in general.

Keywords

oxidative phosphorylation, cytochrome *c* oxidase, COX4, COX6B, isoforms, assembly, supercomplexes, interdependence, integrated stress response

Abstrakt

V mitochondriích, takzvaných ‘buněčných elektrárnách’, probíhá integrální metabolická dráha oxidační fosforylace, jež v buňkách zajišťuje produkci většiny energie. Savčí cytochrom *c* oxidáza, neboli cIV, je nezbytnou terminální oxidázou pro funkci systému oxidační fosforylace, ale také pro regulaci celého procesu k zajištění energie za daných podmínek. cIV je proteinový komplex sestavený z podjednotek kódovaných jaderným i mitochondriálním genomem, proto jeho biogeneze představuje komplikovaný proces vyžadující koordinaci mnoha dějů nezbytných k sestavení plně funkčního enzymu. Mimo to, sestavení cIV z jednotlivých isoform jaderně-kódovaných podjednotek umožňuje funkční nastavení enzymu v závislosti na daných podmínkách. Navzdory dokladům o důležitosti správného sestavení cIV za fyziologických i patologických podmínek, úloha jaderně-kódovaných podjednotek není stále zcela objasněna.

V této práci byly nejprve studovány isoformy podjednotky COX4, které vykazují expresi závislou na tkáni a koncentraci kyslíku. Pro tento účel byly využity HEK293 buněčné modely s výhradní expresí samotných isoform COX4I1 nebo COX4I2. Zajímavým výsledkem bylo zjištění, že COX4I2 izozym vykazoval nižší afinitu ke kyslíku, což může hrát významnou roli v regulaci aktivity cIV za hypoxie a v mechanismu detekce koncentrace kyslíku při fyziologických procesech.

Dále byla prozkoumána úloha podjednotek COX4 a COX6B v procesu sestavování cIV. Pro podjednotku COX4 byla potvrzena role v iniciaci celého procesu, zatímco studium COX6B podjednotky přineslo neočekávané výsledky. Bylo ukázáno, že podjednotka COX6B nehraje roli pouze v pozdních fázích sestavování, jak se dříve předpokládalo, ale rovněž ve stabilizaci časných intermediátů. To bylo prokázáno úplnou deficiencí kompletního cIV při zachování MT-CO1 modulu. Další získané výsledky podporují hypotézu o nestandardním sestavování podjednotek cIV přímo do respirazómu, jež stále představuje velmi neprobádaný proces.

Nakonec buněčné modely s deficitem COX4 a COX6B podjednotek posloužily jako ideální modely ke studiu fenoménu vzájemné závislosti mezi cIV a komplexem I, jakožto i signalizace zajišťující celý proces. Deficience cIV vedla ke snížení komplexu I,

pravděpodobně v důsledku útlumu mitochondriální syntézy proteinů. Funkční nahrazení cIV alternativní oxidázou vedlo ke zlepšení fenotypu, což ukázalo že fenomén vzájemné závislosti je vyvolán změnou funkcí systému oxidační fosforylace, nikoli chybějící strukturní interakcí v důsledku deficitu cIV. V buňkách s deficitem cIV byla vyvolána mitochondriální integrovaná odpověď na stres, která představuje pravděpodobnou signalizační dráhu zajišťující vzájemnou závislost komplexů oxidační fosforylace.

Závěrem, tato práce prozkoumala roli podjednotek COX4 a COX6B cytochrom *c* oxidázy na několika úrovních regulace jak samotné cytochrom *c* oxidázy, tak celého systému oxidační fosforylace.

Klíčová slova

oxidační fosforylace, cytochrom *c* oxidáza, COX4, COX6B, isoformy, asemblace, superkomplexy, vzájemná závislost, integrovaná odpověď na stres

List of Abbreviations

AOX	alternative terminal oxidase
ATP	adenosine triphosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
cI	NADH dehydrogenase
cII	succinate dehydrogenase
cIII	cytochrome <i>bc₁</i> complex
cIV	cytochrome c oxidase
CoQ	coenzyme Q
cV	ATP synthase
ECAR	extracellular acidification rate
FADH	flavin adenine dinucleotide
gRNA	guide RNA
HEK293	human embryonic kidney cell line
HIF-1	hypoxia inducible factor-1
HREs	hypoxia-response elements
IMM	inner mitochondrial membrane
IMS	intermembrane space
ISR	integrated stress response
kDa	kilodaltons
KI	knock-in
KO	knock-out

LFQ-MS	label-free quantification mass spectrometry
MITRAC	mitochondrial translation regulation assembly intermediate of COX
MS	mass spectrometry
mtDNA	mitochondrial deoxynucleic acid
NADH	nicotinamide adenine dinucleotide
nDNA	nuclear deoxynucleic acid
OCR	oxygen consumption rate
OMM	outer mitochondrial membrane
ORE	oxygen responsive element
OXPHOS	oxidative phosphorylation
PAMSCs	pulmonary arterial muscle cells
RC	respiratory chain
RET	reverse electron transfer
ROS	reactive oxygen species
SC	supercomplex
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
WB	western blot
wt	wild type

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1. Introduction

1.1. Oxidative phosphorylation system

Mitochondria are popularly known as ‘the powerhouses of the cell’ because their main role is the synthesis of adenosine triphosphate (ATP), the principal energy-storing molecule, by the oxidative phosphorylation system (OXPHOS) embedded in the inner mitochondrial membrane (IMM). OXPHOS consists of respiratory chain (RC) complexes I (NADH dehydrogenase, cI), II (succinate dehydrogenase, cII), III (cytochrome *bc₁* complex, cIII), and IV (cytochrome *c* oxidase, cIV), plus the complex V (ATP synthase, cV) (Figure 1). Complexes I and II oxidize reduced substrates (NADH and succinate respectively) and transfer electrons through the electron carrier coenzyme Q (CoQ) to cIII. cIII donates electrons to the other mobile electron carrier cytochrome *c*, which shuttles electrons to cIV, the terminal respiratory chain oxidase. cI, cIII, and cIV couple transfer of electrons with proton translocation across the IMM, building the electrochemical (‘proton’) gradient exploited by cV to synthesize ATP (Figure 1).

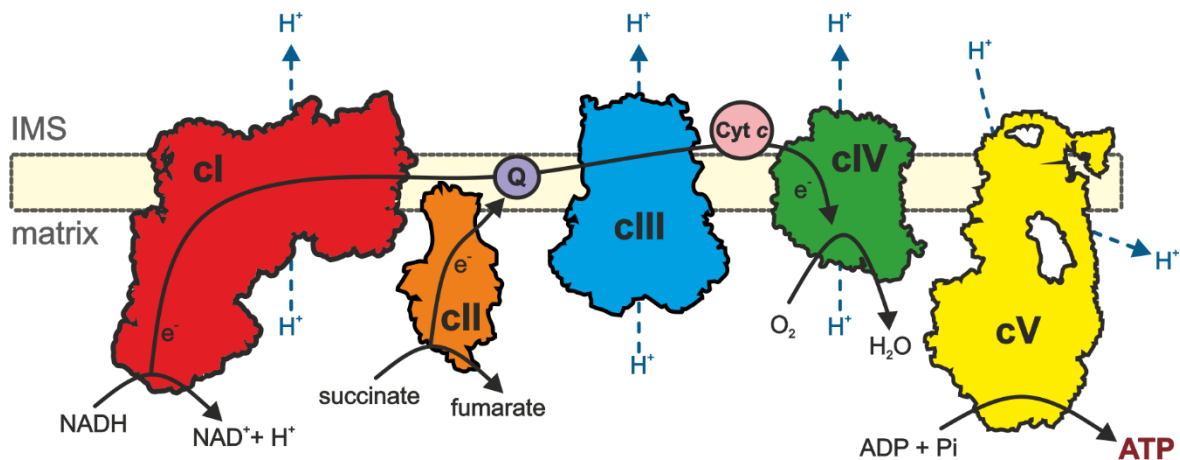


Figure 1. Scheme of the oxidative phosphorylation system function.

Individual OXPHOS complexes are depicted: cI (in red), cII (in orange), cIII (in blue), cIV (in green), and cV (in yellow). Also, electron carriers are shown: coenzyme Q (Q, in violet) and cytochrome *c* (Cyt *c*, in pink).

1.2. Biogenesis of OXPHOS complexes and RC supercomplexes

The biogenesis of individual multimeric OXPHOS enzymes, consisting of up to forty-five subunits in the case of cI (Stroud et al. 2016), is an extremely complex process. Yet another layer of complexity arises from the dual genetic origin of individual subunits. Despite

the transfer of most mitochondrial genes into the nucleus during evolution, thirteen structural subunits (seven of cI, one of cIII, three of cIV, and two of cV) are still encoded in human mitochondrial DNA (mtDNA). The rest of the OXPHOS structural subunits and factors, essential for their biogenesis and activity, are encoded by the nuclear DNA (nDNA) (D'Souza and Minczuk 2018; Signes and Fernandez-Vizarra 2018). Therefore, the expression of individual subunits in both, cytosol and mitochondria (Hallberg and Larsson 2014; Kummer and Ban 2021; Rackham and Filipovska 2022), needs to be strictly coordinated according to the assembly process of OXPHOS complexes to avoid the accumulation of unfolded proteins and nonfunctional assembly intermediates (Figure 2) (Cagin and Enriquez 2015; Dennerlein et al. 2017).

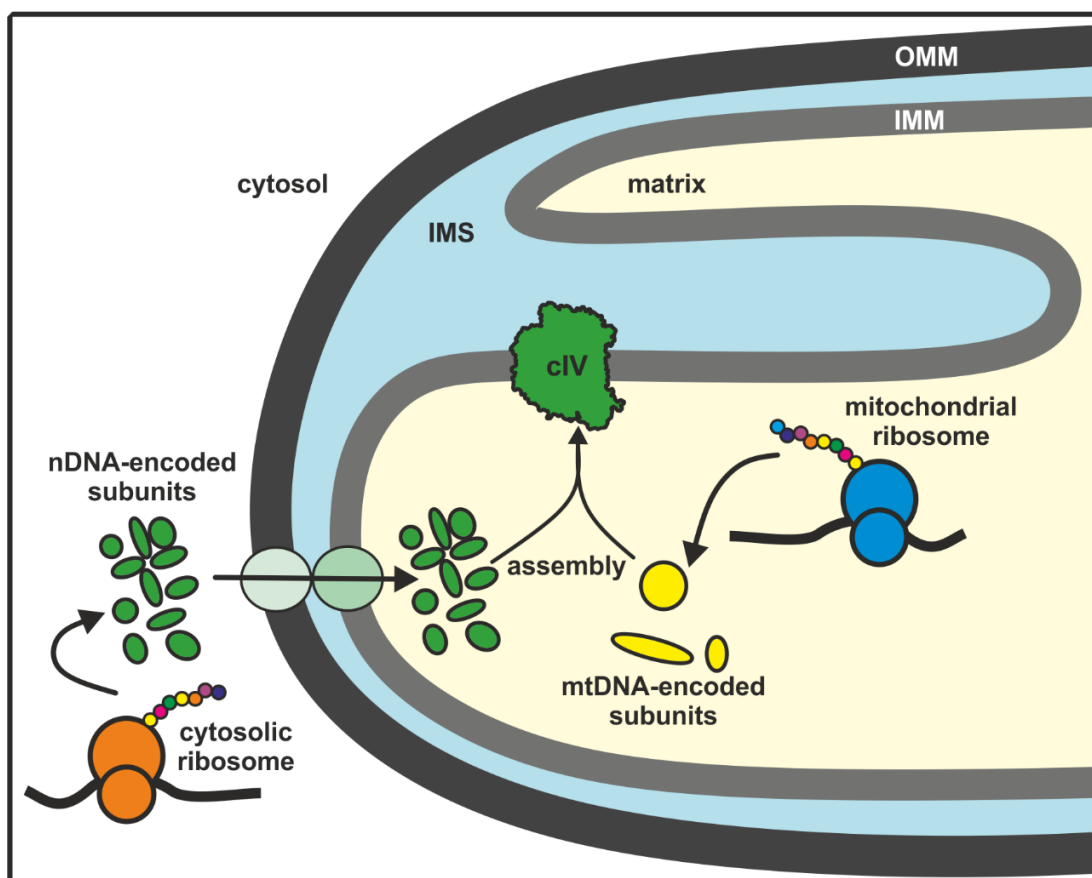


Figure 2. Scheme of the biogenesis of respiratory chain (RC) complexes.

The figure depicts the complexity of the process of RC complexes assembly, which requires synchronization of expression of two independent genomes, the nuclear and mitochondrial ones. Both, cytosolic and mitochondrial translation programs need to synthesize individual subunits of RC complexes to successfully assemble RC complex into fully functional enzyme (cIV, in green, as an example). OMM (outer mitochondrial membrane); IMS (intermembrane space); IMM (inner mitochondrial membrane).

Many processes supported by various assembly factors are indispensable for the *de novo* assembly of individual complexes and alterations in these processes may manifest as human pathology (Fernandez-Vizarra and Zeviani 2021). For example, mutations in the assembly factors of cIV (SURF1, SCO2) are the most common cause of cIV inherited defects (Pecina et al., 2004). Moreover, besides *de novo* assembly, the incorporation of newly imported nuclear-encoded subunits into complete holoenzyme was observed in the case of cI and cIV (Lazarou et al. 2007; Lazarou et al. 2009). Even further, proteolytic ‘recycling’ of the whole matrix-facing domain (N-module) of cI, which is more prone to oxidative damage compared to the rest of the complex, was discovered to maintain functional cI without the need to assemble the complete complex *de novo* (Szczepanowska et al. 2020).

Another regulatory layer in RC complexes biogenesis is represented by the supramolecular structures formed by the combination of cI, cIII, and cIV, so-called supercomplexes (SC) (Schagger and Pfeiffer 2000). In mammalian mitochondria, respirasome (SC I III₂IV) (Althoff et al. 2011; Gu et al. 2016; Letts et al. 2016), SC I III₂ (Letts et al. 2019), SC III₂IV (Vercellino and Sazanov 2021) and the speculative megacomplex (SC I₂III₂IV₂) (Guo et al. 2017) were characterized by cryo-EM. Although several potential roles for SCs were proposed, including improved efficiency of electron transfer through channeling, decrease in ROS production, or involvement in stabilization of individual complexes, the function of SCs remains enigmatic (Milenkovic et al. 2017; Vercellino and Sazanov 2022).

Despite the unclear physiological function, two possible models of SCs assembly were proposed. One of the hypotheses considered the grouping of the complete individual complexes to form SCs (Figure 3A) (Acín-Pérez et al. 2008). The second, and more recent one, presumes the possibility that SCs can be assembled prior to the individual complexes completion (Figure 3B), thus by joining assembly intermediates of RC complexes (Moreno-Lastres et al. 2012). The perspective of simultaneous assembly of individual RC complexes and supercomplexes was supported by further experimental evidence, when the remaining cIV subunits were shown to be connected with SC I III₂ in MT-CO2 (catalytic core subunit of cIV) lacking human cell line without a completely assembled cIV monomer (Lobo-Jarne et al. 2020). Alternatively, a recent study demonstrated that general cIII absence in the human

cell line affects the assembly process of cI (Protasoni et al. 2020). These findings brought a new perspective on the SCs' role not only in the RC complexes stabilization but also as an assembly platform for individual RC complexes.

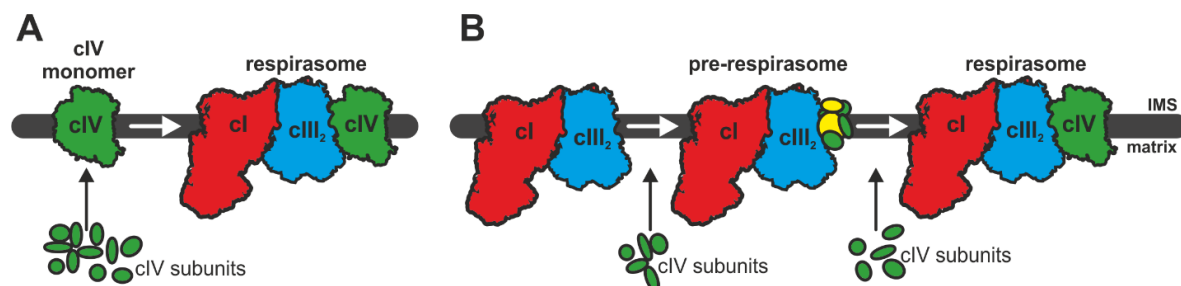


Figure 3. Biogenesis of respiratory chain supercomplexes.

The scheme shows two possible assembly lines for the formation of RC supercomplex, respirasome, composed of one copy of cI, two copies of cIII, and one copy of cIV.

A) Assembly of individual cIV before binding into the respirasome.

B) Simultaneous process of assembly of the individual complex IV and the respirasome.

1.3. Interdependence of respiratory chain complexes

In the past, the major focus was turned on the interplay of individual RC complexes in their function. Then, it has been repeatedly shown that the aberration of one of the complexes may alter the composition and content of the other complexes as well. Thus, RC complexes are not interdependent only by their function, but also by their biogenesis and maintenance.

The relation between cI and cIII represents a widely studied example of RC complexes interdependence. Originally, non-reciprocal cI decrease caused by cIII deficiency was perceived as an outcome of destabilization of cI under cIII absence (Acin-Perez et al. 2004). Later, it was explained as a result of the oxidative damage triggered by an increase of the reduced CoQ to total CoQ (CoQH₂/CoQ) ratio, causing reverse electron transfer (RET) (Guarás et al. 2016). Correspondingly, it was suggested that an increase in reactive oxygen species production alters the assembly or stability of cI and supercomplexes, which leads to the cI level decrease in the model of cIII deficiency (Diaz et al. 2012). However, a recent contradictory study explained cI decrease and accumulation of an inactive 'pre-cI' (without N-module) by disruption of its assembly process due to the lack of fully assembled cIII, which further translates into the absence of all SCs containing cI and cIII (Protasoni et al.

2020). Further, the authors questioned the cI decrease due to the oxidative instability triggered by the high CoQH₂/CoQ ratio, since pharmacological cIII inhibition (antimycin A for 7 days) did not yield a cI decrease (Protasoni et al. 2020).

Moreover, it has been observed that also cIV deficiency diminishes the level of assembled cI, but only in the cases of total cIV absence triggered by early-stage assembly defect (Diaz et al. 2006; Guarás et al. 2016; Hornig-Do et al. 2012; Li et al. 2007). Mild deficiencies of cIV, preserving a residual portion of complete cIV, did not present with cI interdependence (Timon-Gomez et al. 2018). Interestingly, a recent study described that even an incomplete MT-CO1 module of cIV (containing MT-CO1, COX5B, COX7A2 subunits of cIV and HIGD2A) in MT-CO2 deficient cybrid cells can attach to and stabilize SC I III₂, proposing a possibility of an alternative assembly pathway of cIV directly into the SC (Lobo-Jarne et al. 2020). However, it is still not clear, whether the cI level decreases due to impaired cI assembly (Diaz et al. 2006; Li et al. 2007), or the reduced stability of cI triggered by a severe reduction of MT-CO1 (Guarás et al. 2016; Hornig-Do et al. 2012; Lobo-Jarne et al. 2020).

Another possible explanation of the interdependence between cI and cIV biogenesis may involve the MITRAC complex. CIV biogenesis is regulated by assembly-controlled translational plasticity - without the nuclear-encoded subunits of the cIV early assembly stage, translation of the MT-CO1 subunit is stopped, and MT-CO1 occurs as a ribosome-nascent chain complex containing MITRAC component C12ORF62. Thus, new cIV is not assembled and mitochondrial ribosomes may preferentially bind MT-CO1 transcript (Richter-Dennerlein *et al.*, 2016). Moreover, MITRAC15, another component of the MITRAC complex, which also shows ribosome association, is required for the assembly of both complexes, cIV, and cI (Mick *et al.*, 2012).

To sum up, the true nature of cI and cIV interdependence is not fully understood yet, while at the same time represents an important phenomenon underlying combined respiratory defects found in patients suffering from mitochondrial disorders (Fernandez-Vizarra and Zeviani 2021).

1.4. Mitochondrial stress signaling endorses respiratory chain interdependence

As already mentioned, nuclear and mitochondrial expression programs need to be coordinated in the cells to sustain the physiological function of the OXPHOS apparatus. On top of that, specific signaling pathways developed in mammals to mediate the inter-organellar crosstalk under the stress due to the various types of mitochondrial dysfunctions. Such signaling could hypothetically lead to the alteration of nuclear-encoded mitochondrial proteins content, and secondary OXPHOS deficiency – underlying the interdependence.

1.4.1. AMPK-mTORC1 signaling axis

According to the current state of knowledge, energetic deprivation of the cells combined with the higher reactive oxygen species (ROS) production leads to the activation of AMP-dependent kinase (AMPK), which gets phosphorylated and inhibits mTORC1 (mechanistic target of rapamycin complex 1), the nutrient, energy and redox sensor controlling cytosolic protein synthesis (Condon et al. 2021; Hinchey et al. 2018). Active mTORC1 efficiently phosphorylates its main downstream targets, eIF4EBP1 and S6K, therefore supports the initiation of cytosolic translation by releasing eIF4E (eukaryotic initiation factor 4E), to control the expression of the nuclear-encoded mitochondrial components, including the mitochondrial ribosomal proteins (Morita et al. 2013). Thus, AMPK – mTORC1 signaling leading to the attenuation of cytosolic protein synthesis might mediate primary OXPHOS complex shortage towards the secondary OXPHOS deficiency (Figure 4A).

1.4.2. The mitochondrial integrated stress response

In the past, a novel pathway sensing mitochondrial stress in mammals had been characterized. This so-called mitochondrial integrated stress response (ISR) (Figure 4B) can alter the nuclear expression program and for example modulates signaling under mitochondrial deficiencies (Bao et al. 2016; Martínez-Reyes et al. 2012; Quiros et al. 2017; Ryan et al. 2021; Sasaki et al. 2020; Silva et al. 2009). In human cells, activating transcription factor 4 (ATF4), the key ISR player, was described to respond to the variety of mitochondrial stress conditions interconnected by the decrease of the mitochondrial membrane potential (Quiros et al. 2017). Increased ATF4 was shown to activate the expression of many ISR target proteins to reprogram the cellular metabolism to overcome mitochondrial dysfunction

(Bao et al. 2016; Quiros et al. 2017; Ryan et al. 2021). Surprisingly, among the ATF4 targets, even eIF4EBP1 protein has been identified (Bao et al. 2016; Guo et al. 2020; Yamaguchi et al. 2008). eIF4EBP1 binds the eukaryotic translation initiation factor eIF4E to prevent the initiation of cap-dependent cytosolic translation (Haghighat et al. 1995). Importantly, besides the activation of ATF4, cells undergoing mitochondrial stress presented a decrease in the mitochondrial ribosomal proteins content coupled with the attenuation of mitochondrial translation (Quiros et al. 2017). However, the trigger of the molecular mechanism of mitochondrial ISR remained unknown.

Recently, two independent studies (Fessler et al. 2020; Guo et al. 2020) described the role of the OMA1-DELE1-HRI signaling pathway in the initiation of mitochondrial ISR. Stress-induced mitochondrial protease OMA1, possibly due to the decrease in the mitochondrial membrane potential, was found to cleave IMM-bound DELE1 protein, which delocalizes from mitochondria to the cytosol to activate HRI kinase (Fessler et al. 2020; Guo et al. 2020). HRI kinase then phosphorylates eIF2 α that on one side inhibits cytosolic synthesis of most proteins, and on the other hand enhances ATF4 translation (reviewed by Pakos-Zebrucka et al. 2016).

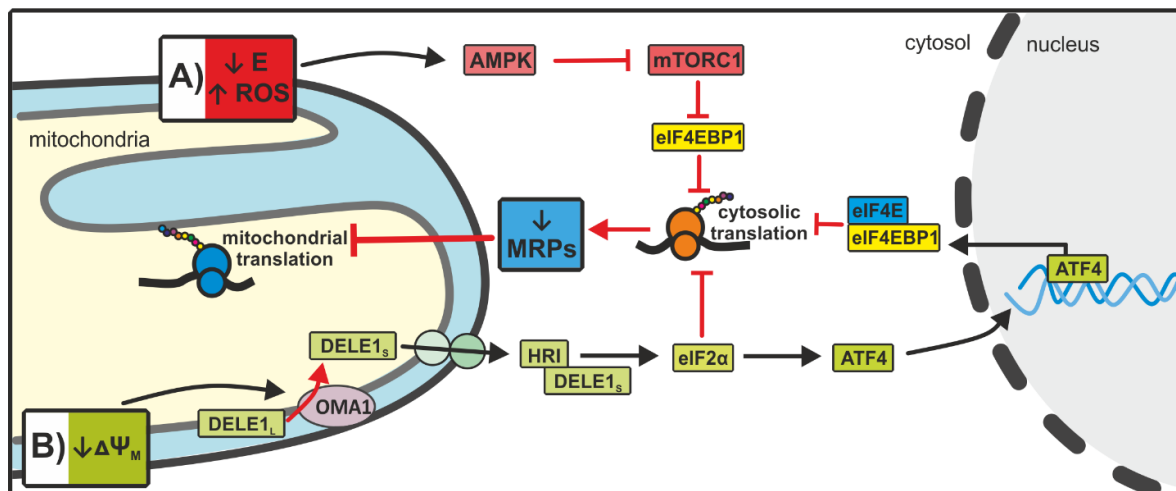


Figure 4. Scheme of the mitochondrial stress signaling.

The scheme shows the signaling pathways from the mitochondrial stressor towards the attenuation of mitochondrial protein synthesis. See the text for the description.

(A) AMPK-mTORC1 signaling axis.

(B) Mitochondrial integrated stress response.

Therefore, ISR represents a possible signaling pathway interconnecting primary OXPHOS deficiency with the mitochondrial protein synthesis attenuation, resulting in a secondary OXPHOS complex deficiency. However, future studies are necessary to fully comprehend the complexity of signaling needed to adapt mitochondrial metabolism under OXPHOS deficiencies.

1.5. Cytochrome *c* oxidase structure and function

Cytochrome *c* oxidase (cIV, COX), the terminal enzyme of RC, accepts electrons from cytochrome *c* and catalyzes the reduction of the terminal electron acceptor, the oxygen, to form water molecules (Figure 5). Further, cIV contributes to the electrochemical (‘proton’) gradient formation. The importance of cIV in mammalian RC is not given only by its irreplaceable function as the terminal RC oxidase, but also by cIV role in the regulation of the entire RC function, predisposed by a large decrease of Gibbs free energy during cIV turnover (Villani and Attardi 2000).

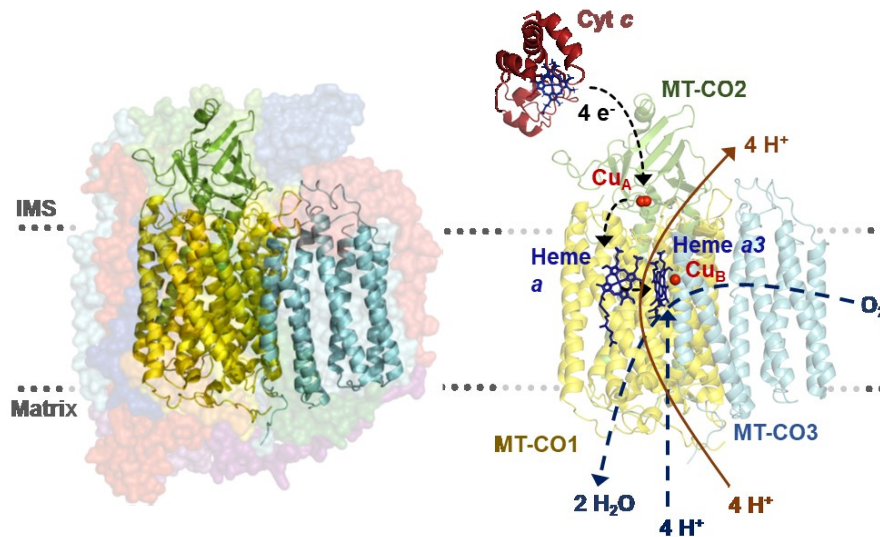


Figure 5. cIV structure and catalytic function (Čunátova et al. 2020).

Left: Cartoon representation of the core subunits of cIV (MT-CO1 (yellow), MT-CO2 (green), and MT-CO3 (blue)) wrapped by eleven nDNA-encoded subunits (semi-transparent, surface mode).

Right: Catalytic core subunits of cIV (MT-CO1 (yellow), MT-CO2 (green), and MT-CO3 (blue)) and cytochrome *c* (red). The scheme depicts electron transfer from cytochrome *c* (Cyt *c*) through the Cu_A center localized in the MT-CO2 subunit, and Heme *a* to the binuclear center formed by heme *a*₃ and Cu_B centers in the MT-CO1 subunit. In the binuclear center, oxygen is reduced to the water molecules, utilizing protons transferred to the binuclear center (dashed blue lines). Proton pumping across the inner mitochondrial membrane is depicted (orange line). The structure of cIV and cytochrome *c* were prepared using PDB files 5Z62 (cIV) and 2N9J (cytochrome *c*). IMS (intermembrane space).

Mammalian cIV can be found in diverse forms – as a monomer, a dimer, and also taking part in various RC SCs (typically in SC III₂IV, and respirasome - SC I III₂IV). Even though the composition of cIV in its different forms, and various tissues, may differ (Ikeda et al. 2013; Lapuente-Brun et al. 2013; Timon-Gomez et al. 2020; Vercellino and Sazanov 2021), the essential composition remains consistent. cIV monomer consists of three mtDNA-encoded subunits (MT-CO1, MT-CO2, and MT-CO3) that shape a core part of the enzyme responsible for cIV catalytic function, and is wrapped by eleven nDNA-encoded subunits (COX4, COX5A, COX5B, COX6A, COX6B, COX6C, COX7A, COX7B, COX7C, COX8, and NDUFA4) (Kadenbach and Merle 1981; Pitceathly and Taanman 2018; Tsukihara et al. 1996). nDNA-encoded subunits do not directly participate in the catalytic function of cIV but are necessary for its assembly, association within RC SCs, stabilization, and regulation to fine-tune its function according to specific conditions in diverse tissues (Čunátova et al. 2020). Moreover, several proteins were found to associate with cIV, although not being present in cIV monomer regularly. Typically, cIV associated proteins (HIGD1A, MNRR1)

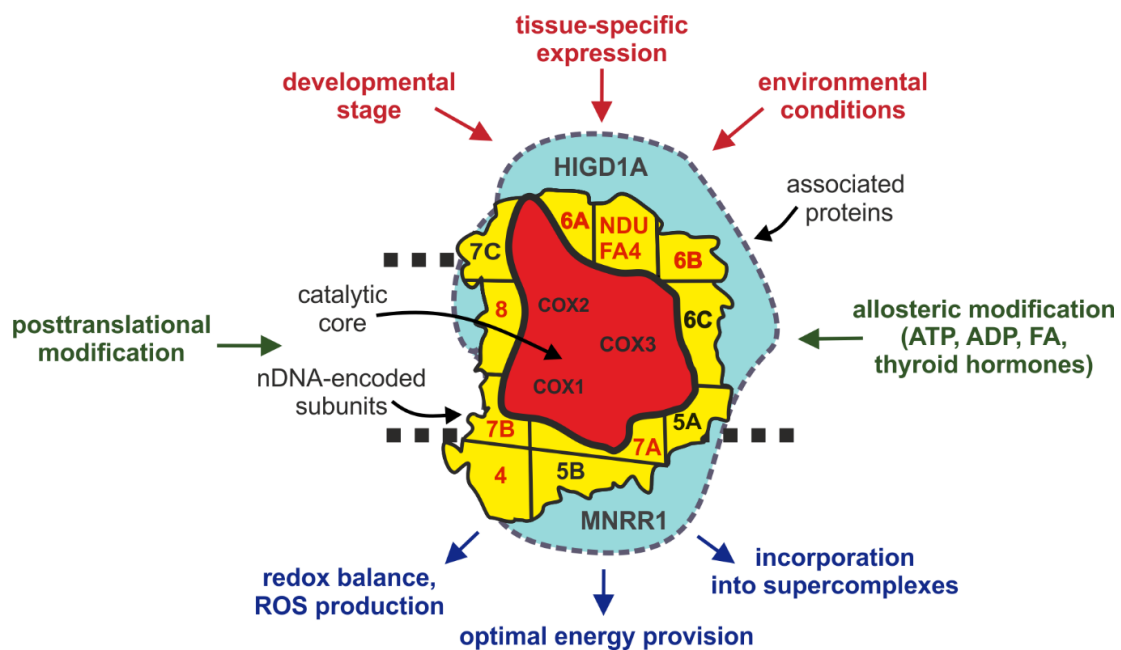


Figure 6. Diagram of three levels of cIV composition (Čunátova et al. 2020).

The first level represents catalytic core subunits (in red). **The second level** presents nDNA-encoded subunits in the stoichiometric ratio (in yellow), with stress on subunits with multiple isoforms (red font). **The third level** shows associating proteins in a sub-stoichiometric ratio (in light blue). Conditions affecting the cIV composition (upper, in red font) and regulatory mechanisms altering the cIV function according to its composition (side, in green) together tune the function and interaction of cIV within other OXPHOS complexes (bottom, dark blue).

are present in sub-stoichiometric ratio compared to the common cIV subunits, and their role can be found in the regulation of cIV catalytic function, or association into SCs (Figure 6) (Aras et al. 2015; Čunátova et al. 2020; Grossman et al. 2017; Hayashi et al. 2015).

1.5.1. Assembly of cytochrome c oxidase

De novo assembly of cIV is a finely regulated process comprising the participation of dozens of nDNA-encoded ‘assembly factors’ indispensable for the completion of a fully active enzyme (Fontanesi et al. 2006). Originally, the process of cIV assembly was perceived as a sequential addition of individual cIV subunits (Figure 7A), defined by rate-limiting steps, which can be documented by the detection of assembly intermediates (Nijtmans et al. 1998). The assembly process begins with MT-CO1 synthesis, representing the S1 intermediate that is extended by COX4 and COX5A subunits that are indispensable for cIV assembly (S2 intermediate). Intermediate S3 is formed after subunits MT-CO2, MT-CO3, COX5B, COX6C, COX7C, and COX8 join. The assembly process continues with the addition of subunits COX7A and COX6B (intermediate S4*) and is finalized by COX6A, forming a complete holoenzyme (Fornuskova et al. 2010).

However, the view of sequential assembly has been challenged by the modular model (Figure 7B), which states that individual modules, and not free subunits, assemble into the complete enzyme in a linear process (Signes and Fernandez-Vizarra 2018; Timón-Gómez et al. 2018; Vidoni et al. 2017). Individual mtDNA-encoded subunits represent cores of the independent modules, and they are accompanied by respective nDNA-encoded subunits in a process secured by a group of specific chaperons and assembly factors. Initially, the MT-CO1 module is formed by the connection of the initiating COX4-COX5A subcomplex with MT-CO1, which matures in the MITRAC complex (mitochondrial translation regulation assembly intermediates of cytochrome *c* oxidase) (Dennerlein and Rehling 2015; Mick et al. 2012; Vidoni et al. 2017). The second module consists of MT-CO2, which undergoes copper insertion assisted by SCO1, SCO2, COX16, and COA6 chaperones, and subunits COX5B, COX6C, COX7B, and COX8A (Aich et al. 2018; Maghool et al. 2019; Pacheu-Grau et al. 2020; Soma et al. 2019; Stiburek et al. 2005). In the end, the third module containing MT-CO3, COX6A, COX6B, and COX7A subunits joins the nascent cIV under the assistance of HIGD2A, and the addition of NDUFA4 finalizes the assembly of complete and fully

functional cIV (Hock et al. 2020; Signes and Fernandez-Vizarra 2018; Timon-Gomez et al. 2020).

Except for *de novo* cIV assembly, incorporation of nDNA-encoded subunits COX6A, COX6B, and COX7A directly into complete cIV monomer, or cIV associating in the SCs, has been suggested (Lazarou et al. 2009). Moreover, subunits COX4, COX6A, COX6B, COX6C, and COX7A preferentially associated with SC I III₂ in patient mitochondria (Lazarou et al. 2009). This finding was verified by other studies presenting the importance of the MT-CO1 subunit to facilitate the incorporation of cIV subunits into SC I III₂ under

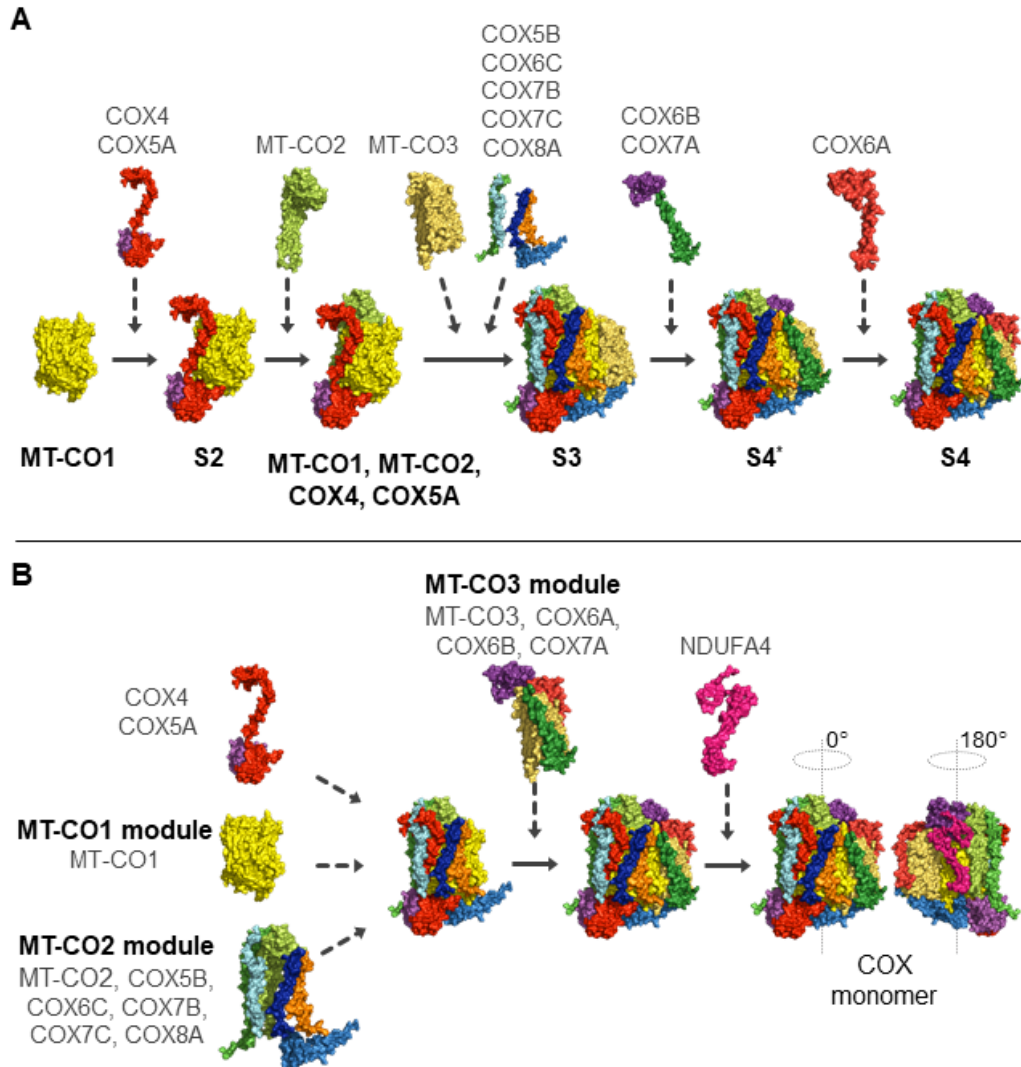


Figure 7. Two alternative views on cIV monomer assembly (Čunátova et al. 2020). Schemes depict the original idea of a sequential assembly of individual cIV subunits (A), and the novel modular model of cIV assembly (B). Structural images were prepared using PDB file 5Z62.

cIV deficiency (Lobo-Jarne et al. 2020), and by the relevance of regulation of this process by the HIGD2A factor (Timon-Gomez et al. 2020).

Although the assembly of mammalian cIV has been widely studied in the past, investigation of diverse cIV deficiency models remains fundamental to fully comprehend the overall process. Further, with the increasing evidence grows the importance to study non-canonical assembly pathways of cIV components directly into the respirasome.

1.5.2. Nuclear-encoded subunits, and their isoforms

Nuclear-encoded subunits represent an evolutionary innovation in the structure and function of eukaryotic cIV (Das et al. 2004; Pierron et al. 2012) and their origin, with a high probability, is not in the mtDNA (Szklarczyk and Huynen 2010). The role of the mammalian nDNA-encoded subunits is not in the enzyme catalysis itself, but rather in the assembly, SCs formation, cIV stabilization, and fine-tuning of the cIV function (Čunátová et al. 2020; Sinkler et al. 2017). In mammals, only four nDNA-encoded subunits out of eleven (COX5A, COX5B, COX6C, and COX7C) are present within a single protein variant. Interestingly, the remaining nDNA-encoded subunits (COX4, COX6A, COX6B, COX7A, COX7B, COX8, and NDUF44) show more than one variant, so-called isoforms, which are encoded by distinct genes that most probably evolved following a gene or whole-genome duplication (Blomme et al. 2006; Little et al. 2010). Subsequently, individual isoforms went through sub-

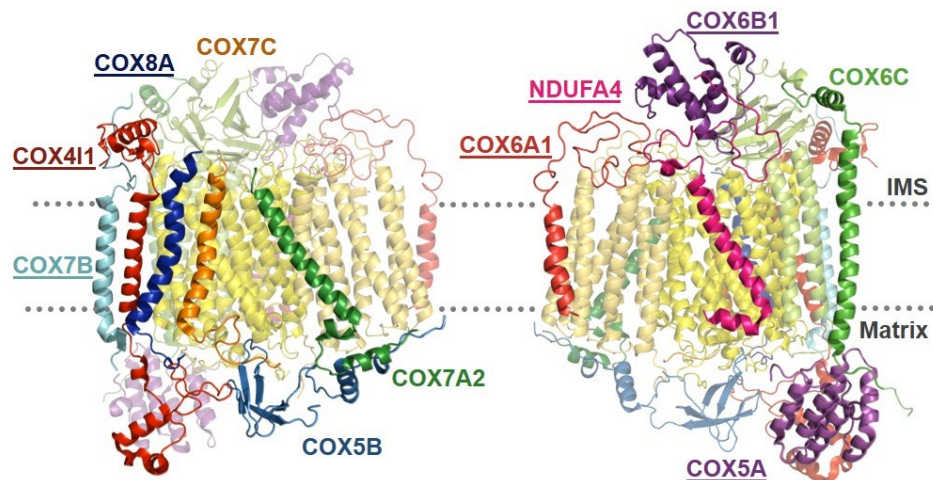


Figure 8. Organization of nuclear-encoded subunits in cIV monomer (Čunátová et al. 2020). The figure shows mutually rotated images of cIV monomer with the individually denoted nDNA-encoded subunits. Human pathologies-associated subunits are stressed (underlined). Structural images were prepared using PDB file 5Z62.

functionalization (Lynch et al. 2001; Pierron et al. 2012), which distinguished their impact on cIV function and maintenance (reviewed by Čunátová et al. 2020; Sinkler et al. 2017). Even though they only indirectly participate in cIV catalytic activity, the importance of nDNA-encoded subunits in the cIV function can be demonstrated by discoveries of rare mutations in six nDNA-encoded subunits causing hereditary human diseases like encephalomyopathy and cardiomyopathy (Figure 8) (reviewed by Čunátová et al. 2020).

1.5.2.1. Role of COX4 subunit and its isoforms

The largest nDNA-encoded subunit COX4 possesses a transmembrane helix neighboring with the catalytic subunit MT-CO1, and a large N-terminal domain-oriented towards mitochondrial matrix (Tsukihara et al. 1996). Moreover, the intermembrane space-facing C-terminal domain of the COX4 subunit has a role in the docking of cytochrome *c* on the binding site formed by MT-CO2 (Sampson and Alleyne 2001). These properties of the COX4 subunit predispose its regulatory function in the modulation of cIV activity and RC function in general. Moreover, the mammalian COX4 subunit is present in two isoforms (Hüttemann et al. 2001), with tissue- and oxygen-specific expression. In mammals, COX4I1 represents an ubiquitously expressed isoform, while the second isoform, COX4I2, expression was found especially in the lung, heart, brain (Hüttemann et al. 2001), and skeletal muscle (Schiffer et al. 2016). Even though the two isoforms share a high level of homology, differences can be found especially in the N-terminus, where the COX4I2 isoform possesses three cysteine residues (Cys40, Cys54, Cys108) hypothetically capable of disulfide bonds formation under oxidative stress (Hüttemann et al. 2001). Therefore, COX4 isoforms represent yet another level to fine-tune cIV function according to specific conditions.

Indeed, the matrix domain of COX4 contains an ATP binding site that senses the energetic status of the cell to adjust the enzyme turnover by allosteric inhibition of cIV function, the so-called ‘second mechanism of respiratory control’ (Arnold and Kadenbach 1997; Bender and Kadenbach 2000; Kadenbach and Arnold 1999; Lee et al. 2002; Lee et al. 2005; Ludwig et al. 2001). Allosteric inhibition can be abolished by phosphorylation of Ser58 by mitochondrial PKA, which is positively regulated by carbonate originating from the Krebs cycle. Therefore, this mechanism serves to coordinate metabolic pathways to the current needs of the cell (Acín-Pérez et al. 2011). However, this regulation has been suggested only

for the COX4I1 isoform, since the homologous region of the COX4I2 isoform contains negatively charged residues disabling ATP binding, and lacks regulating Ser58 residue (Hüttemann et al. 2001).

Expression of COX4I2 isoform was found increased under hypoxia in several mammalian cell types (Aras et al. 2013; Fukuda et al. 2007; Horvat et al. 2006; Hüttemann et al. 2007), which express COX4I1 under the standard partial pressure of oxygen. The molecular mechanism of the hypoxic switch of COX4 isoforms was studied and two hypothetical mechanisms were described. The first one suggests a broadly known pathway of hypoxia-inducible factor 1 (HIF-1), which can bind the hypoxia-response element found in the COX4I2 promoter sequence (Fukuda et al. 2007). The alternative signaling comprises discovered oxygen response element (ORE), also found in the COX4I2 promoter sequence, which binds the activators (RBPJ, CHCHD2) for hypoxic activation of COX4I2 gene expression, or the repressor (CXXC5) to attenuate COX4I2 transcription in normoxia (Aras et al. 2013; Hüttemann et al. 2007).

The possible alteration of cIV function based on the COX4 isoform present in the complex has been extensively studied in diverse models. In HeLa and HEK293 cell lines, the hypoxic expression of the COX4I2 isoform led to an increase in cIV activity and mitochondrial ROS production reduction (Fukuda et al. 2007). Correspondingly, higher cIV activity coupled with the presence of COX4I2 isoform was also found in astrocytes, possibly as an outcome of missing regulation by allosteric inhibition (Horvat et al. 2006; Singh et al. 2009). Another study questioned the absence of allosteric inhibition in COX4I2 containing isozyme, but at the same time supported the idea of higher cIV activity with COX4I2 isoform using the COX4I2 knock-out mice model (Hüttemann et al. 2012). On the other hand, a recent publication presented COX4I1 isozyme to be more active in glioblastoma cells (Oliva et al. 2015). Even though many studies have been published on the effect of individual COX4 isoforms on cIV function, their findings are often contradictory, with the absence of basic research including a simplified experimental model with a well-characterized proportion of individual isoforms.

The physiological relevance of COX4 isoforms switch has been in the focus for about a decade. COX4I2 isoform was primarily found crucial for hypoxic pulmonary

vasoconstriction in a study using a model of mouse COX4I2 knock-out (Hüttemann et al. 2012; Sommer et al. 2017). Moreover, glomus cells in the carotid bodies of mice showed higher expression of COX4I2 isoform that was coupled with HIF-2 α -dependent expression increase of another two abnormal isoforms of cIV nDNA-encoded subunits, COX8B and NDUFA4L2. It has been suggested that this atypical composition of cIV works as an oxygen sensor in a process of acute oxygen sensing in carotid bodies (Gao et al. 2017; Moreno-Dominguez et al. 2019). Oxygen sensing in both processes, hypoxic pulmonary vasoconstriction, and acute oxygen sensing, is interconnected with hypoxic redox status changes and reactive oxygen species (ROS) production in mitochondria. However, the molecular mechanism of oxygen sensing triggering the whole signaling was not clarified yet.

1.5.2.2. Role of COX6B subunit and its isoforms

COX6B is a nDNA-encoded subunit exclusively present in the intermembrane space-facing part of cIV, which was perceived as one of the subunits joining cIV assembly in the very late stages (Fornůsková et al. 2010; Hock et al. 2020; Signes and Fernandez-Vizarra 2018; Timon-Gomez et al. 2020). COX6B mediates tight interaction between two monomers of cIV in the dimer and participates in the architecture of the cytochrome *c* binding site (Tsukihara et al. 1996). An initial hint on the COX6B function was provided by experiments showing that its dissociation mediated by mild solubilization from cIV isolated from the bovine heart results in disruption of the dimer, which is coupled to doubled enzyme activity (Kadenbach and Hüttemann 2015; Weishaupt and Kadenbach 1992). Two rare COX6B1 missense mutations (Arg20Cys, Arg20His) identified as a genetic cause of childhood encephalopathies (Abdulhag et al. 2015; Massa et al. 2008), caused destabilization of subunit's interaction with the enzyme. In contrast to the original solubilization experiment, COX6B dissociation resulted in decreased cIV activity, likewise, knock-down of COX6B1 expression manifested as cIV deficiency (Massa et al. 2008). These conflicting data highlight the need for systematic dissection of the COX6B function within the cIV enzyme.

While ubiquitous mammalian isoform COX6B1 occurs in all cell types and tissues, expression of the later identified isoform COX6B2 is strictly restricted to testes (Hüttemann et al. 2003). COX6B2 extends the group of testis-specific mitochondrial proteins, such as the ANT4 isoform of adenine nucleotide translocator (Dahout-Gonzalez 2006) or cytochrome *c*

identified in rodents (Hennig 1975). Given that COX6B participates in the formation of the cytochrome *c* binding site, it is tempting to speculate that these two isoform pairs could have been established by co-evolution. Either way, the testis isoform of cytochrome *c* is not conserved in primate lineage (Hüttemann et al. 2003). The two COX6B isoforms display high similarity, but a striking difference in the primary sequence is the presence of three COX6B2-specific tyrosine residues (Tyr33, Tyr39, Tyr58) that are conserved in mammals. It was speculated that they may represent one of the multiple targets of a global cAMP-dependent signaling burst triggered during sperm capacitation, crucial for the activation of sperm motility (Balbach et al. 2020; Krzyzosiak et al. 2000). Importantly, two independent high-throughput proteomic studies detected a link between decreased COX6B2 content and aberrant sperm motility (Cao et al. 2018). Despite these indications on the importance of COX6B2 for sperm physiology, it has not been explored in detail to date, leaving a significant knowledge gap from the perspectives of both cIV biogenesis/function and male fertility research.

Interestingly, COX6B2 was recently identified in a high-throughput screen as one of the cancer testis antigens, proteins with occurrence normally restricted to testes, whose expression is aberrantly switched on in certain types of cancers (Maxfield et al. 2015). Indeed, expression of COX6B2 was later associated with a worse prognosis of lung adenocarcinoma (Cheng et al. 2020) and prostate cancer ductal carcinoma (Nie et al. 2020). Also, COX6B2 as a putative tyrosine phosphorylation target should be considered - the notorious involvement of kinase mutations driving lung tumorigenesis (Oberndorfer and Mullauer 2018) might be involved in hijacking COX6B2 expression and regulation.

Apparently, the COX6B subunit remains surprisingly poorly characterized, with inconclusive data about COX6B function in cIV maintenance, and extends to the role of the COX6B2 isoform.

2. Aims of the study

The **general aim** of the present thesis was to study the role of nuclear-encoded subunits of cIV, COX4, and COX6B, in the biogenesis and maintenance of RC complexes and mitochondrial metabolism as a whole.

The **specific aims** were to study:

1. how do the COX4 subunit isoforms, COX4I1 and COX4I2, alter cIV structure and function.
2. what is the role of the COX6B1 subunit in cIV biogenesis and maintenance.
3. how does the cIV deficiency, caused by COX4 or COX6B1 knock-out, trigger changes in the other RC complexes biogenesis, the so-called phenomenon of interdependence.

3. List of publications

Research results summarized in the listed publications (two original articles), together with the results included in the thesis, served as the basis of the present thesis. Individual publications are attached at the end of this thesis (Chapter 9).

Loss of COX4I1 Leads to Combined Respiratory Chain Deficiency and Impaired Mitochondrial Protein Synthesis.

Čunátová K, Reguera DP, Vrbacký M, Fernández-Vizarra E, Ding S, Fearnley IM, Zeviani M, Houštěk J, Mráček T, Pecina P., Cells. 2021 Feb 10;10(2):369.

Impact factor: 6,600

Author's contribution: Investigation (cell culture, SDS-PAGE/WB, BN-PAGE/WB, measurement of metabolic fluxes, SILAC/complexome profiling, pulse-chase analysis), original draft preparation, review, editing, and funding acquisition of the study evaluating the impact of COX4 isoforms on function and biogenesis of oxidative phosphorylation system.

Summary: Even though the interdependence of cI on cIV has been studied in the past, and cI level decrease was ascribed to the alteration of cI assembly or stability in cIV deficient cells, the conclusive explanation of this phenomenon was not described yet. To shed more light on the cI and cIV interdependence, we utilized the HEK293 cellular model of COX4 knock-out with total cIV absence, resulted from the blockade of cIV assembly initiation. cIV deficiency was followed by a severe cI deficiency observable at the level of cI subunits content, as well as at the native cI level. All the respiratory chain supercomplexes were lost in the COX4 deficient cells. Metabolic labeling of mtDNA-encoded OXPHOS subunits revealed a decrease in mitochondrial protein synthesis of cIV and cI subunits, which was connected to a decrease in mitochondrial ribosomal proteins. Further, cI assembly intermediates were detected by complexome profiling analysis, suggesting alteration of cI biogenesis.

Cytochrome c Oxidase Subunit 4 Isoform Exchange Results in Modulation of Oxygen Affinity.

Pajuelo Reguera D, Čunátová K, Vrbacký M, Pecinová A, Houštěk J, Mráček T, Pecina P., Cells. 2020 Feb 14;9(2):443.

Impact factor: 6,600

Author's contribution: Investigation (cell culture, preparation of CRISPR knock-out models, SDS-PAGE/WB, BN-PAGE/WB, measurement of metabolic fluxes, measurement of cIV activity), original draft preparation, review, editing, and funding acquisition of the study evaluating the functional role of COX4 isoforms.

Summary: The COX4 subunit has two isoforms, COX4I1 and COX4I2, with an oxygen-dependent expression that possibly fine-tunes cIV and OXPHOS function according to the current conditions. However, the mechanism behind is not fully understood. Therefore, we prepared HEK293 cell line-based models with a sole expression of either COX4I1 or COX4I2 isoform on the knock-out background. Both isoforms were able to restore the cIV defect observed in COX4 lacking cells, while cIV subunits content and native complexes level were comparable between the models. Correspondingly, the activity of cIV, respiratory rate, and affinity to cytochrome *c* did not differ. Interestingly, the preferential mitochondrial ATP production in COX4I2-containing cells and lower oxygen affinity were found, supporting the hypothesis of the COX4I2 isoform's role in oxygen sensing.

During the course of my Ph.D. studies, I also co-authored the below-stated publications, which were not included in the present thesis.

Role of cytochrome c oxidase nuclear-encoded subunits in health and disease.

Čunátová K, Reguera DP, Houštěk J, Mráček T, Pecina P.

Physiological Research. 2020 Dec 22;69(6):947-965.

Impact factor: 1,880

Author's contribution: Original draft preparation, review, and editing of the review article summarizing the function of disease-associated nuclear-encoded subunits of cytochrome *c* oxidase.

TMEM70 facilitates the biogenesis of mammalian ATP synthase by promoting subunit c incorporation into the rotor structure of the enzyme.

Kovalčíková J, Vrbacký M, Pecina P, Tauchmannová K, Nůsková H, Kaplanová V, Brázdová A, Alán L, Eliáš J, Čunátová K, Kořínek V, Sedlacek R, Mráček T, Houštěk J.

FASEB J. 2019 Dec;33(12):14103-14117.

Impact factor: 5,192

Author's contribution: Investigation (cell culture, preparation of CRISPR knock-out model of ATP5F1B utilized in the present thesis).

4. Methods

4.1. Preparation and cultivation of HEK293 cellular models

COX4I1/COX4I2 (4dKO) and ATP5F1B (β KO) HEK293 (ATCC® CRL-1573™) based knock-out (KO) models were generated in our previous studies, as described (Čunátová et al. 2021; Kovalčíková et al. 2019). COX6B1 KO model (6BKO) was established using the commercially available eSpCas9-GFP system (ESPCAS9GFP-1EA, Merck, USA), which shows improved specificity and is less prone to off-targets when compared to wild type Cas9. Single-cell clones were obtained by fluorescence-activated cell sorting (FACS) based on the medium intensity of the GFP signal and individual cell lines were then screened by SDS-PAGE/Western blot (WB) for a total absence of COX6B1 protein, and indel mutations in the CRISPR-Cas9 cleavage site resulting in the premature STOP codon were verified by Sanger sequencing. For a rescue experiment, a pcDNA3.1+ mammalian expression vector (Thermo Fisher Scientific, USA) with human COX6B1 full-length cDNA followed by C-terminal FLAG-tag was transfected into 6BKO (clone 2) using Metafectene Pro (Biontex Laboratories GmbH, Germany). Stably transfected cells (6BKO^{compl}) were selected by 2 mg/mL G418, starting 48h after transfection. For alternative oxidase (AOX) expression in 4dKO, 6BKO (clone 2), and β KO, pcDNA3.1+ mammalian expression vector (Thermo Fisher Scientific) with full-length AOX (from *Aspergillus nidulans*) cDNA followed by C-terminal HA-tag was transfected using Metafectene Pro (Biontex Laboratories GmbH). Stably transfected cells were selected by 1 mg/mL (4dKO, β KO) and 2 mg/mL (6BKO) G418, starting 48h after transfection. HEK293 cells were cultivated at 37 °C and 5% CO₂ atmosphere in DMEM/F-12 medium (Thermo Fisher Scientific, 31331-028) supplemented with 10% (v/v) FBS (Thermo Fisher Scientific, 10270-106), 40 mM HEPES, antibiotics (100 U/mL penicillin + 100 µg/mL streptomycin, Thermo Fisher Scientific, 15140-122) and 50 µM uridine.

4.2. SDS-PAGE

For proteins separation under denaturing conditions, tricine - sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Standard SDS samples were prepared from frozen cellular pellets, as described in detail (Pajuelo Reguera

et al. 2020; Schagger 2006). To examine the phosphorylation status of AMPK α , S6K, S6, and eIF2 α proteins, cells were washed with ice-cold PBS and harvested in ice-cold RIPA buffer (150 mM NaCl, 1 % Nonidet NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8.0) with Phosphatase inhibitor cocktail (1:200, Sigma-Aldrich P5726), ReadyShield® Protease and Phosphatase Inhibitor Cocktail (1:100, Sigma-Aldrich PPC2020) and benzonase (1:1000, Merck). Then, samples were centrifuged (10 000 g, 15 min), supernatant was mixed with SLB buffer (sample lysis buffer; 2 % (v/v) 2-mercaptoethanol, 4 % (w/v) SDS, 50 mM Tris (pH 7.0), 10 % (v/v) glycerol, 0.02 % Coomassie Brilliant Blue R-250) and samples were incubated for 10 min at 65 °C. Samples (20-30 μ g of protein) were separated on 10 % gel (for OPA-1 forms separation) or 12 % gel using the Mini-PROTEAN III apparatus (Bio-Rad, USA). Experiments were performed at least three times in order to assess the statistical significance of the results.

4.3. Native electrophoresis

For the separation of native protein complexes, blue-native (BN-PAGE) electrophoresis (Wittig et al. 2006) was performed. Mitochondrial pellets were isolated from freshly harvested cells by hypotonic shock followed by differential centrifugation (Bentlage et al. 1996), and then isolated mitochondrial pellets were solubilized by mild non-ionic detergent digitonin (6 g detergent / 1 g protein), preventing dissociation of supercomplexes. 30 μ g of finalized samples (Pajuelo Reguera et al. 2020; Wittig et al. 2006) were subjected to 4 – 13 % polyacrylamide gradient gel using Mini-PROTEAN III apparatus (Bio-Rad). Native electrophoresis analysis was performed in at least two independent experiments.

4.4. Western blot and immunodetection

SDS-PAGE or BN-PAGE separated proteins were transferred on polyvinylidene difluoride (PVDF) membrane (Immobilon FL 0,45 μ m, Merck) using semi-dry electroblotting (0.8 mA/cm², 1 hour) in Transblot SD apparatus (Bio-Rad). For immunodetection, as described in detail (Pajuelo Reguera et al. 2020), following primary and secondary antibodies were used: NDUFA9 (Abcam 14713, UK), NDUFB8 (Abcam 110242), NDUF3 (Abcam 110246), SDHA (Abcam 14715), UQCRC2 (Abcam 14745), UQCRC2 (Proteintech 14742-1-AP, USA), MT-CO1 (Abcam 14705), MT-CO2 (Abcam 110258), COX4I1 (Abcam 14744), COX5A (Abcam 110262), COX6B1 (Abcam 110266),

COX6C (Abcam 110267), ATP5F1B (Abcam 14730), ATP5MC1 (Abcam 180149), FLAG (Merck F1804), HA-tag (Thermo Fisher Scientific 26183), MRPL48 (Proteintech 14677-1-AP), ATF4 (Cell Signaling Technology 11815S, USA), eIF4EBP1 (Cell Signaling Technology 9644S), LC3B (Abcam 48394), OPA-1 (BD Biosciences 612606, USA), H3 (Cell Signaling Technology 14269S), AMPK α (Cell Signaling Technology 5831S), P-AMPK α T172 (Cell Signaling Technology 2535S), S6K (Cell Signaling Technology 2708S), S6K T389 (Cell Signaling Technology 9234S), S6 (Santa Cruz Biotechnology 74576), P-S6 S235/236 (Santa Cruz Biotechnology 293144, USA), eIF2 α (Proteintech 20499-1-AP), P-eIF2 α S51 (Cell Signaling Technology 3398S) and citrate synthase (Abcam 129095). Corresponding infra-red fluorescent secondary antibodies (Alexa Fluor 680, Thermo Fisher Scientific; IRDye 800, LICOR Biosciences, USA) were used. The signals were detected on the fluorescence scanner Odyssey (LI-COR Biosciences). The resulting signals were analyzed and quantified by Image Lab software (Bio-Rad).

4.5. Label-free quantification mass spectrometry analysis

Proteomic analysis was performed on a service basis by the Proteomics Service Laboratory at the Institute of Physiology and the Institute of Molecular Genetics of the Czech Academy of Sciences. Label-free quantification mass spectrometry analysis (LFQ-MS) of wt and 6BKO (Figure 1D) was performed in biological triplicate (three clones), technical duplicate. LFQ-MS analysis of wt, 4dKO, 6BKO, and β KO cells, and 4dKO, 6BKO, and β KO cells expressing AOX was performed in technical triplicates. Briefly, cellular pellets (100 μ g of protein) were processed according to the protocol for in-solution trypsin digestion (Čunátová et al. 2021). About 1 μ g of peptide digests were separated on a 50 cm C18 column using 2.5 h elution gradient and analyzed in a DDA mode on Orbitrap Exploris 480 (Thermo Fisher Scientific) mass spectrometer. The resulting raw files were processed in MaxQuant (v. 1.5.3.28) (Tyanova et al. 2016a) with the label-free quantification (LFQ) algorithm MaxLFQ (Cox et al. 2014). Downstream analysis was performed in Perseus (v. 1.5.3.1) (Tyanova et al. 2016b).

4.6. Complexome profiling

Isolated mitochondria from wild type, 6BKO, and 6BKO cells expressing AOX were solubilized by digitonin (6 g detergent / 1 g protein) and samples for BN-PAGE separation

were prepared as described (4.3. Native electrophoresis). 50 µg of finalized samples were subjected to 4 – 13 % polyacrylamide gradient gel using Mini-PROTEAN III apparatus (Bio-Rad). The gel was fixed (1 methanol: 1 dH₂O; 5% acetic acid), washed in dH₂O, and excised in 48 slices. Slices were cut to 1 mm³ cubes and transferred to a 96-well filter plate (Multiscreen Solvinert MSRLN0450, 0.45 µm pore size PTFE membrane, Merck Millipore). In-gel digestion was performed according to the protocol (Shevchenko et al. 2006) modified for a 96-well filter plate processed on a vacuum manifold. Briefly, reduction by DTT was followed by iodoacetamide alkylation, destaining, and trypsin digestion. Tryptic peptides were extracted, dried, dissolved, desalted by 1-layer C18 StageTips, and transferred to a U-shaped 96-well plate suitable for the Ultimate 3000 nanoUHPLC autosampler. Peptides were separated on a 15 cm C18 column using 30 min elution gradient and analyzed in a DDA mode on Orbitrap Exploris 480 MS. Raw files were processed in MaxQuant (v. 1.6.17.0). The iBAQ values of each protein detected in the individual slices were normalized to the protein with the highest intensity and were visualized in the heatmaps using Microsoft Excel and Prism 8 (GraphPad Software, La Jolla California USA).

4.7. Pulse-chase analysis

In-vivo metabolic pulse-chase labeling by ³⁵S-methionine + ³⁵S-cysteine using Express Protein Labeling Mix, [³⁵S] (PerkinElmer, USA) was performed to study the synthesis and turnover of mtDNA-encoded proteins. Analysis of wt, 4dKO, 6BKO, and βKO cells, and 4dKO, 6BKO, and βKO cells expressing AOX was performed in two independent experiments. Cells were labeled for 3 hours in methionine- and cysteine-free DMEM with anisomycin (100 µg/mL) and the radioactive labeling mix (100 µCi/mL). Then, the non-labeled methionine and cysteine (final concentration 250 µM) were added for 15 min incubation. Afterwards, cells were washed with non-labeled methionine (250 µM) and cysteine (250 µM) containing PBS, and “pulse” samples were harvested. Parallel dishes with labeled cells were incubated for 24h in a complete non-labeling DMEM medium and then harvested as the “chase” samples. SDS samples were prepared from the cell pellets and were separated (70 µg protein) by 16 % gel using a large format Hoefer™ SE 600 Chroma Vertical Electrophoresis System (Thermo Fisher Scientific). After electrophoresis, gel was fixed by CBB staining solution (40 % (v/v) methanol, 8 % (v/v) acetic acid, 0.05 % (w/w) Coomassie Brilliant Blue R-250) and dried on Whatmann CHR 3 mm paper. Radioactivity was detected

by exposing the gel to Storage Phosphor Screen BAS-IP SR 2025 E for 14 days (GE Healthcare), which was then scanned by Amersham Typhoon biomolecular imager (GE Healthcare, USA). Detected signals were quantified by Image Lab software (Bio-Rad).

4.8. Measurement of metabolic fluxes

Seahorse Extracellular Flux (XF) Analyzer (Agilent Technologies, USA) was used to measure intact mitochondrial respiration and glycolytic rate in parallel, as described (Čunátová et al. 2021; Pajuelo Reguera et al. 2020). Briefly, cells ($2 - 3.5 \times 10^4$) were seeded in pentaplicates on poly-L-lysine coated wells of a 24-well measuring plate and incubated overnight in standard conditions. Before the measurement, wells were washed with 1 mL of XF Assay Medium Modified DMEM (with 10 mM glucose, pH 7.4, 37 °C), and then 500 μ L of the same medium with 0.2 % (w/w) BSA was added for an incubation (37 °C, 30 min). Basal metabolic rate with glucose as a substrate and rates after subsequent additions of the following substrates and inhibitors were recorded: oligomycin (Oligo, 1 μ M), FCCP (FCCP, 1 μ M), and a mixture of rotenone (Rot, 1 μ M), antimycin A (AA, 1 μ g/mL), 2-deoxyglucose (2DG, 100 mM) and Hoechst (5 μ g/mL). Measured rates were normalized to the cell count based on nuclei staining by Hoechst dye directly after the measurement. Images of whole wells were captured by Cytation 3 Cell Imaging Reader (BioTek, USA) and analyzed using Gen5 software (BioTek). Measurements were performed in at least three independent experiments to assess the statistical significance of the results. Measurement of metabolic fluxes was performed by RNDr. Alena Pecinová, Ph.D.

4.9. Measurement of mitochondrial ROS level

Mitochondrial ROS production was measured by monitoring 1 μ M CM-H2DCFDA (chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate, Thermo Fisher Scientific) fluorescence (Pajuelo Reguera et al. 2020). Cells were seeded on poly-L-lysine coated 24-well plates (3×10^5). On the second day, cells were incubated in parallel with either CM-H2DCFDA (“basal”) or CM-H2DCFDA and FCCP (“FCCP”, 1 μ M) for 2 h at 37 °C. The fluorescence increase was recorded by the Infinite M200 plate reader (Tecan Group Ltd., Switzerland) at 495/525 nm, and the difference between “basal” and “FCCP” CM-H2DCFDA fluorescence was calculated and attributed to mitochondrial ROS production (Mráček et al. 2006). ROS measurement was performed at least three times in order to assess

the statistical significance of the results. Measurement of mitochondrial ROS level was performed by Guillermo Puertas-Frias MSc.

5. Results

5.1. COX6B subunit is indispensable for cIV assembly and function

In our previous studies, we have inspected the role of cIV structural subunit COX4, and its individual isoforms (COX4I1, COX4I2), in the assembly and function of cIV (Čunátová et al. 2021; Pajuelo Reguera et al. 2020). Moreover, we explored the consequences of cIV deficiency affecting even the other OXPHOS complexes maintenance possibly through the attenuation of mitochondrial protein synthesis (Čunátová et al. 2021). In order to dissect detailed signaling mechanisms behind OXPHOS complexes interdependence, we wanted to include also a less severe model of cIV deficiency than COX4 lacking cells. We intended to construct a model, where the initial assembly intermediates of cIV (especially subcomplex formed by subunits MT-CO1 – COX4 – COX5A) would be present to contrast with COX4 lacking cells, where assembly does not proceed to that stage. For this reason, we constructed the HEK293 cell line with CRISPR knock-out of COX6B1 (6BKO) which was previously considered as one of the MT-CO3 module components, representing one of the later assembling subunits of cIV (Timon-Gomez et al. 2018; Vidoni et al. 2017).

In contrast with this (and similarly to our previous model of 4dKO), we showed that COX6B1 KO causes total cIV deficiency, as indicated by the complete absence of the MT-CO2 subunit (Figure 9A) that can be rescued by the transgenic expression of COX6B1 construct (6BKO^{comp}). Functional analysis (by Seahorse XFe24) showed complete impairment of mitochondrial respiration in 6BKO compared to the wild type (wt), indicating cIV dysfunction that can be rescued by COX6B1 complementation (Figure 9B). Detailed analysis of the steady-state level of cIV subunits in three 6BKO clones by SDS-PAGE/WB (Figure 9C) revealed significant downregulation of numerous cIV subunits. MT-CO1 and COX6C subunits were significantly decreased and MT-CO2, one of the catalytic core subunits, was completely lost in 6BKO, similar to the 4dKO model. Interestingly, the content of COX4I1 and COX5A subunits, forming the early assembly intermediate together with MT-CO1, was not different from the wt, suggesting preservation of the early assembly intermediate of cIV in 6BKO (Figure 9C). This finding was confirmed and extended by whole proteome analysis of three 6BKO clones in comparison with the wt using label-free

quantification mass spectrometry (LFQ-MS), which showed a significant reduction in the level of the majority of cIV subunits in COX6B1 lacking cells (Figure 9D).

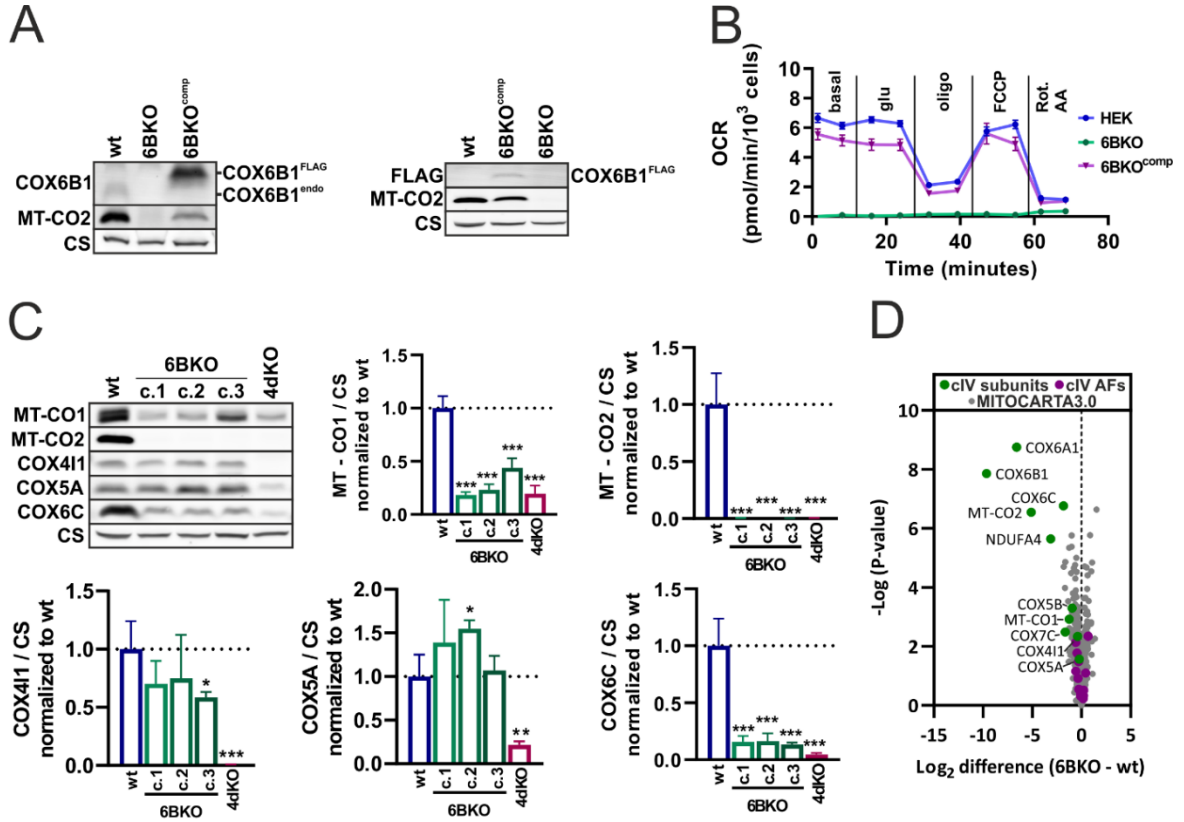


Figure 9. Impact of COX6B1 knock-out on cIV composition.

(A) Representative Western blot analysis of the steady-state levels of COX6B1 and MT-CO2 subunits in whole cell-lysates of HEK293 wt, 6BKO and 6BKO^{comp}. Citrate synthase (CS) antibody was used as a loading control.

(B) Measurement of cellular oxygen consumption rate (OCR) by Seahorse XF Bioenergetic Analyzer in wt, 6BKO and 6BKO^{comp} (basal respiration, 10 mM glucose, oligomycin, FCCP, rotenone and antimycin A).

(C) Representative Western blot analysis of the steady-state levels of cIV subunits in whole cell-lysates of wt, 6BKO and 6BKO^{comp}. Citrate synthase (CS) antibody was used as a loading control. Graphs represent quantification of cIV subunits signal normalized to CS. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; ** <0.002; *** <0.001.

(D) Label-free quantification - mass spectrometry analysis (LFQ-MS) of wt and three 6BKO clones. Volcano plot depicts differential contents of MitoCarta3.0 annotated proteins (grey), cIV subunits (green) and cIV assembly factors (cIV AFs, purple).

Using blue native electrophoresis (BN-PAGE), complete disappearance of fully assembled cIV and accumulation of several cIV assembly intermediates (MT-CO1_{module}, IV_{sub}) were observed in 6BKO clones (Figure 10). The most prominent assembly

intermediate composed of subunits MT-CO1, COX4, and COX5A corresponds to the assembled MT-CO1 module, just before the supposed incorporation of the absent MT-CO2. Based on the slower migration in BN gel, this intermediate should be slightly larger than the MITRAC subcomplex in 4dKO. Strikingly, MT-CO1 signal was detected in 6BKO clones even in the respirasome resembling molecular weight band, corresponding to the top band after immunodetection of cI by NDUFA9 antibody. This implicates the existence of SC formed by cI, the dimer of cIII and at least MT-CO1, but more likely cIV assembly intermediate containing other remaining cIV subunits (SC I III₂IV_{sub}). Generally, the level of cI was decreased in 6BKO clones, and in addition to the I III₂IV_{sub} mentioned above was also detected in association with cIII dimer (SC I III₂), similarly to 4dKO.

Taken together, the COX6B1 subunit was found essential for proper cIV maintenance and function, since its loss surprisingly results in severe cIV deficiency comparable to the COX4 lacking cells. However, in contrast to 4dKO, early-assembly intermediates and SC I III₂IV_{sub} are present in COX6B deficient cells.

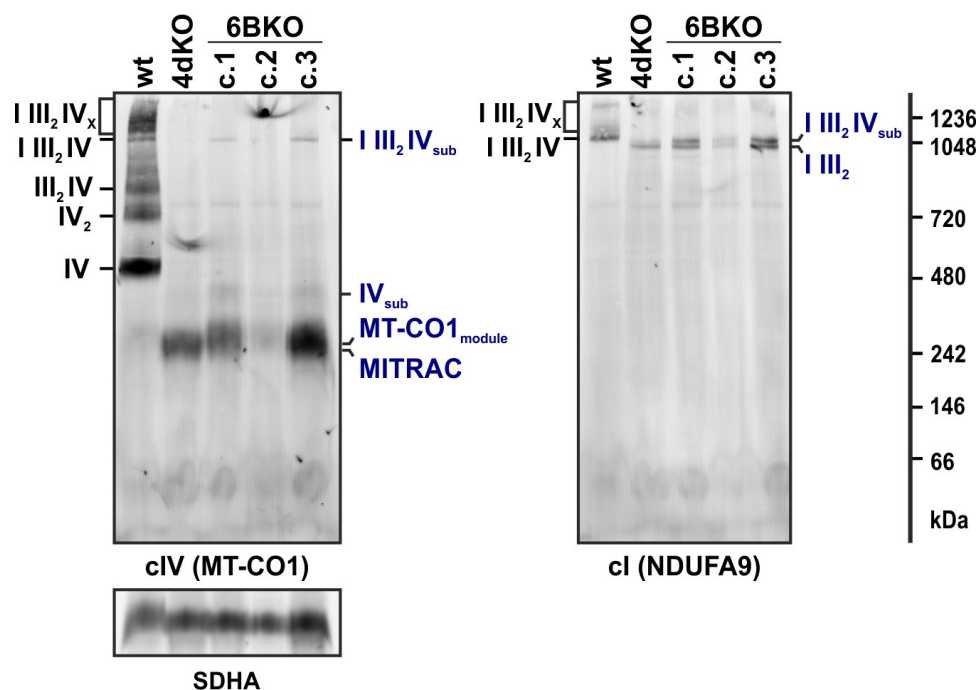


Figure 10. Impact of COX6B1 knock-out on supercomplexes composition.

Digitonin solubilized mitochondria (30 µg of protein) of wt, 4dKO and three 6BKO clones were subjected to blue-native (BN)-PAGE to detect OXPHOS complexes: complex I using NDUFA9 antibody and complex IV using MT-CO1 antibody. Antibody against cII (SDHA) was used as a loading control.

5.2. cIV and cV deficiency in HEK293 cells promotes cI decrease coupled with insufficient ETC electron flux, partially restored by AOX expression in cIV deficient models

To examine the impact of cIV deficiency on the other OXPHOS complexes, we previously established the HEK293 cell-line based model by introducing CRISPR/CAS9 knock-out (KO) of cIV early assembling structural subunit COX4 (4dKO) (Čunátová et al. 2021; Pajuelo Reguera et al. 2020). In the current study, we utilized further two models of cellular OXPHOS deficiency. First was COX6B1 knock-out (6BKO), which represents a model of severe cIV deficiency, but preserves the initial assembly intermediate formed by subunits MT-CO1, COX4, and COX5A (Figure 10). The second model was a knock-out of the ATP5F1B gene (β KO) encoding an essential cV subunit, to represent a respiratory chain-independent model of OXPHOS deficiency. For a general characterization of the mitochondrial proteome of OXPHOS deficient models, we performed LFQ-MS. Similar to our recent publication (Čunátová et al. 2021), diminished level of cIV and cV subunits in cIV and cV deficient cells respectively was accompanied by a general secondary decrease of cI subunits in all the models (4dKO, 6BKO, and β KO), compared to the wild type (wt) cells. Moreover, β KO cells presented with a secondary decrease in cIV subunits content (Figure 11A).

Alternative oxidase (AOX) works as CoQ:O₂ oxidoreductase and is naturally expressed in plants and in some fungi to bypass cIII and cIV (Young et al. 2013). Expression of AOX in the mouse and human cells was previously used to restore CoQ redox balance and OXPHOS function in RC dysfunction models (Guarás et al. 2016; Protasoni et al. 2020). We asked whether the substitution of cIV function as a terminal oxidase by AOX recovers the electron flux through ETC (as depicted in Figure 11B). For this purpose, we stably introduced AOX (HA-tagged, from *Aspergillus nidulans*) (Protasoni et al. 2020) in cIV and cV deficient cells (as documented in Figure 11C). Assessment of metabolic phenotype of the models by parallel measurement of mitochondrial respiration and glycolytic rate confirmed a complete absence of oxygen consumption (OCR) in cIV deficient cells and a significant decrease of OCR in β KO cells, compared to the wt (Figure 11D). The reported inability of mitochondrial ATP production in cIV and cV deficient models was compensated by increased extracellular

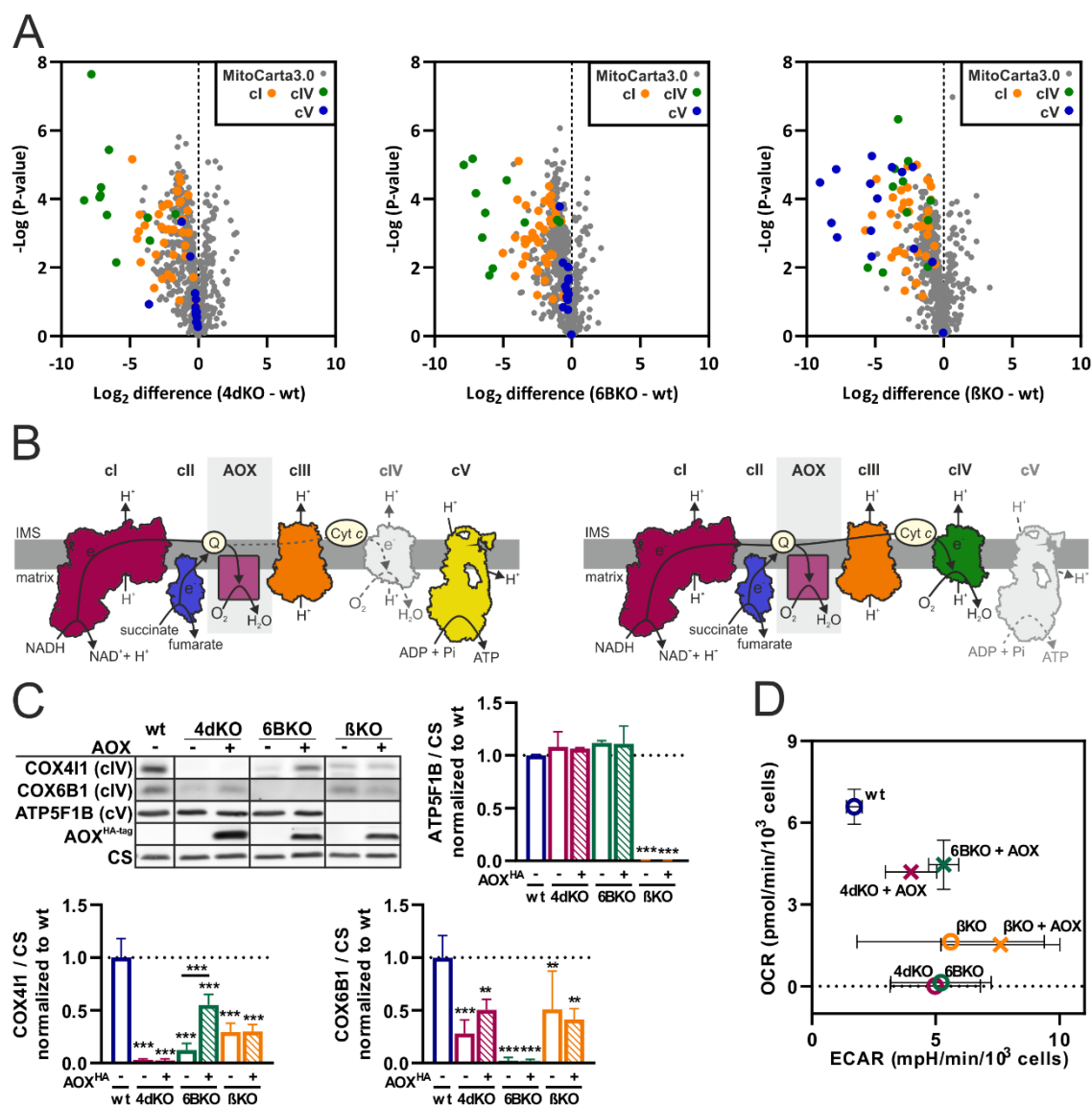


Figure 11. Characterization of cIV and cV deficient HEK293 cells with and without AOX expression.

(A) Label-free quantification - mass spectrometry analysis (LFQ-MS) of wt, 4dKO, 6BKO and βKO cells. Volcano plots depicting differential contents of MitoCarta3.0 annotated proteins in between wt and 4dKO, 6BKO and βKO respectively. Individual circles represent subunits of cI (orange), cIV (green), cV (blue) and MitoCarta3.0 annotated proteins (grey).

(B) Scheme of oxidative phosphorylation system depicting its function in cIV deficient cells (left) and cV-lacking cells (right) with possible substitution of cIV function by AOX (in the light grey box).

(C) Representative Western blot analysis of the steady-state levels of cIV subunits (COX4I1, COX6B1), cV subunit ATP5F1B and HA-tagged AOX (AOX^{HA}-tag) in whole cell-lysates of wt, 4dKO, 6BKO and βKO cells (-), and 4dKO, 6BKO and βKO cells expressing AOX (+). Citrate synthase (CS) antibody was used as a loading control. Graphs represent quantification of COX4I1, COX6B1 and ATP5F1B signal normalized to CS. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: ** < 0.002; *** < 0.001.

(D) Metabolic phenotype of wt, 4dKO, 6BKO and β KO cells (circle), and 4dKO, 6BKO and β KO cells expressing AOX (cross) represented as a phenogram combining parallel measurement of cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) by Seahorse XF Bioenergetic Analyzer with 10 mM glucose as a substrate.

acidification rate (ECAR), indicating a higher glycolytic rate providing ATP by the substrate-level phosphorylation. AOX expression in cIV deficient models led to significant increase of OCR, therefore partial recovery of electron flux through the RC. However, the unchanged ECAR rate after AOX expression in cIV deficient models reflects the insufficient formation of the electrochemical gradient to utilize for mitochondrial ATP production by cV. On the contrary, cV deficiency triggered attenuation of mitochondrial respiration that was not improved by AOX expression, since OXPHOS performance is primarily limited by the inability to utilize membrane potential (Figure 11D).

Therefore, mitochondrial respiration dysfunction in cIV deficient cells can be partially restored by AOX expression without significant improvement in mitochondrial ATP provision, while AOX expression in cV lacking cells is not beneficial.

5.3. AOX expression restores cI only in cIV deficient cells

To investigate the effect of AOX expression on the overall steady-state proteins content, we performed proteomic (LFQ-MS) analysis of cIV (4dKO, 6BKO) and cV (β KO) deficient cells with and without expression of AOX, in comparison to the wt cells. The deficiency models showed a decreased level of MitoCarta3.0 annotated proteins, with the most prominent decrease in the OXPHOS subunits compared to the wt. AOX expression in cIV deficiency models resulted in a significant increase in both MitoCarta3.0 and OXPHOS proteins, while AOX expression in the cV lacking model did not improve its phenotype (Figure 12A). To further follow OXPHOS subunits' steady-state level in detail, we combined the analysis of representative OXPHOS subunits by SDS-PAGE/WB (Figures 12B, 12C) and LFQ-MS data analysis (Figures 13A, 13B). SDS-PAGE/WB analysis revealed that the deficiency models show the most prominent decrease in the amount of representative cI (NDUFA9, NDUFB8, NDUFS3) and cIV (MT-CO1, MT-CO2) subunits compared to the wt (Figures 12B, 12C). Corresponding results were obtained by the proteomics data analysis, where the cI and cIV subunits were also generally decreased (Figure 13). cV lacking cells showed additional downregulation in all the cV subunits, while cIV deficient models

presented only a specific decrease of mtDNA-encoded subunits of cV (MT-ATP6, MT-ATP8) (Figures 13A, 13B). Even though the amount of UQCRC2 subunit of cIII was significantly decreased in the deficiency models (Figures 12B, 12C), in general, cIII subunits were not significantly altered as showed by the average fold change from LFQ-MS (Figures 13A, 13B). cII subunits were not altered in any of the models (Figures 12B, 12C, 13A, 13B).

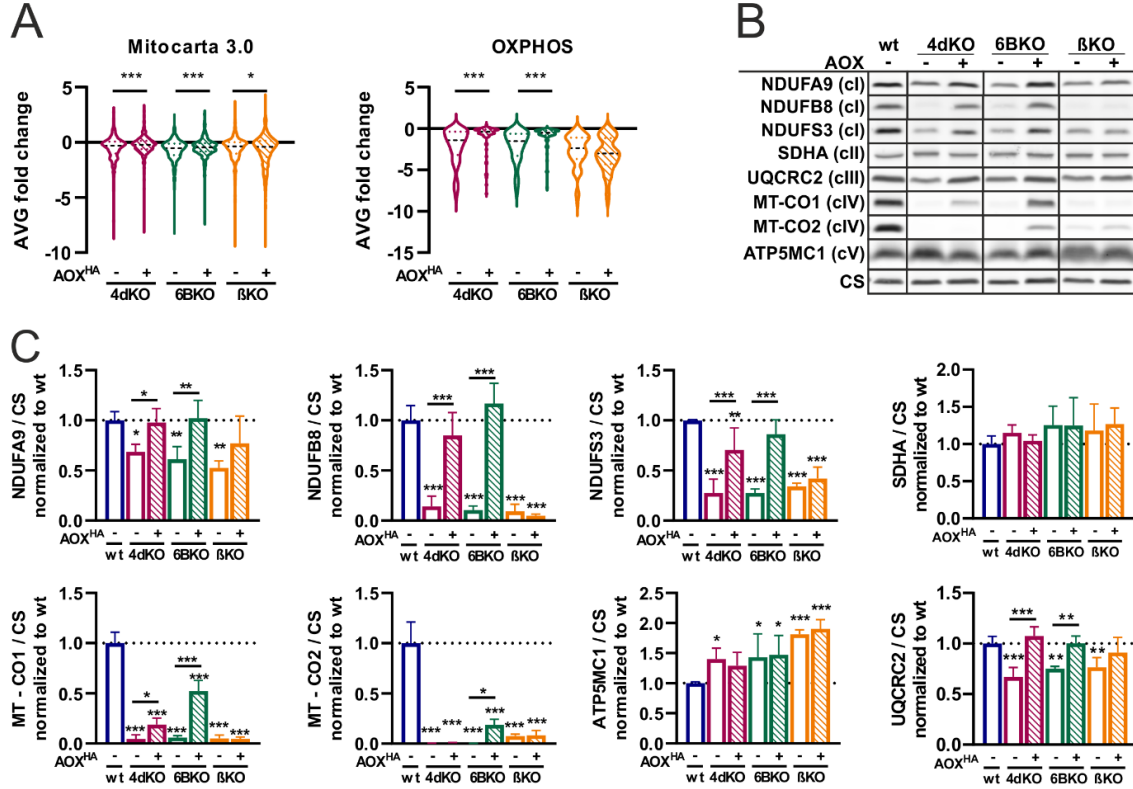


Figure 12. Analysis of OXPHOS subunits steady-state level by SDS-PAGE/WB.

(A) LFQ-MS analysis of wt, 4dKO, 6BKO and β KO cells (-), and 4dKO, 6BKO and β KO cells expressing AOX (+). Violin plots depict average fold-change of MitoCarta3.0 annotated proteins and OXPHOS complexes subunits. Statistically significant differences between individual KO and its AOX expressing counterpart were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; *** <0.001.

(B) Representative Western blot analysis of the steady-state levels of OXPHOS complexes subunits in whole cell-lysates of HEK293 wt, 4dKO, 6BKO and β KO cells (-), and 4dKO, 6BKO and β KO cells expressing AOX (+). Citrate synthase (CS) antibody was used as a loading control.

(C) Graphs represent quantification of OXPHOS subunits signal normalized to CS. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; ** <0.002; *** <0.001.

Interestingly, the content of analyzed cI and cIV structural subunits was significantly increased by AOX expression in cIV deficient cells (Figures 12B, 12C, 13A, 13B), while not improved in cV lacking cells. One of the differences between cIV deficient models was a

significant increase in the level of MT-CO2 subunit of cIV after AOX expression in 6BKO that was not observed in the 4dKO model (Figures 12B, 12C, 13A). Moreover, subunits COX6C and COX7A2 were also notably increased after AOX expression only in 6BKO, but not in the 4dKO model (Figure 13B).

Based on the results of our previous study (Čunátová et al. 2021), we reasoned that the prominent changes in the OXPHOS subunits steady-state level are presumably reflected in the composition of native OXPHOS complexes as well. We also expected possible changes between the cIV lacking models (4dKO, 6BKO) due to the differential restoration of MT-CO2, COX6C, and COX7A2 subunits after AOX expression. Therefore, we performed BN-PAGE/WB analyses to compare the level of individual OXPHOS complexes and supercomplexes composition in wt, cIV (4dKO, 6BKO) deficient models with and without

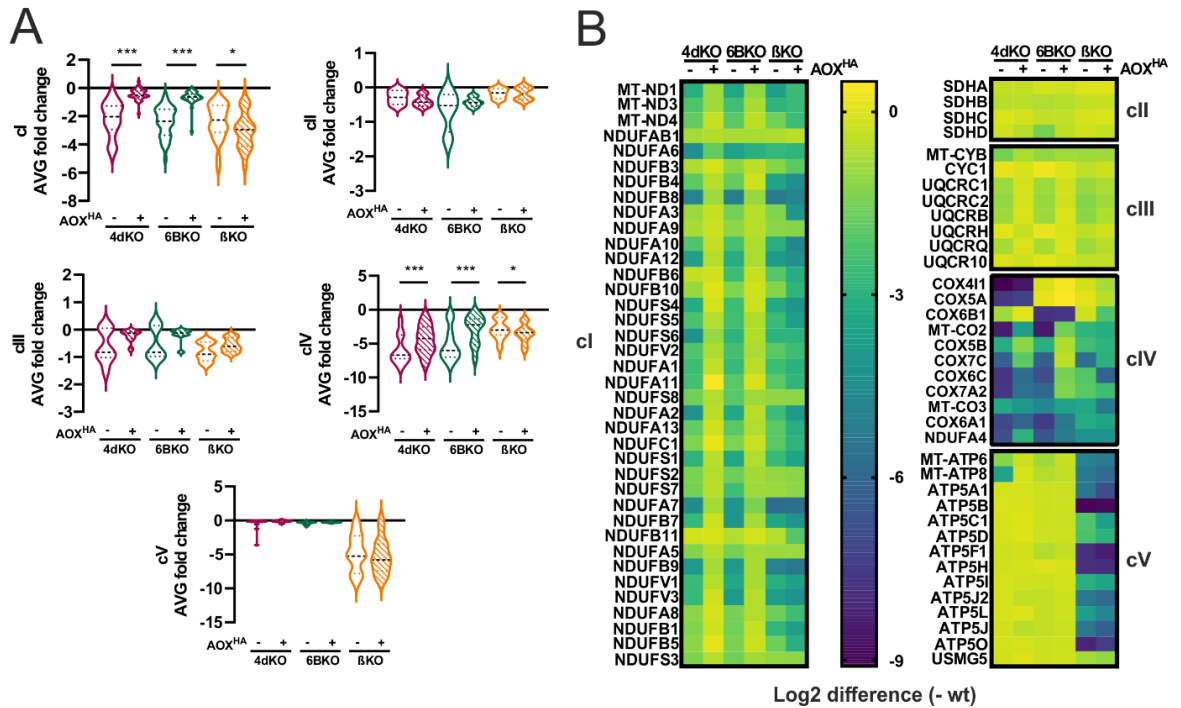


Figure 13. Proteomic analysis of OXPHOS subunits steady-state level.

(A) LFQ-MS analysis of wt, 4dKO, 6BKO and β KO cells (-), and 4dKO, 6BKO and β KO cells expressing AOX (+). Violin plots depicting average fold-change of individual OXPHOS complexes subunits (cI, cIII, cIV, cV). Statistically significant differences between individual KO and its AOX expressing counterpart were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; *** <0.001.

(B) LFQ-MS analysis of wt, 4dKO, 6BKO and β KO cells (-), and 4dKO, 6BKO and β KO cells expressing AOX (+) represented as a heat-map.

expression of AOX and cV lacking cells (β KO) (Figure 14). As expected, the level of cI (detected by NDUFA9 antibody) was pronouncedly decreased in all its native forms in 4dKO, 6BKO, and β KO compared to the wt, being present only in the supercomplex formed by cI and cIII dimer (SC I III₂). Respirasome and higher molecular weight SCs were undetectable due to the loss of cIV in all its forms in 4dKO and 6BKO. Only assembly intermediate representing MITRAC (4dKO) or MT-CO1 module (6BKO) subcomplexes were detected by MT-CO1 antibody. Strikingly, all native forms of complexes I and IV were severely diminished in β KO (Figure 14).

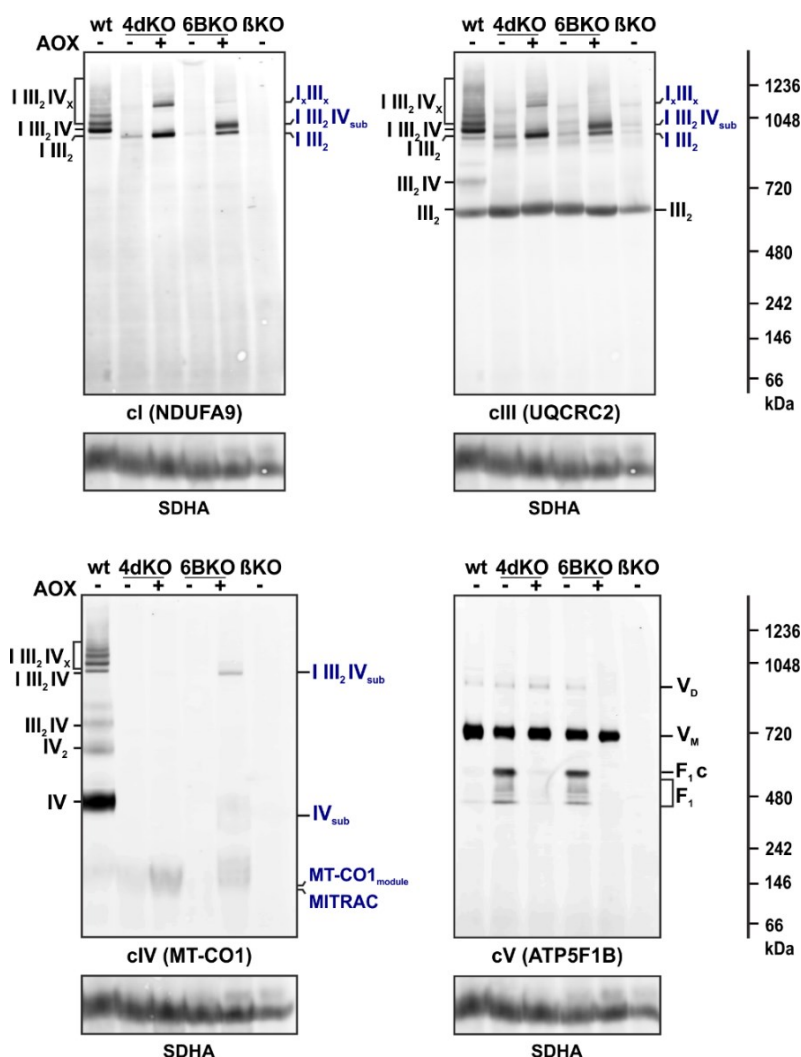


Figure 14. Analysis of OXPHOS native supercomplexes composition.

Digitonin solubilized mitochondria (30 μ g of protein) of wt, cIV (4dKO, 6BKO) deficient models with (+) and without (-) expression of AOX and cV lacking cells (β KO) were subjected to blue-native (BN)-PAGE to detect OXPHOS complexes: complex I using NDUFA9 antibody, complex III using UQCRC2 antibody, complex IV using MT-CO1 antibody and complex V using ATP5F1B antibody. Antibody against cII (SDHA) was used as a loading control.

The AOX expression in cIV deficient cells resulted in a pronounced increase of the level of SC I III₂ with an appearance of higher molecular weight SC formed by cI and cIII (SC I_xIII_x). Assembly intermediate representing MITRAC complex was accumulated in 4dKO after AOX expression. Interestingly, a low level of cIV assembly intermediate formed by the remaining subunits (IV_{sub}) was observed in 6BKO cells expressing AOX, pointing to the stabilization of this subcomplex lacking the COX6B subunit only under the conditions of the partial ETC function. In addition, AOX expression in 6BKO resulted in the formation of yet another SC formed by cI, cIII, and remaining cIV subunits (SC I III₂IV_{sub}), resembling the incomplete respirasome (SC I III₂IV). The level of native cIII (UQCRC2 antibody) was mildly decreased in 4dKO, 6BKO, and βKO with apparent redistribution towards cIII dimer (SC III₂), while improved by AOX expression in cIV lacking cells, appearing in the SCs with cI and remaining cIV subunits. Levels of cV (ATP5F1B antibody) monomer and dimer were not changed in cIV deficient cells. However, F₁ domain subassemblies of cV were detected in cIV KOs, disappearing after AOX expression (Figure 14).

To characterize the precise composition of the stabilized cIV assembly intermediates and SC I III₂IV_{sub} in COX6B lacking cells with and without AOX expression, we performed complexome profiling analysis, which combines native separation of RC complexes and supercomplexes with mass spectrometry analysis of individual slices cut from the gel (Figures 15A, 15B). Compared to the wt, which showed uniform distribution of 13 detected cIV subunits into all the cIV forms, 6BKO presented only 8 subunits, predominantly early assembling ones, accumulated in the assembly intermediate (Figure 15B). Surprisingly, few cIV subunits in SC I III₂IV_{sub} could be detected by a sensitive complexome profiling analysis even in 6BKO. Apparently, more of the cIV subunits were present in higher quantities after being stabilized in SC I III₂IV_{sub} of 6BKO expressing AOX, lacking only a few late assembling cIV subunits. Besides the presence of early intermediates in 6BKO with AOX, IV_{sub} subassembly, which contained almost all cIV subunits was also detected. Notably, detected subunits in IV_{sub} span all three assembly modules. The reduction of the cI level in 6BKO was further reflected by the increased content of its assembly intermediates relative to the assembled complex (I III₂) compared to the wt, and the presence of the cI assembly intermediate in association with cIII and cIV subunits COX4I1, COX5A and COX7A2L (pre-I III₂IV_{sub}) lacking matrix facing domain necessary for its catalytic function (N-module).

(B) Heat-map representation of relative iBAQ values of individual subunits of cI and cIII detected in all three analyzed models: wt, 6BKO, and 6BKO with AOX. In the case of cIV, all the detected subunits are shown. N-module of cI and individual modules of cIV (MT-CO1, MT-CO2, and MT-CO3) are highlighted by a red mark on the right (N, CO1, CO2, CO3). Data are organized according to the increasing molecular weight, from the left to the right.

Both these signs of cI deficiency were improved after AOX expression in 6BKO when the cI level increased and the complex was stabilized in the respective SCs (Figure 15B).

To sum up, the secondary decrease in the steady-state level of cI subunits in cIV and cV deficient models is translated to the native cI level, while being improved by partial restoration of electron flux by AOX expression in cIV deficient cells, but not in the cV lacking cells.

5.4. cIV and cV deficiency results in mitochondrial ribosomal proteins decrease and mitochondrial translation attenuation reversible by AOX expression only in cIV lacking cells

Further analysis of the LFQ-MS dataset revealed a decrease of mt-DNA encoded OXPHOS subunits in both, cIV and cV lacking cells, which was compensated by AOX expression only in cIV deficient models (Figure 16A). To determine whether the mtDNA-encoded proteins were decreased as an effect of mitochondrial protein synthesis attenuation, as we suggested in our previous study (Čunátová et al. 2021), or faster degradation rate, we performed pulse-chase *in vivo* ³⁵S Met + ³⁵S Cys metabolic labeling (Figure 16B).

Pulse-chase experiment showed that cIV deficient cells present mainly with the decrease in pulse signal of cIV (MT-CO1, MT-CO2+MT-CO3) and cI (MT-ND1, MT-ND2, MT-ND3+MT-ND6) mtDNA-encoded subunits, while cIII (MT-CYB) and cV (MT-ATP6, MT-ATP8) subunits showed rather increased or unchanged pulse signal compared to the wt (Figures 16B, 16C, 16D). The deficiency model of cV showed an even more pronounced decrease in the pulse of cI and cIV with an additional significant decrease of cV mtDNA-encoded subunits, while the cIII signal remained unchanged. Comparable to the steady-state OXPHOS protein levels (Figures 12B, 13B), AOX expression resulted in an increase of newly synthesized mtDNA-encoded proteins of cIV and cI in the cIV deficient cells, while AOX expression in cV lacking model did not improve its phenotype (Figures 16B, 16C). The only profound difference between 4dKO and 6BKO models was a significant increase in the

pulse level of MT-CO2+MT-CO3 in 6BKO after AOX expression (Figure 16B), indicating stabilization of the MT-CO2 subunit in subcomplexes and supercomplex described

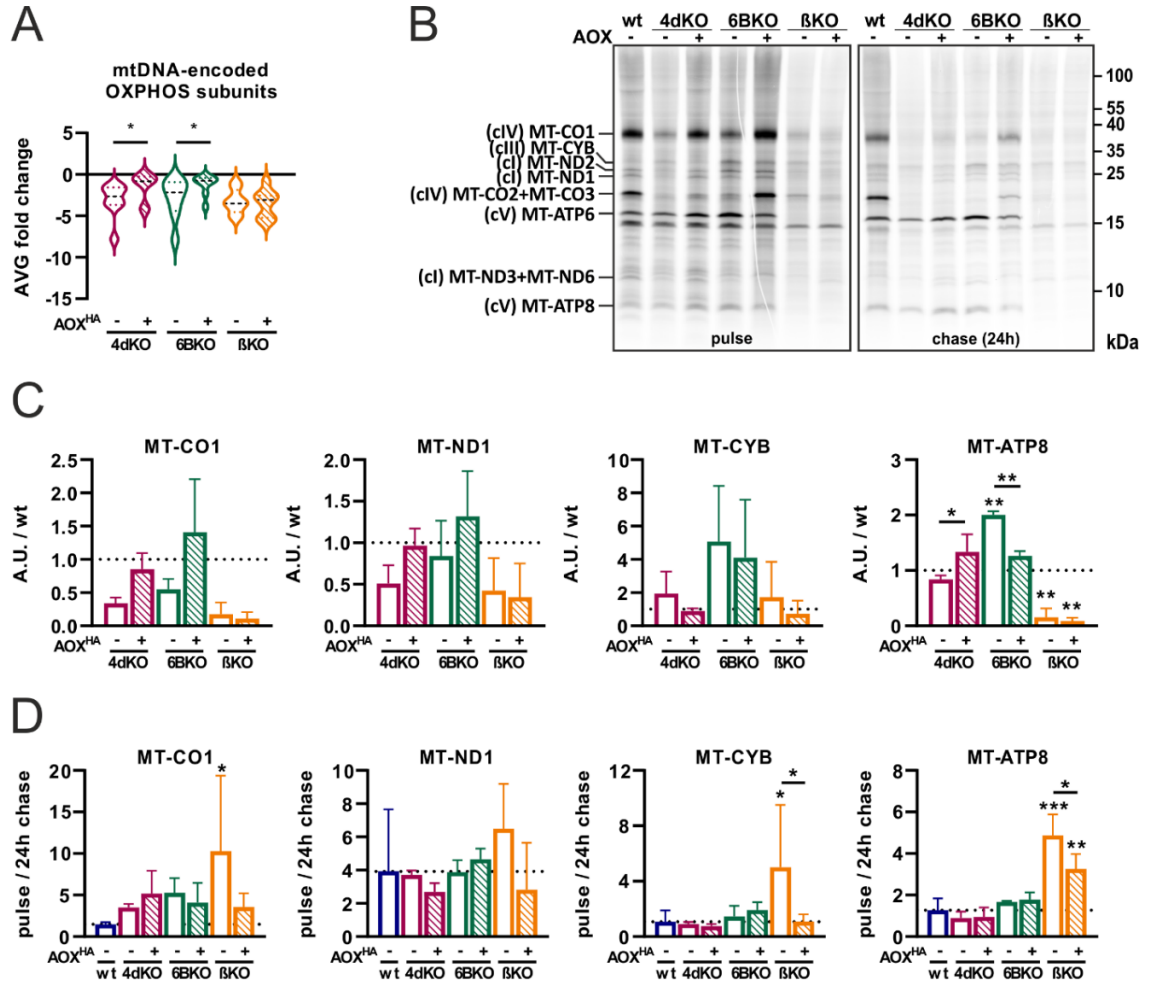


Figure 16. Attenuation of mitochondrial protein synthesis.

(A) LFQ-MS analysis of wt, 4dKO, 6BKO and β KO cells (-), and 4dKO, 6BKO and β KO cells expressing AOX (+). Violin plots depict average fold-changes of mtDNA-encoded proteins. Statistically significant differences between individual KO and its AOX expressing counterpart were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * < 0.033.

(B) ^{35}S *in vivo* labeling of mtDNA-encoded OXPHOS subunits. Representative image of two independent experiments showing autoradiographic detection of labeled proteins in 60 μg protein of whole cell lysates analyzed by SDS-PAGE.

(C) Quantification of pulse ^{35}S *in vivo* labeling signal of representative cIV (MT-CO1), cI (MT-ND1), cIII (MT-CYB) and cV (ATP8) subunits normalized to Coomassie staining. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * < 0.033; ** < 0.002.

(D) Quantification of pulse to 24h chase ratio of ^{35}S *in vivo* labeling signal of representative cIV (MT-CO1), cI (MT-ND1), cIII (MT-CYB) and cV (ATP8) subunits. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * < 0.033; ** < 0.002; *** < 0.001.

A

4dKO 6BKO BKO AOX^{HA}

MRPs, mtLSU Log₂ difference (- wt)

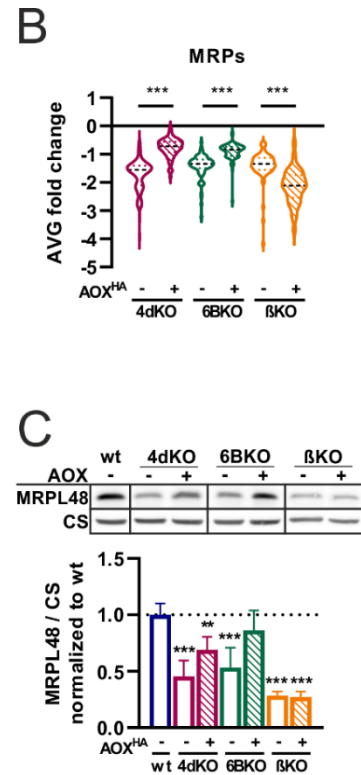
MRPL10
MRPL11
MRPL12
MRPL13
MRPL14
MRPL15
MRPL16
MRPL17
MRPL18
MRPL19
MRPL20
MRPL21
MRPL23
MRPL24
MRPL27
MRPL28
MRPL3
MRPL30
MRPL32
MRPL34
MRPL35
MRPL37
MRPL38
MRPL39
MRPL4
MRPL40
MRPL41
MRPL42
MRPL43
MRPL44
MRPL45
MRPL46
MRPL47
MRPL48
MRPL49
MRPL50
MRPL51
MRPL52
MRPL53
MRPL54
MRPL55
MRPL57
MRPL9

MRPs assembly
Log₂ difference (- wt)

MRPS10
MRPS11
MRPS12
MRPS14
MRPS15
MRPS16
MRPS17
MRPS18A
MRPS18B
MRPS18C
MRPS21
MRPS22
MRPS23
MRPS24
MRPS25
MRPS26
MRPS27
MRPS28
MRPS30
MRPS31
MRPS33
MRPS34
MRPS35
MRPS36
MRPS5
MRPS6
MRPS7
MRPS9

MRPS assembly
Log₂ difference (- wt)

RNMTL1
MTG1
DDX28
DHX30
AFG3L2
SPG7
TFB1M
NSUN4
ERAL1
GRSF1
MPV17L2
GTPBP10



(A) LFQ-MS analysis of wt, 4dKO, 6BKO and β KO cells (-), and 4dKO, 6BKO and β KO cells expressing AOX (+). Heat-maps depict average fold-change of MRPs forming large subunit of mitochondrial ribosome (mtLSU), small subunit (mtSSU), and assembly factors of mitochondrial ribosome.

(C) Representative Western blot analysis and of the steady-state levels of MRPL48 in whole cell lysates of HEK293 wt, 4dKO, 6BKO and β KO cells (-), and 4dKO, 6BKO and β KO cells expressing AOX (+). Citrate synthase (CS) antibody was used as a loading control. Graph represents quantification of MRPL48 signal normalized to CS. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: ** <0.002; *** <0.001.

cIV subunits of both cIV and cV deficient models show both reduced pulse signals (Figures 16B, 16C) as well as an increase in the pulse to chase ratio (Figures 16B, 16D). This means they are not only synthesized at a slower pace (similarly to other mtDNA-encoded proteins) but that they also have faster turnover. In the case of the cV lacking model, also cV subunits (MT-ATP6, MT-ATP8) pulse to chase ratios were elevated.

Lower mitochondrial protein synthesis rate in the deficiency models was accompanied by a significant decrease in the level of mitochondrial ribosomal proteins (MRPs) forming both, large and small, mitochondrial ribosome subunits (Figures 17A, 17B, 17C). Moreover, proteins involved in the assembly of mitochondrial ribosomes were downregulated as well (Figure 17A), determining a general decrease in mitochondrial ribosomes content in the deficiency models. In concordance with previous data, AOX expression had ameliorating effect only in the case of cIV deficient cells, where the level of MRPs was significantly increased (Figure 17A, 17B, 17C).

To conclude, loss of cIV or cV in HEK293 cells lead not only to the specific decrease of native cI but is accompanied by a decreased content of mitochondrial ribosomes resulting in specific attenuation of cI and cIV mtDNA-encoded subunits synthesis. This phenotype is reversible by partial restoration of ETC function by AOX expression, but only in the cIV deficient cells. This finding brings up the question, of which particular mechanism interconnects primary OXPHOS deficiency with the MRPs decrease and specific mitochondrial protein synthesis attenuation to affect the level of cI secondarily.

5.5. AMPK - mTORC1 axis is not a determinant for OXPHOS complexes interdependence

To examine whether the inability of mitochondrial ATP production together with the supposed increase in ROS production of cIV and cV deficient models lead to the activation of AMPK (Figure 18A) and if it is reverted in cIV deficient cells by AOX expression the same as the level of cI, we determined the ratio of phosphorylated AMPK by SDS-PAGE/WB analysis (Figure 18B). The level of AMPK (T172) phosphorylation was significantly increased only in 4dKO with and without AOX expression, as well as in the cV deficient model expressing AOX, while AMPK phosphorylation level in 6BKO with or without AOX showed no difference compared to the wt. We also examined the level of ROS production,

which displayed a significant increase only in 4dKO, β KO, and β KO expressing AOX. Interestingly, an increase of ROS in 4dKO was reverted by AOX expression, therefore the

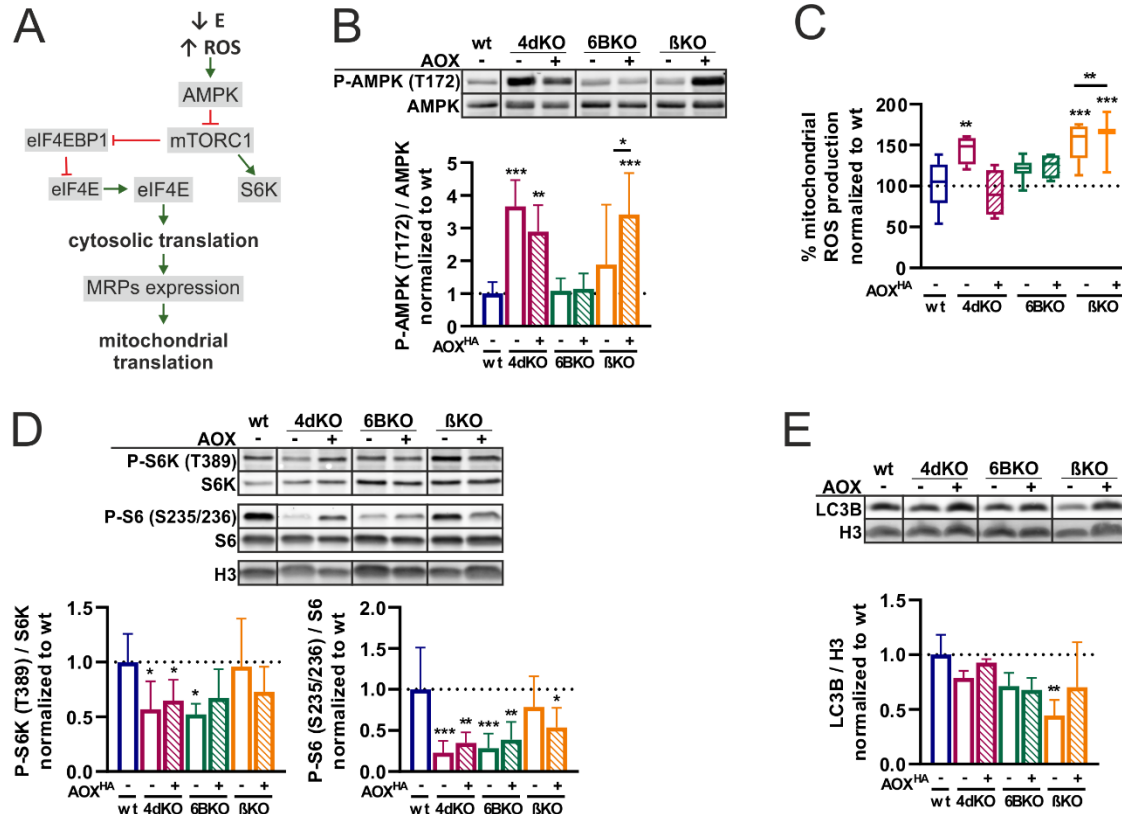


Figure 18. Questioning the role of AMPK-mTORC1 signaling in OXPHOS complexes interdependence.

(A) Scheme of AMPK-mTORC1 axis signaling.

(B) Representative Western blot analysis of the AMPK phosphorylation (T172) in whole cell-lysates of HEK293 wt, 4dKO, 6BKO and β KO cells, and 4dKO, 6BKO and β KO cells expressing AOX (+). Graph represent quantification of phosphorylated (T172) to total AMPK ratio normalized to the wt. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; ** <0.002; *** <0.001.

(C) Mitochondrial ROS production normalized to wt. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: ** <0.002; *** <0.001.

(D) Representative Western blot analysis of the S6K1 (T389) and S6 ribosomal protein (S235/236) phosphorylation in whole cell-lysates of HEK293 wt, 4dKO, 6BKO and β KO cells, and 4dKO, 6BKO and β KO cells expressing AOX (+). Histone H3 (H3) antibody was used as a loading control. Graphs represent quantification of phosphorylated to total S6K1 or S6 ratio normalized to the wt. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; ** <0.002; *** <0.001.

(E) Representative Western blot analysis of the steady-state levels of LC3B in whole cell-lysates of HEK293 wt, 4dKO, 6BKO and β KO cells, and 4dKO, 6BKO and β KO cells expressing AOX (+). Histone H3 (H3) antibody was used as a loading control. Graph represents quantification of LC3B signal normalized to H3. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; ** <0.002; *** <0.001.

AMPK phosphorylation was in this case ROS independent. ROS level in 6BKO with and without AOX was comparable to the wt (Figure 18C).

Next, we explored the possibility of mTORC1 inactivation (Figure 18A) in cIV and cV deficiency models by AMPK signaling-independent pathways. Thus, we analyzed the phosphorylation status of one of the active mTORC1 main targets, S6K1, which is critically phosphorylated at T389, and while active, phosphorylates its target S6 ribosomal protein. However, the S6K1 phosphorylation level was significantly decreased only in 4dKO, 4dKO with AOX, and 6BKO compared to the wt, being unchanged in the other models. In the case of S6 ribosomal protein phosphorylation (S235/236), β KO was the only model with an unchanged pattern compared to the wild type, while it was significantly decreased in the other deficiency models (Figure 18D). Despite the general trend of mTORC1 target phosphorylation decrease in cIV and cV deficient cells, no significant amelioration of this phenotype was observed after AOX expression in cIV deficient cells, thus questioning the role of mTORC1 signaling in OXPHOS complexes interdependence. Also, we assessed the level of autophagy by the autophagosome marker, LC3B, since autophagy represents a universal outcome of mTORC1 inactivation (Figure 18E). Interestingly, LC3B was slightly decreased in all the models. In β KO, LC3B level was even significantly decreased. This may implicate a higher requirement to save energy at the expense of less stringent quality control.

To sum up, our results do not suggest a dominant role of the AMPK – mTORC1 signaling axis in the OXPHOS complexes interdependence regulation.

5.6. Primary cIV and cV deficiency results in integrated stress response

To test if the MRPs decrease and a secondary OXPHOS deficiency may be the result of ISR signaling (Figure 19A), we analyzed the phosphorylation status of eIF2 α (S51) and the content of ATF4 and eIF4EBP1 in wt cells and OXPHOS deficiency models with and without AOX expression by SDS-PAGE/WB (Figure 19B). The level of eIF2 α phosphorylation was elevated in both cIV and cV deficient models and was decreased to the wt level in cIV lacking cells after AOX expression, but not in cV deficiency. Compared to the wt cells, ATF4 steady-state level was increased in cIV and cV deficient models, while dropped after AOX expression in cIV models, yet again not in cV KO (Figure 19B). Correspondingly with the levels of ATF4, eIF4EBP1 protein showed a similar pattern being

increased in cIV and cV deficient models, while decreased after AOX expression in cIV models, but not in cV lacking cells (Figure 19B).

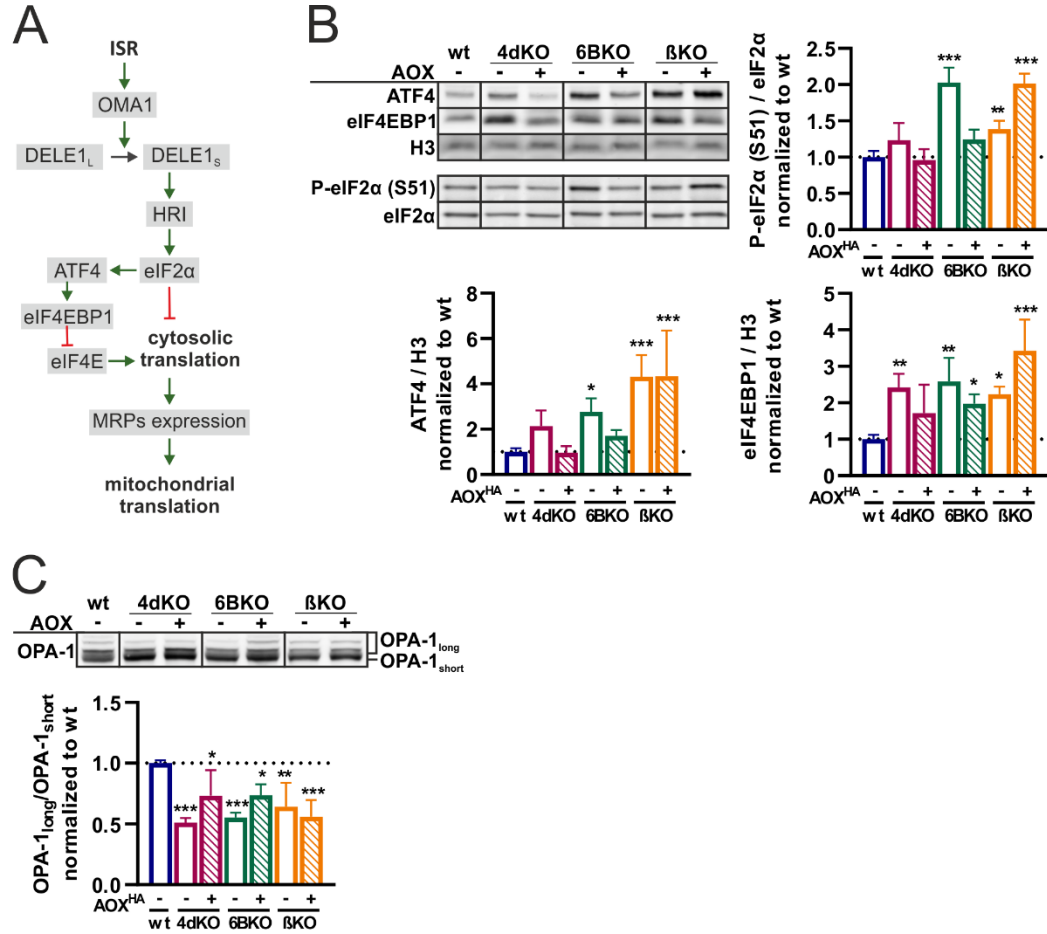


Figure 19. cIV and cV deficiency trigger integrated stress response.

(A) Scheme of possible OMA1-DELE1-HRI signaling towards integrated stress response (ISR).

(B) Representative Western blot analysis of the levels of ATF4 and eIF4EBP1, and eIF2α phosphorylation (S51) in whole cell-lysates of HEK293 wt, 4dKO, 6BKO and βKO cells, and 4dKO, 6BKO and βKO cells expressing AOX (+). Graph on the top represent quantification of phosphorylated (S51) to total eIF2α ratio normalized to the wt. Graphs on the bottom represent quantification of ATF4 and eIF4EBP1 signals normalized to H3. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; ** <0.002; *** <0.001.

(C) Representative Western blot analysis of the steady-state levels of long (OPA-1_{long}) and short (OPA-1_{short}) OPA-1 in whole cell-lysates of HEK293 wt, 4dKO, 6BKO and βKO cells, and 4dKO, 6BKO and βKO cells expressing AOX (+). Graph represents quantification of OPA-1_{long} to OPA-1_{short} ratio. Statistically significant differences were calculated using One-way ANOVA in GraphPad Prism 8. Asterisks (*) represent p-value: * <0.033; ** <0.002; *** <0.001.

Moreover, we analyzed levels of short and long OPA-1, since it is cleaved by OMA1 as one of its substrates. Interestingly, the ratio of long to short OPA-1 was significantly decreased in cIV and cV lacking cells, showing a higher OPA-1 cleavage rate, presumably

due to higher OMA1 activity. After AOX expression, the long to short OPA-1 ratio was increased in cIV KO models but remained unchanged in cV KO (Figure 19C).

Taken together, both cIV and cV deficiencies trigger ISR signaling most likely through the OMA1-DELE1-HRI pathway, putatively attenuating cap-dependent cytosolic translation to decrease MRPs level.

6. Discussion

The process of mitochondrial oxidative phosphorylation is essential for energy provision in all mammalian organisms and has been widely studied since the chemiosmotic hypothesis was defined by Petr Mitchell in 1966 (Mitchell 2011). The terminal enzyme of the respiratory chain, cytochrome *c* oxidase, plays an important role in this process, not only by coupling the electron transfer to oxygen with the proton gradient formation but also in terms of regulation of the overall process (Villani and Attardi 2000). The proper composition of cIV is crucial for the physiological function of the whole OXPHOS, therefore the biogenesis of this multimeric complex composed of fourteen subunits with dual genetic origin has to be precisely synchronized (Signes and Fernandez-Vizarra 2018). Within mammalian OXPHOS, cIV is unique in the number of nuclear-encoded subunits which exist as tissue- or condition-specific isoforms. This opens up the quite complex landscape, which allows for fine-tuning of the cIV function (Čunátova et al. 2020). On top of that, other OXPHOS complexes do not depend on cIV just by their function, but also by their maintenance. Both aspects – the impact of cIV subunit composition on cIV structure and function, as well as cIV role in the overall maintenance of the OXPHOS system, have been addressed in this thesis.

6.1. COX4I1 and COX4I2 isoforms of COX4 subunit represent a sophisticated switch in the oxygen sensing

In mammals, the largest nuclear-encoded subunit of cIV, COX4, was found to be present in two distinct isoforms, whose expression is regulated in a tissue- and oxygen-dependent manner (Hüttemann et al. 2001). In standard conditions, all tissues contain COX4I1 isoform, while COX4I2 isoform expression to a higher extent was detected predominantly in the lung, heart, and brain (Hüttemann et al. 2001), and more recently also in skeletal muscle (Schiffer et al. 2016). However, in various mammalian cell types, the hypoxic switch from COX4I1 to COX4I2 isoform expression was described (Aras et al. 2013; Fukuda et al. 2007; Horvat et al. 2006; Hüttemann et al. 2007). Despite the numerous studies exploring the effect of isoforms switch on the cIV function and its physiological impact in various models, a conclusive explanation of the mechanism was not reached. To elucidate specific differences between COX4I1 and COX4I2 isozyme structure and function,

HEK293 cell-line based models with exclusive isoform expression (4I1KI, 4I2KI) were created on the background of knock-out of both isoforms, COX4I1 and COX4I2 (4dKO) (Pajuelo Reguera et al. 2020).

Knock-out of COX4 isoforms resulted in complete cIV loss (Čunátová et al. 2021). Reintroduction of either of the isoforms (COX4I1 or COX4I2), restored cIV content to a comparable extent, although in both knock-in models was the MT-CO2 content (a good proxy for cIV content) cut by half when compared to the wild type cells with exclusive COX4I1 expression. Moreover, mass spectrometry analysis of immunocaptured cIV from 4I1 and 4I2 KI did not show any significant difference in the cIV subunits composition. Interestingly, similar composition and level of cIV in all its native forms could be detected by BN-PAGE/WB analysis, which rules out the probability of differential formation of supercomplexes incorporating individual cIV isozymes that could underlie functional differences between COX4 isoforms. Thus, 4I1KI and 4I2KI models were found as ideal models to uncover specific cIV functional differences between the two isozymes (Pajuelo Reguera et al. 2020).

Contrary to the already contradictory findings on functional differences of cIV isozymes published (Fukuda et al. 2007; Horvat et al. 2006; Hüttemann et al. 2012; Oliva et al. 2015; Singh et al. 2009), analysis of neither cIV activity, affinity to cytochrome *c*, nor the respiratory capacity of permeabilized cells showed the significant functional difference between COX4 isozymes, while the decrease in these parameters compared to wild type cells was given by the lower spare capacity of cIV in COX4I1 and COX4I2 KI models. However, intact cells' measurement of metabolic fluxes revealed a mild inclination of the COX4I2 KI model towards mitochondrial ATP production than glycolysis after the addition of glucose, compared to COX4I1 KI. This finding is in concordance with measurements of NAD⁺/NADH ratio and ROS production, when COX4I2 KI conferred a higher portion of oxidized NADH and lower ROS production under normoxia (Pajuelo Reguera et al. 2020). This preference could be explained by the hypothesized inability of allosteric cIV inhibition in COX4I2 isozyme, due to the differences in the primary structure of COX4I2 compared to COX4I1 protein (Acín-Pérez et al. 2011; Hüttemann et al. 2001).

Strikingly, affinity to the oxygen, measured as the partial oxygen pressure at the half-maximal respiratory rate (p_{50} value), differed between the isozymes. COX4I2 containing cIV showed a two-fold higher p_{50} value, meaning lower affinity to the oxygen, compared to both COX4I1 KI and wild type (Pajuelo Reguera et al. 2020). This could be of physiological relevance since COX4I2 was found as a fundamental component in the processes of hypoxic pulmonary vasoconstriction (HPV), the homeostatic response to alveolar hypoxia, preventing blood flow to poorly ventilated areas in the lung. It was previously shown that HPV is completely absent in COX4I2 knock-out mice (Moreno-Dominguez et al. 2019; Sommer et al. 2017). One of the current hypotheses on the mechanism of HPV triggering suggests that it is controlled by hypoxia-induced cellular membrane depolarization, acting downstream of increased superoxide production in mitochondria (Sommer et al. 2017). This could be indeed explained by the specific COX4I2 isoform property, the lower oxygen affinity. COX4I2 isozyme activity could be prematurely limited by the low oxygen concentration under hypoxia, and consequently could promote higher ROS production by the upstream OXPHOS components (Pajuelo Reguera et al. 2020). Nevertheless, in pulmonary arterial smooth muscle cells (PASMC), oxygen affinity was found unchanged in COX4I2 knock-out mice (Sommer et al. 2017). However, this biochemical phenotype could have been masked due to incomplete penetrance of COX4I2 isoform *in-vivo*. Therefore, if the proposed mechanism should be relevant under physiological conditions with mixed COX4 isoform expression, superoxide production would have to originate from a respiratory supercomplex structure containing the hypoxia-limited COX4I2 isozyme, while COX4I1 isozymes would be operating normally (Figure 20).

In further support of COX4I2 role in oxygen sensing, its expression was found to be indispensable for acute oxygen sensing in glomus cells of carotid bodies. In this case, a specialized setup of cIV nDNA-encoded subunits isoforms is present - besides COX4I2, also the non-canonical COX8B and NDUFA4L2 isoforms of nDNA-encoded subunits were identified in the cIV (Gao et al. 2017; Moreno-Dominguez et al. 2019; Zhou et al. 2016). Decreased oxygen affinity was suggested as the necessary property of the modified COX complex in an integrated model of carotid body hypoxic response (Ortega-Sáenz et al. 2020). Moreover, substoichiometric COX accessory protein HIGD1C was recently identified as another crucial player in glomus cells and was characterized to work in concert with the

COX4I2 isoform to ensure the oxygen sensing properties (Timón-Gómez et al. 2021). In summary, a specific pattern of COX components in oxygen-sensing cells supports our overall idea of characteristic nDNA-encoded subunits isoforms composition of cIV to fine-tune cIV function for specific physiological conditions (Čunátova et al. 2020).

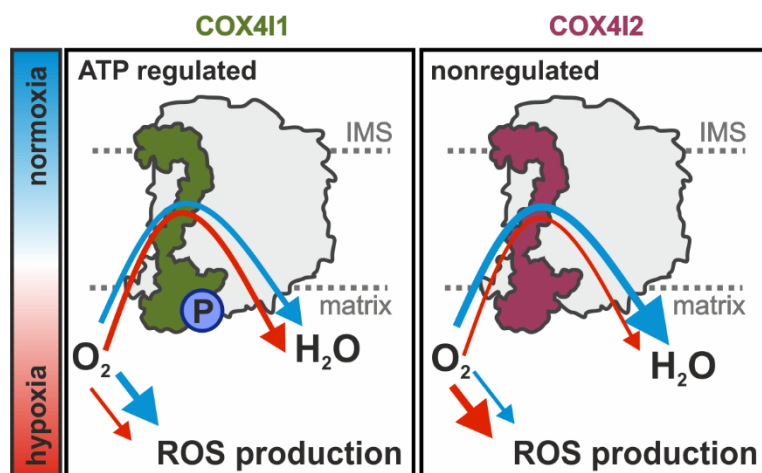


Figure 20. Model of functional implication of COX4 isoforms switch (Pajuelo Reguera et al. 2020).

Oxygen consumption rates under normoxia (blue) or hypoxia (red) in COX4I1 (left) or COX4I2 isozyme. ‘P’ depicts Ser58 phosphorylation site of COX4I1 isoform.

6.2. Role of subunits COX4 and COX6B in the cIV assembly process

Since many cases of mitochondrial diseases were found to be caused by aberrations of cIV assembly factors (Fernandez-Vizarra and Zeviani 2021; Pecina et al. 2004) and the nuclear-encoded subunits themselves (Čunátova et al. 2020), resulting in insufficient cIV assembly, the fidelity of cIV assembly had proved itself fundamental. The whole process was originally viewed as a sequential process of addition of individual subunits (Fornůsková et al. 2010). Later, a novel model of the modular assembly was defined, which perceives the assembly of three individual modules, each composed of one mtDNA-encoded subunit plus nDNA-encoded subunits, before the finalization of complete cIV (Signes and Fernandez-Vizarra 2018; Timón-Gómez et al. 2018; Vidoni et al. 2017). Moreover, recent studies showed data proposing non-canonical *de novo* assembly line of cIV not into the monomer, as previous models described, but directly into the RC supercomplex, the respirasome (Lobo-Jarne et al. 2020; Timon-Gomez et al. 2020). However, the role of individual nDNA-encoded subunits in the assembly process still needs to be refined in diverse knock-out models of cIV subunits and assembly factors to fully understand it. To shed more light on cIV assembly,

knock-out models of COX4 (4dKO) and COX6B (6BKO) subunits were created in the HEK293 cell line and their effect on cIV subunits steady-state level, as well as on the native forms of cIV, were conclusively characterized combining electrophoretic and mass spectrometry techniques.

COX4 subunit was previously shown to be indispensable for cIV early assembly steps (Nijtmans et al. 1998; Signes and Fernandez-Vizarra 2018). Correspondingly, the loss of the COX4 subunit led to a dramatic decrease in the steady-state content of most of the cIV subunits, as documented by both, electrophoretic and mass spectrometry, approaches (Čunátová et al. 2021). Notably, catalytic core subunit MT-CO1 was significantly decreased, while MT-CO2 was lost completely. The level of the COX5A subunit, which is the first subunit interacting with COX4 to jointly approach MT-CO1 in the very first step of cIV assembly, was also diminished. These findings were supported by the native electrophoresis and complexome profiling analysis, showing the absence of cIV in all of its native forms. The only native complex present was MT-CO1 containing intermediate resembling the early MITRAC complex, awaiting the addition of COX4-COX5A intermediate, which is absent and cannot be incorporated (Čunátová et al. 2021). This proved the inability of MT-CO1 module maturation in COX4 lacking cells, disabling the progression of cIV assembly in general. Therefore, the COX4 subunit was unequivocally established as an essential component in the initiation of the cIV assembly process (Čunátová et al. 2021).

On the other side, COX6B has always been suggested as a late-stage assembly subunit (Fornůsková et al. 2010), taking a part in the MT-CO3 module (Hock et al. 2020; Timon-Gomez et al. 2020). This was further corroborated by the detection of, albeit reduced, still fully assembled cIV in COX6B1 patient samples with a decreased amount of COX6B1 protein (Massa et al. 2008). Unexpectedly, COX6B knock-out cells presented with an analogical overall decrease in the level of cIV subunits as we observed in the 4dKO model (Figures 12B, 12C, 13A, 13B). The major difference between these two is the preservation of COX4 and COX5A subunits in 6BKO, reflected by the stabilization of a higher molecular weight assembly intermediate formed by subunits MT-CO1-COX4-COX5A, i.e. MT-CO1 module (Figures 14, 15). Higher molecular weight intermediates, containing a residual portion of MT-CO2 subunit, were present in the 6BKO model, yet only in trace quantities

detected only by a sensitive complexome profiling analysis. Moreover, a few cIV subunits (COX4, MT-CO2, COX7A2, and COX7A2L) were detected comigrating with incomplete SC resembling respirasome (SC I III₂IV_{sub}). But cIV monomer or dimer, which would only lack the COX6B subunit, were not detected (Figure 15B).

Both cIV deficient models, 4dKO and 6BKO, showed an inability to assemble fully-functional cIV, as documented by measurement of oxygen consumption (Čunátová et al. 2021). However, alternative oxidase (AOX) expression in cIV deficient models partially restored the electron flux (Figure 11D). AOX expression increased the level of MT-CO1 (Figures 12B, 12C), which was explained by boosted synthesis of mtDNA-encoded subunit MT-CO1 that was profoundly decreased in both cIV deficient models without AOX expression (Figure 16B, 16C). Further, the MT-CO2 subunit was detected even after 24 hours chase in the metabolic labeling experiment (Figure 16B), and correspondingly, its steady-state level was significantly increased in 6BKO expressing AOX (Figures 12B, 12C, 13A, 13B). As a consequence, cIV assembly intermediates were stabilized in individual models after AOX expression, being present at higher steady-state levels (Figures 12B, 12C, 13B). In particular, the level of MT-CO1 containing intermediate corresponding to the early MITRAC complex was increased in 4dKO (Figure 14).

Strikingly, in 6BKO after AOX expression, not only the level of the MT-CO1 module was increased, but cIV subunits detected across all the three assembly modules were further stabilized in a later intermediate (IV sub2) and in SC I III₂IV_{sub} (Figures 14, 15A, 15B). Complexome profiling analysis showed that even incomplete modules (MT-CO2 module missing COX7B, COX7C, and COX8A; MT-CO3 module missing COX6A1 and COX6B1) can be assembled into these entities. Therefore, under standard conditions, COX6B subunit loss unexpectedly leads to stalled cIV assembly. This can be caused by the destabilization of individual assembly intermediates, or by attenuation of mitochondrial protein synthesis, which ultimately leads to a decrease of mtDNA-encoded cIV subunits, followed by their enhanced clearance (Figure 16D). Interestingly, the yeast ortholog of the human COX6B subunit, *COX12*, was found to have a function in MT-CO2 maturation, assisting the copper delivery to form the Cu_A prosthetic group (Ghosh et al. 2016). Thus, there is a possibility that the mammalian COX6B subunit might have a similar function since MT-CO2 was

undetectable in 6BKO. The fact that its level is significantly increased after AOX expression suggests that favorable conditions for MT-CO2 maturation are restored, allowing its incorporation into assembly intermediates and stabilization even in the absence of the COX6B subunit (Figures 12B, 12C, 15B, 16). However, future research is needed to inspect this hypothesis in detail, particularly to dissect the effects of dysregulated redox conditions due to RC dysfunction or the shortage of any specific role of COX6B1 in MT-CO2 maturation (Figure 21).

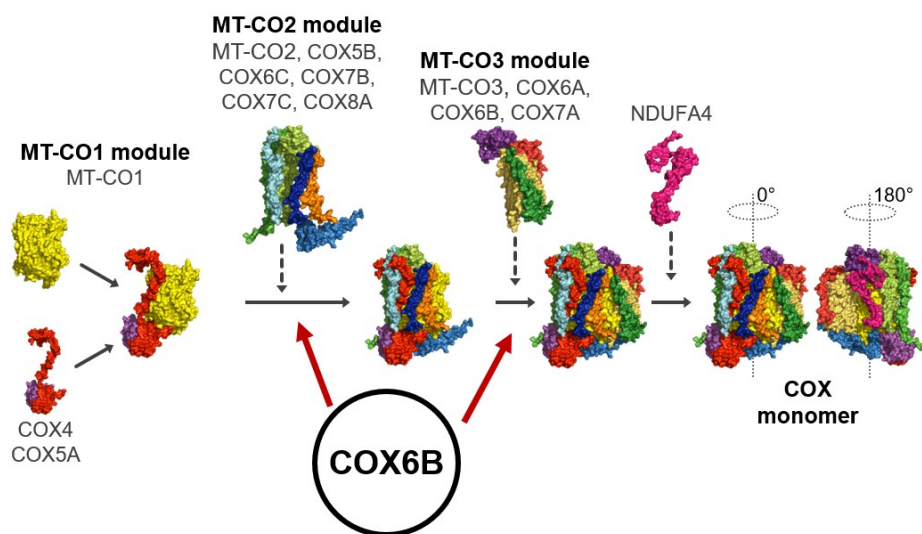


Figure 21. Scheme of possible involvement of COX6B subunit in cIV assembly.

Finally, complexome profiling revealed the presence of cI assembly intermediate without N-module (matrix facing domain) in contact with cIII dimer in 4dKO (Čunátová et al. 2021), while this intermediate in 6BKO contained even cIV subunits COX4-COX5A (pre-I III₂IV_{sub}), favoring the hypothesis of non-canonical *de novo* assembly of cI and cIV directly into the respirasome (Lobo-Jarne et al. 2020; Timon-Gomez et al. 2020). According to the present results, the addition of cIV subunits seems to be supported by the presence of an initial assembly intermediate formed by subunits MT-CO1, COX4, and COX5A, since the MT-CO1 signal was detected in the corresponding band only in 6BKO after AOX expression, but not in 4dKO (Figure 14). However, the absence of SC I III₂IV_{sub} in 4dKO could be also explained by a lower level of recovered MT-CO1 compared to 6BKO, which may be below the detection limit (Figure 12B, 12C). The possibility of SC I III₂IV_{sub} presence in 4dKO with AOX should be further explored, since MT-CO1 together with cIV subunits COX5B

and COX7A2 was previously detected within SC I III₂IV_{sub} in MT-CO2 lacking cells (Lobo-Jarne et al. 2020).

6.3. Interdependence of cIV and cI correlates with mitochondrial protein synthesis attenuation

Many mitochondrial disorders caused by a defect of one of the OXPHOS complexes manifest secondary deficiency of another complex in terms of its content and function (Fernandez-Vizarra and Zeviani 2021). One of the examples of such interdependence is the relation between cIV and cI, as documented by several studies showing decreased content of cI only in severe cIV deficiency models (Diaz et al. 2006; Guarás et al. 2016; Hornig-Do et al. 2012; Li et al. 2007; Lobo-Jarne et al. 2020). Even though interdependence represents a hot topic in the current mitochondrial research, the real cause of cI decline remains speculative. For this reason, 4dKO (Čunátová et al. 2021) and 6BKO represent the ideal models to compare the extent of cI interdependence and to search for its trigger, since they are both models of severe cIV deficiency, with assembly stalled at slightly different points.

As expected, a decrease in cI subunits' steady-state level was evident from the data obtained by both LFQ-MS (Figure 11A) and SDS-PAGE/WB (Figure 12B, 12C) in 4dKO (Čunátová et al. 2021). Interesting findings brought the analysis of the 6BKO model with the later assembly-stage blockade, which contains assembled MT-CO1 module. Even in the case of preservation of this assembly intermediate, an interdependent decrease of cI subunits content was observed (Figures 11A, 12B, 12C). The decrease in individual cI subunits in cIV lacking cells was reflected by the level of cI in its native forms, as only a residual portion of cI was observed exclusively incorporated into the SC I III₂ and lower assembly intermediates of cI, including I III₂ without N-module of cI in 4dKO, or pre-I III₂IV_{sub} in 6BKO, as described earlier. These intermediates were detected in wild type cells as well, although in a lower relative ratio to the fully assembled complex, pointing to the relevance of *de novo* assembly in physiological conditions (Figures 14, 15A, 15B) (Čunátová et al. 2021). Therefore, the possibility of early cI assembly dysregulation through a disabled progression of cIV assembly, reflected by MT-CO1 being stuck within the MITRAC complex (Mick et al. 2012; Richter-Dennerlein et al. 2016), seems to be refuted by our data.

The interdependence in the case of cIV deficiencies seems to be specific for cI because other OXPHOS complexes subunits steady-state content was not dramatically changed (Figure 13A). The level of native cII and cV was not altered, except F₁ domain subassemblies of cV present, while the cIII level was mildly decreased and redistributed towards the form of cIII dimer in cIV deficient models (Figure 14). The only change observed in cIV deficient cells was decreased content of mtDNA-encoded subunits of not only cI and cIV but also of cIII and cV (Figures 13B, 16A) (Čunátová et al. 2021). However, the impact on the native cIII and cV levels was negligible (Figure 14). Indeed, decreased level of cIV and cI mtDNA-encoded subunits in cIV deficient models was explained by attenuated mitochondrial protein synthesis. Nevertheless, cIII and cV subunits did not show altered translational levels after three hours of labeling (Figures 16B, 16C). However, there is a possibility that shorter labeling would uncover slower synthesis of cIII and cV mtDNA-encoded subunits, which cannot be observed in a longer period due to the extraordinary longevity of the proteins forming these complexes, compared to the faster turnover of the cI and cIV (Bomba-Warczak et al. 2021). In comparison to cIV mtDNA-encoded subunits, the other complexes did not show a sign of faster turnover compared to the wt, which rules out the possibility of faster degradation of cI to affect its level secondarily (Figures 16B, 16D). Therefore, mitochondrial protein synthesis attenuation represents a solid explanation for the specific decrease in overall cI level (Čunátová et al. 2021), which was further illustrated by the observed decrease in mitochondrial ribosomal proteins level, as well as factors indispensable for mitochondrial ribosome assembly (Figure 17) (Čunátová et al. 2021).

In the previous study concerning OXPHOS complexes interdependence, highly reduced CoQ status was proposed as a trigger for cI destabilization caused by the reverse electron transfer in cIII and cIV deficient models. Expression of alternative oxidase (AOX) led to an increase in cI level in both deficiency models, explained by a restoration of electron flux through cI and cII, and oxidation of CoQ by AOX (Guarás et al. 2016). To test, whether AOX expression ameliorates cI level decrease and its hypothetical cause, mitochondrial protein synthesis, cIV deficient models with AOX expression were prepared (Figures 11B, 11C, 11D). A general significant increase in steady-state content of cI subunits was evident after AOX expression in both cIV deficient models (Figures 12, 13), as well as the level of cI in its various native forms described earlier (Figures 14, 15). Since cI could be stabilized in SC

I III₂ IV_{sub} after AOX expression in 6BKO, which was also identified in a residual amount in 6BKO, it is unlikely that cI decrease is caused by cIV loss in terms of cI assembly, but is rather triggered by a declined rate of mitochondrial protein synthesis. Indeed, one of the effects of AOX expression was also an increase in the cI mtDNA-encoded subunits protein synthesis coupled with a significant elevation of mitochondrial ribosomal proteins content (Figures 16, 17).

To conclusively examine the cI interdependence cause, the HEK293 cell-line based model of cV deficiency, with knock-out of subunit ATP5F1B, was included in this study (Kovalčíková et al. 2019). Because cV does not participate in any RC supercomplex, it was found as a useful tool to test, whether cI interdependence occurs also in an OXPHOS deficiency independent of supercomplexes formation, as recently published in the case of cII deficient cells (Hart et al. 2022). Indeed, total cV deficiency resulted in a decreased steady-state level of cV, cIV, and cI as well (Figures 11A, 12, 13), which was reflected by a dramatic decrease of native cI and cIV following the loss of cV (Figure 14). Comparable to cIV deficient cells, cV defect also presented with diminished mitochondrial protein synthesis of cI, cIV, and cV mtDNA-encoded subunits (Figure 16) that was coupled to the significant decrease in mitochondrial ribosomal proteins content (Figure 17). Therefore, even cV deficient cells showed signs of interdependence between OXPHOS complexes connected with mitochondrial protein synthesis attenuation, despite its absence in supercomplexes. AOX expression in cV lacking cells did not result in cI and mitochondrial translation amelioration, which could be subscribed to the inability to restore electron flux through being under persisting respiratory control owing to the inability to utilize proton gradient due to cV absence.

In summary, severe cIV and cV deficiencies lead to a specific decrease in cI level, which could be explained by associated attenuation of mitochondrial protein synthesis due to diminished level of mitochondrial ribosomes (Čunátová et al. 2021). This interdependence phenotype could be reversed by AOX expression, although only in cIV deficient cells, while did not have any positive effect on the cV lacking model.

6.4. Mitochondrial integrated stress response suspected of the mitochondrial protein synthesis attenuation towards OXPHOS complexes interdependence

After the connection of cI interdependence with aberrant mitochondrial protein synthesis, the question arose about the molecular mechanism sensing the OXPHOS dysfunction and signaling towards mitochondrial ribosomal proteins decrease.

One of the possible triggers of such signaling could involve sensing of energetic deprivation and an increase in ROS production provoked by the OXPHOS deficiency through the AMPK-mTORC1 signaling pathway (Condon et al. 2021; Hinchey et al. 2018) to decrease cytosolic protein synthesis of nDNA-encoded mitochondrial proteins, among them mitochondrial ribosomal proteins (Morita et al. 2013). However, the findings of the present study did not confirm the AMPK role in the interdependence signaling, since neither AMPK phosphorylation, ROS level, nor mTORC1 targets phosphorylation corresponded to the interdependence phenotype observed in individual models (Figure 18). Further, examination of the autophagy level by the LC3B marker resulted in a decrease in all cIV and cV deficient models compared to the wild type, disproving the relevance of mTORC1 signaling, and mitophagy itself, as a cause of OXPHOS complexes interdependence (Figure 18D).

Recent research on the mammalian mitochondrial stress signaling accented the importance of the integrated stress response (ISR) pathway, which was found to be responsible for metabolic adaptation under stress conditions evoked by mitochondrial DNA depletion (Bao et al. 2016), or inhibition of OXPHOS (Martínez-Reyes et al. 2012; Quiros et al. 2017; Silva et al. 2009), mitochondrial translation (Quiros et al. 2017; Sasaki et al. 2020), mitochondrial import, Krebs cycle (Ryan et al. 2021), etc. Mitochondrial ISR initiation has been described to involve mitochondrial OMA1 protease induced by a decrease in the mitochondrial membrane potential, which transmits the signal towards cytosolic HRI kinase through DELE1 protein (Fessler et al. 2020; Guo et al. 2020). To test, whether OMA1 could be activated in cIV and cV deficient models and inactivated after AOX expression in cIV lacking cells only, thus reflecting the interdependence phenotype, the level of OMA1 cleavage target, OPA-1, was characterized (Figure 11C). Indeed, the long to short OPA-1 ratio correlated with the cI interdependence, possibly corresponding to OMA1 activity. This is in an agreement with previously published results of higher long OPA-1 processing in the

cells with destabilized OXPHOS complexes, and mitochondrial membrane potential decrease, induced by respective inhibitors (actinonin, FCCP) (Quiros et al. 2017).

Downstream ISR targets are eIF2 α phosphorylation by HRI kinase, leading to inhibition of the cytosolic cap-dependent translation initiation (Pakos-Zebrucka et al. 2016). At the same time, translation of specific targets is induced, including the key player of the mitochondrial ISR, activating transcription factor ATF4. ATF4 activates the expression of its target genes, including eIF4EBP1 (Bao et al. 2016; Guo et al. 2020; Yamaguchi et al. 2008). However, the ATF4 pathway was also shown to be activated independently on eIF2 α kinases, including HRI (Quiros et al. 2017). Besides, mitochondrial stress triggered by mitochondrial dysfunction was not only connected with ISR signaling alone but even with an ATF4-independent decrease in the mitochondrial ribosomal proteins level (Quiros et al. 2017). To address the mitochondrial ISR as a highly probable signaling pathway towards OXPHOS complexes interdependence, eIF2 α phosphorylation, as well as the level of ATF4 and eIF4EBP1 proteins, were analyzed by SDS-PAGE/WB (Figure 11B). Compared to the wild type, all three tested steps of ISR signaling, eIF2 α phosphorylation, ATF4, and

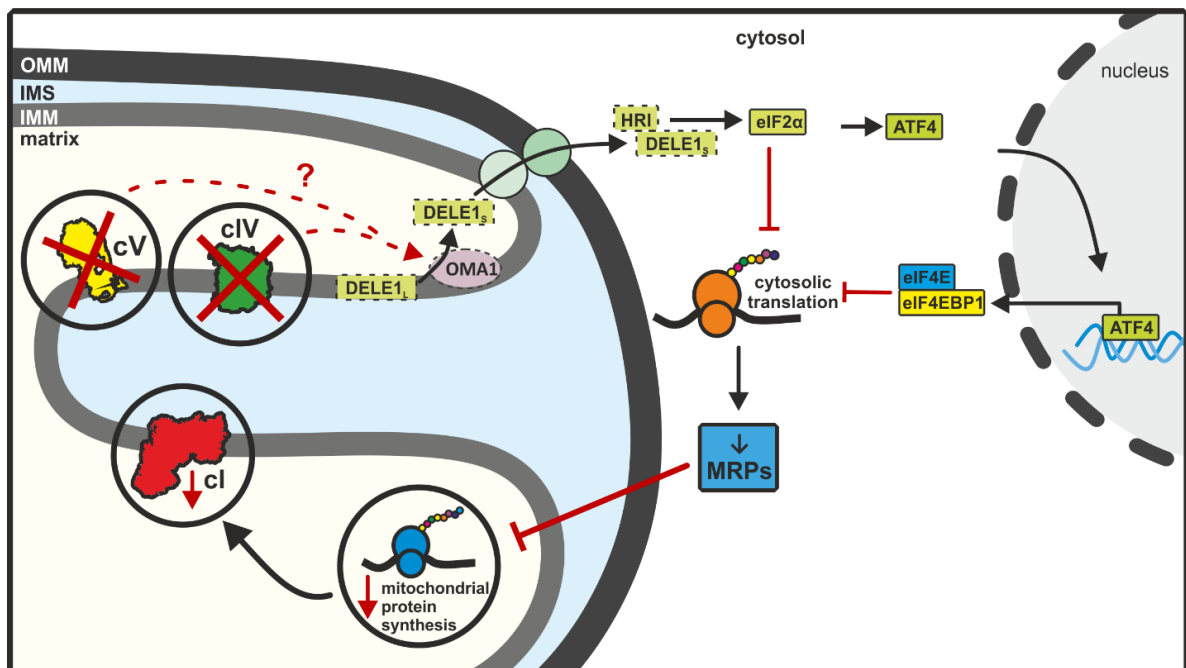


Figure 22. Scheme of hypothetical OXPHOS complexes interdependence signaling.

Individual parts of the presumable ISR signaling ensuring OXPHOS complexes interdependence are depicted. Parts experimentally approved in this thesis are shown bounded by a full line, while the presumable ones are presented in a dashed line.

eIF4EBP1 levels, were increased in cIV and cV deficient cells, with a trend of decrease after AOX expression only in cIV lacking cells.

Altogether, the presented data support the involvement of the mitochondrial ISR signaling in the OXPHOS complexes interdependence through a decrease in the mitochondrial ribosomal proteins level and attenuation of mitochondrial protein synthesis (Figure 22). Nevertheless, follow-up research is necessary to specifically address the trigger of the mitochondrial ISR in OXPHOS deficient cells.

7. Conclusions

The present thesis has aimed to push the knowledge boundaries of cIV composition impact on its function and the function of the whole OXPHOS system.

Firstly, our focus was turned on the role of COX4 subunit isoforms in the modulation of cIV structure and function. Both isoforms COX4I1 or COX4I2 on the knock-out background showed the capability to assemble complete functional cIV in all its native forms and conferred comparable activity of cIV, affinity to cytochrome *c*, and respiratory capacity. The preference for mitochondrial ATP provision with glucose as a substrate was presented in COX4I2 isozyme-containing cells, suggesting the absence of allosteric cIV inhibition compared to the COX4I1-containing enzyme. The most striking finding was a decreased affinity to the oxygen of the COX4I2 isozyme, which could explain the role of the COX4I2 isoform in the physiologically relevant oxygen-sensing mechanisms (Pajuelo Reguera et al. 2020).

Secondly, the role of nuclear-encoded subunits of cIV, COX4 and COX6B, was studied in the context of the cIV assembly process. COX4 subunit was proved fundamental in the initiation of the cIV assembly process (Čunátová et al. 2021). Interestingly, exclusive COX6B subunit participation in the later stages of cIV assembly was disproved, while the role of stabilization even in the early assembly steps following MT-CO1 module completion was proposed. Further, the results favor the importance of non-canonical *de novo* assembly of RC complexes directly into the respirasome.

Last, but not least, the phenomenon of the interdependence of cIV and cI was studied in cIV deficient models. The decrease of cI induced by the cIV defect was connected with the mitochondrial protein synthesis attenuation due to the decreased content of mitochondrial ribosome (Čunátová et al. 2021). cIV function replacement by alternative oxidase resulted in the amelioration of both cI level and mitochondrial protein synthesis rates, pointing out that the trigger is rather functional than structural. Finally, we provided evidence that mitochondrial integrated stress response is involved in the signaling of interdependence between OXPHOS deficiencies.

8. References

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9. Publications

Attachment 1: Loss of COX4I1 Leads to Combined Respiratory Chain Deficiency and Impaired Mitochondrial Protein Synthesis.

Čunátová K, Reguera DP, Vrbacký M, Fernández-Vizarra E, Ding S, Fearnley IM, Zeviani M, Houštěk J, Mráček T, Pecina P.
Cells. 2021 Feb 10;10(2):369.

Attachment 2: Cytochrome c Oxidase Subunit 4 Isoform Exchange Results in Modulation of Oxygen Affinity.

Pajuelo Reguera D, Čunátová K, Vrbacký M, Pecinová A, Houštěk J, Mráček T, Pecina P.
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Article

Loss of COX4I1 Leads to Combined Respiratory Chain Deficiency and Impaired Mitochondrial Protein Synthesis

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Abstract: The oxidative phosphorylation (OXPHOS) system localized in the inner mitochondrial membrane secures production of the majority of ATP in mammalian organisms. Individual OXPHOS complexes form supramolecular assemblies termed supercomplexes. The complexes are linked not only by their function but also by interdependency of individual complex biogenesis or maintenance. For instance, cytochrome *c* oxidase (cIV) or cytochrome *bc1* complex (cIII) deficiencies affect the level of fully assembled NADH dehydrogenase (cI) in monomeric as well as supercomplex forms. It was hypothesized that cI is affected at the level of enzyme assembly as well as at the level of cI stability and maintenance. However, the true nature of interdependency between cI and cIV is not fully understood yet. We used a HEK293 cellular model where the COX4 subunit was completely knocked out, serving as an ideal system to study interdependency of cI and cIV, as early phases of cIV assembly process were disrupted. Total absence of cIV was accompanied by profound deficiency of cI, documented by decrease in the levels of cI subunits and significantly reduced amount of assembled cI. Supercomplexes assembled from cI, cIII, and cIV were missing in COX4I1 knock-out (KO) due to loss of cIV and decrease in cI amount. Pulse-chase metabolic labeling of mitochondrial DNA (mtDNA)-encoded proteins uncovered a decrease in the translation of cIV and cI subunits. Moreover, partial impairment of mitochondrial protein synthesis correlated with decreased content of mitochondrial ribosomal proteins. In addition, complexome profiling revealed accumulation of cI assembly intermediates, indicating that cI biogenesis, rather than stability, was affected. We propose that attenuation of mitochondrial protein synthesis caused by cIV deficiency represents one of the mechanisms, which may impair biogenesis of cI.

Keywords: mitochondria; OXPHOS; cI; COX; cIV; COX4; knock-out; cIV assembly; complex I; biogenesis interdependency; complexome profiling; mitochondrial protein synthesis

1. Introduction

Energetic needs of mammalian organisms fully depend on ATP produced by oxidative phosphorylation (OXPHOS), a major metabolic pathway localized in the inner mitochondrial membrane. OXPHOS complexes are encoded by both mitochondrial (mtDNA) and nuclear (nDNA) DNA. Complex I (NADH dehydrogenase, cI) contains 7 out of 45 subunits encoded by mtDNA, complex II (succinate dehydrogenase, cII) contains 4 nuclear-encoded subunits, complex III (cytochrome *bc1* complex, cIII) contains 1 out of 10 subunits encoded by mtDNA, and complex IV (cytochrome *c* oxidase, cIV) contains 3 out of 14 subunits encoded by mtDNA. Regulation of OXPHOS capacity may therefore

occur at various levels of mtDNA expression, including mRNA maturation, mRNA stability, translational coordination, ribosomal biogenesis, and translation itself [1–5]. Expression and posttranslational modifications of individual subunits in cytosol and mitochondria need to be coordinated by signaling crosstalk between nucleus and mitochondria [6]. Moreover, for proper OXPHOS function, many enzyme cofactors are needed and their homeostasis and efficient incorporation into apoenzyme are important [7]. Individual subunits and cofactors must assemble to produce the mature and functional enzymes, with a myriad of so-called “assembly factors” involved. In addition, respiratory chain complexes form supramolecular assemblies termed as supercomplexes, adding yet another level of complexity in OXPHOS biogenesis. The most abundant supercomplex in mammalian mitochondria was named as respirasome, because it is composed by all the elements capable of transferring electrons from NADH to oxygen, i.e., one copy of complex I, dimer of complex III, and one copy of cIV (I III₂IV) [8]. Its structure has been recently solved with overall resolution at 5.8 Å [9] or 5.4 Å [10].

Mammalian cIV consists of a catalytic core composed of three mtDNA-encoded subunits: MT-CO1, MT-CO2, and MT-CO3, surrounded by 11 different regulatory subunits encoded in nucleus: COX4, COX5A, COX5B, COX6A, COX6B, COX6C, COX7A, COX7B, COX7C, COX8, and NDUFA4, which is currently also considered as part of the cIV, discovered on the basis of genetic [11] and structural data [12]. Six of the nuclear-encoded subunits exist as developmental stage or tissue-specific isoforms [13]. In case of COX4 subunit specifically, alternative isoforms COX4I1 and COX4I2 are expressed by independent genes. COX4I1 represents the ubiquitous isoform, while COX4I2 is mainly lung-specific and its expression is further regulated by oxygen levels [13]. It was recently shown that COX4I2 is necessary for hypoxic pulmonary vasoconstriction [14], and that COX4 isoform exchange may modulate cIV affinity to oxygen [15].

Biogenesis of cIV is a complicated and highly regulated process that was at first described as sequential incorporation of cIV subunits through four distinct assembly intermediates S1–S4, reflecting rate-limiting steps in enzyme biogenesis [16]. The cIV assembly mechanism is not spontaneous, because numerous accessory nuclear DNA-encoded proteins are needed to build the holoenzyme. Their function is required for all steps of the process and most of them are evolutionary conserved from lower to higher eukaryotes [17,18]. Currently, cIV assembly is considered to be modular, with independent maturation of three mtDNA-encoded subunits accompanied by addition of specific sets of nuclear-encoded subunits [4,19]. MT-CO1 maturation occurs within the MITRAC complexes (mitochondrial translation regulation assembly intermediates of cytochrome *c* oxidase) before binding of an assembly initiator subcomplex containing nuclear-encoded subunits COX4 and COX5A, the first ones to be assembled together [20]. The MT-CO2 module then associates with nascent cIV upon MT-CO2 copper metallization by the specific SCO1/SCO2, COX16, and COA6 chaperones [21–25]. Addition of the MT-CO3 module, with the help of HIGD2A, finalizes cIV assembly [4,26,27].

Complex I is the largest OXPHOS complex, which in mammals consists of 45 subunits, including 14 core subunits homologous to prokaryotic cI components, and numerous accessory subunits that are indispensable for enzyme assembly and stabilization [5]. The biogenesis of the complex is characterized by pre-assembly of individual modules that reflect enzyme functional units, aided by specific assembly factors [4]. The membrane arm containing the mtDNA-encoded subunits ND1–ND6 first associates with the ubiquinone (CoQ)-binding module. The assembly is completed by attachment of the soluble N-module responsible for electron transfer between NADH and CoQ.

It has been repeatedly found that deficiency in one of the complexes may in turn affect the other ones. Therefore, complexes are linked not only by their function, but also by interdependency in individual complex formation or maintenance. Notably, deficiency of cIII has been frequently shown to cause decrease of cI content and activity. This

occurrence was interpreted as a consequence of cI destabilization in the absence of its supercomplex partner [28–33]. However, some studies convincingly show that the interdependency is associated with impairment in cI maturation, which can be at least partially attributed to essential association of both these complexes within the respirasome precursor during their assembly [34,35]. Although less frequently, it has been shown that cIV deficiency also affects the level of assembled cI, both non-associated and within supercomplexes. Interestingly, this phenomenon seems to occur only in rare cases with complete absence of cIV caused by defect in early phases of MT-CO1 maturation/assembly [36,37]. These studies indicate that cI is affected at the level of complex assembly, suggesting some degree of interdependency between biogenesis of these respiratory chain complexes. In contrast, another reported case of cI-cIV interdependency postulated that cIV loss due to nonsense mutations in MT-CO1 gene led to increased activity of the mitochondrial m-AAA protease AFG3L2, which in turn impaired the stability of complex I [38]. Importantly, numerous cases of milder cIV assembly defects with at least minimal residual portion of assembled cIV display no effect on cI [39]. Recently, a study using 143B Δ MT-CO1 and Δ MT-CO2 cybrid cells demonstrated that even an incomplete, non-canonical MT-CO1 submodule containing subunits COX5B and HIGD2A can associate with the I III₂ supercomplex and stabilize it, thus maintaining the content of these respiratory complexes [40].

Here, we explored the mechanisms of cIV-cI interdependency by interfering with the earliest phases of cIV assembly using COX4 loss-of-function cellular models. We report that removal of COX4 subunit in HEK293 results in complete cIV deficiency. Furthermore, the impairment of the earliest steps in cIV assembly also causes profound deficiency of complex I, which is associated with decreased synthesis of mtDNA-encoded cI subunits as well as reduced content of mitochondrial ribosomal proteins. We propose that mitochondrial protein synthesis attenuation may represent yet another mechanism of cIV-cI biogenesis interdependency.

2. Materials and Methods

2.1. Cell Culture and Models

Preparation of HEK293 (ATCC CRL-1573)-based cellular models, i.e., COX4I1, COX4I2, and COX4I1/4I2 gene knock-outs and cells expressing COX4I1 or COX4I2 on the background of COX4I1/4I2 knock-out (KO) HEK293 was described in detail in our previous study [15]. Briefly, COX4I1 and COX4I2 knock-outs were introduced by CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) knock-out technology employing Cas9-D10A paired nickase with two chimeric RNA duplexes [41]. To obtain double knock-out of both isoforms (COX4I1/4I2 KO), we subsequently knocked out COX4I2 and COX4I1. For this study, two individual clones of each knock-out was used (COX4I1 KO 1 and 2, COX4I2 KO 1 and 2, COX4I1/4I2 KO 1 and 2). For rescue experiments, COX4I1/4I2 KO 2 served as a background generating cell lines with stable expression of COX4I1 (COX4I1 KI) or COX4I2 (COX4I2 KI), using full length complementary DNA (cDNA) constructs in pcDNA3.1⁺ with C-terminal FLAG (DYKDDDDK) tag.

Cells were cultivated under standard conditions (37 °C, 5% CO₂ atmosphere in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Thermo Fisher Scientific, 31331-028), supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, 10270-106), 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 μ M uridine, and antibiotics (100 U/mL penicillin + 100 μ g/mL streptomycin (Thermo Fisher Scientific, 15140-122)).

2.2. SDS-PAGE

Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for separation of proteins on the basis of their different molecular weights under

denaturing conditions. SDS-PAGE samples were prepared from frozen aliquots of harvested cellular pellets according to [42], to a final concentration 2–5 mg protein/mL. Then, samples were sonicated and incubated for 20 min at 40 °C. Samples (10–50 µg of protein as indicated) were separated on 12% gel using Mini-PROTEAN III apparatus (Bio-Rad, Hercules, CA, USA).

2.3. Native Electrophoresis

Blue-native (BN)-PAGE electrophoresis [43] was used to separate native protein complexes. For protein complex solubilization, mild non-ionic detergent digitonin, which prevents dissociation of supercomplexes, was used; thus, OXPHOS complexes and their supercomplexes could be detected. Mitochondria from HEK293 were released by hypotonic shock and isolated using differential centrifugation as previously described [44] and then stored as dry pellets at -80 °C. Isolated mitochondrial pellets were solubilized by digitonin (6 g detergent/g of protein) and prepared for analysis according to [43]. Samples were separated on 5–16% polyacrylamide gradient gel using Mini-PROTEAN III apparatus (Bio-Rad).

2.4. Western Blot (WB) and Immunodetection

Proteins separated by electrophoresis were transferred onto PVDF (polyvinylidene difluoride) membrane (Immobilon FL 0.45 µm, Merck) by semi-dry electroblotting (0.8 mA/cm², 1 h) using Transblot SD apparatus (Bio-Rad). Immunodetection was performed as described in [15]. Primary and secondary antibodies used for immunodetection are listed in Table S1. Signal detection was performed using fluorescence scanner Odyssey (LI-COR Biosciences, Lincoln, NE, USA). Signals detected by scanner were quantified by software AIDA Image Analyzer (Raytest Isotopenmessgeräte GmbH, Germany).

2.5. Mass Spectrometry—Label-Free Quantification (MS LFQ)

Biological duplicates (COX4I1 KO clones 1 and 2, COX4I2 KO clones 1 and 2, COX4I1/4I2 KO clones 1 and 2) were harvested and MS LFQ analysis was performed in technical duplicates, as described previously [45]. Briefly, cell pellets (100 µg of protein) of wild-type HEK293 cell line, COX4I1, COX4I2, and COX4I1/4I2 KO clones were solubilized by sodium deoxycholate (final concentration 1% (w/v)), reduced with TCEP ([tris(2-carboxyethyl)phosphine]), alkylated with MMTS (S-methyl methanethiosulfonate), digested sequentially with Lys-C and trypsin, and extracted with ethylacetate saturated with water, as previously described. Samples were desalted on Empore C18 columns, dried in Speedvac, and dissolved in 0.1% trifluoroacetic acid (TFA) + 2% acetonitrile. About 1 µg of peptide digests were separated on a 50 cm C18 column using 2.5 h elution gradient and analyzed in a data dependent acquisition (DDA) mode on an Orbitrap Fusion Tribrid (Thermo Fisher Scientific) mass spectrometer. Resulting raw files were processed in MaxQuant (v. 1.5.3.28) with label-free quantification (LFQ) algorithm MaxLFQ. Downstream analysis and visualization were performed in Perseus (maxquant.org/perseus/, v. 1.5.8.5). LFQ dataset is publicly available in the PRIDE repository (<https://www.ebi.ac.uk/pride>), accession number PXD023367 (accessed on February 15th 2021).

2.6. Stable isotope labeling using amino acids in cell culture (SILAC)/Complexome Profiling

Wild-type and COX4I1/4I2 KO clone 2 cells were cultivated in “heavy” (¹⁵N- and ¹³C-labeled Arg and Lys) and in “light” (¹⁴N and ¹²C Arg and Lys) DMEM (Thermo Fisher Scientific). Unlabeled (“light”) cell line was mixed equally with labeled (“heavy”) one and mitochondria-enriched fraction was isolated [46]. Mitochondrial pellets were solubilized by digitonin and Bis/Tris BN-PAGE was performed using 3–12% polyacrylamide gradient gel. The gel was stained, fixed, and excised in 64 slices. Slices were in-gel digested by trypsin and analyzed by tandem mass spectrometry [19,47]. For the protein identification,

peptide quantification and plain text export, Proteome Discoverer software (Thermo Fisher Scientific), and Mascot peptide search engine (Matrix Science) were used. For further data evaluation, Python code was used to separate unlabeled and labeled peptides into two datasets and R code was used to determine the relative peptide intensity for each protein according to the most frequently occurring representative peptide in both unlabeled and labeled forms (for details see [29]). The relative peptide intensity profile of each protein was normalized to the slice with the highest intensity from the average of duplicate experiments and was visualized in heatmaps using Microsoft Excel and Prism 8 (GraphPad Software, La Jolla California USA). Complexome profiling dataset is publicly available in the CEDAR database [48] (<https://www3.cmbi.umcn.nl/cedar/browse/>), accession number CRX25 (accessed on December 22nd 2020).

2.7. Mitochondrial Protein Synthesis Pulse-Chase Analysis

To study synthesis and turnover of mtDNA-encoded proteins, we essentially employed the method of *in vivo* metabolic pulse-chase protein labeling by ³⁵S-methionine + ³⁵S-cysteine as described [49], using Express [³⁵S] Protein Labeling Mix (PerkinElmer, USA). For this experiment, wild-type HEK293 and two independent clones of COX4I1 KO, COX4I2 KO, and COX4I1/4I2 KO were analyzed in two independent experiments. Cells were labeled in DMEM, without methionine and cysteine, in the presence of 100 µg/mL of anisomycin and the radioactive labeling mix with 100 µCi/mL for 3 hours. Afterwards, 20 µL of the mixture of “cold” methionine and cysteine (non-labeled Met and Cys) was added into each well (final concentration 250 µM) and incubated for a further 15 min. Then, cells were washed two times with phosphate buffered saline PBS containing 250 µM cold methionine and 250 µM cold cysteine. One aliquot of labeled cells was immediately harvested (“pulse” sample). Parallel dishes of labeled cells (“chase” samples) were further incubated in complete non-labeling DMEM medium for 24 hours. Cellular pellets were later used for SDS-PAGE analysis using large format Hoefer SE 600 Chroma Vertical Electrophoresis System (Thermo Fisher Scientific). The 70 µg protein aliquots of each sample were separated by 16% gel. Following the electrophoretic run, gel was fixed in staining solution (40% (v/v) methanol, 8% (v/v) acetic acid, 0.05% (w/w) Coomassie Brilliant Blue R-250) and dried onto Whatmann CHR 3 mm paper. Radioactivity was detected by exposing the gel to Storage Phosphor Screen BAS-IP SR 2025 E for 14 days (GE Healthcare), which was then scanned by FX Molecular Imager (Bio-Rad). Signals detected by scanner were quantified by software AIDA Image Analyzer.

2.8. Measurement of Metabolic Fluxes

Parallel measurement of mitochondrial respiration and glycolytic rate was performed using Seahorse Extracellular Flux (XF) Analyzer (Agilent Technologies, USA), as described previously [15]. We analyzed wild-type HEK293 (*n* = 5 biological replicates, measurement on different days), COX4I1 clone 1 (*n* = 5), COX4I2 KO clone 1 (*n* = 4), and COX4I1/COX4I2 clone 1 (*n* = 3). Briefly, 1 day prior to measurement, 3.10⁴ cells were seeded in pentaplicates in poly-L-lysine-coated wells of measuring plate, and incubated overnight under standard cultivation conditions. For the measurement, the cells were washed with 1 mL of XF Assay Medium Modified DMEM containing 10 mM glucose as a substrate (pH 7.4, 37 °C), and 500 µL of the same medium with 0.2% (w/w) BSA was added; then, the microplate was incubated at 37 °C for 30 min. Meanwhile, XFe24 Sensor Cartridge was prepared by injection of substrates and inhibitors to record basal metabolic rate with glucose and after subsequent additions reaching final concentrations of 1 µM oligomycin (Oligo); 1 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP); and inhibitor cocktail of 1 µM rotenone, 1 µg/mL antimycin A, and 100 mM 2-deoxyglucose (Rot+AA+2DG). For precise normalization of rates according to cell counts, immediately after Seahorse measurements, we stained cell nuclei by Hoechst dye (final concentration 5 µg/mL, diluted in FluoroBrite DMEM Media). Images of whole wells were

acquired by Cytation 3 Cell Imaging Reader (BioTek, Winooski, VT, USA) and analyzed using software Gen5 (BioTek) to obtain cell counts for each well.

3. Results

3.1. Decreased Steady-State Content of cIV and cI Subunits in Cells Lacking COX4I1

We decided to elucidate how the complete knockout of the COX4 subunit interferes with biogenesis of cIV as well as other OXPHOS complexes. To study this, we utilized COX4I1 and COX4I2 genetic knock-outs in HEK293, and two clones of each mutant (COX4I1, COX4I2, and COX4I1/4I2 with loss of both isoforms) were used in subsequent experiments.

The steady-state content of five representative cIV subunits (COX4I1, MT-CO1, MT-CO2, COX5A, and COX6C) was examined by denaturing SDS-PAGE followed by Western blot analysis and immunodetection in two representative clones of each COX4I2 KO, COX4I1 KO, and COX4I1/4I2 KO in comparison with wild-type (wt) HEK293. Representative WB images are shown in Figure 1a, where COX4I1 and MT-CO2 were undetectable, COX5A and COX6C only barely detectable, and MT-CO1 significantly decreased in COX4I1 KO and double COX4I1/4I2 KO (Figure 1a,b, Figure S1a). In contrast, COX4I2 KO cells showed a similar pattern to the wt. Densitometric quantification revealed that relative levels of studied cIV subunits in COX4I2 KO clones ranged between 55 and 120% of controls with significant decrease of MT-CO2 content when data were normalized to actin levels (Figure S1a). However, upon normalization to citrate synthase levels (CS, marker of mitochondrial mass), cIV subunits content was unaffected (Figure 1b). Importantly, COX4I1 KO in HEK293 cells did not trigger expression of the alternative isoform COX4I2 (Figure 1a). The functionality and specificity of the antibodies used to detect COX4I1 or COX4I2 was confirmed using positive control samples from COX4I1/4I2 KO transfected with the cDNA of the corresponding genes—knock-in (KI) of either COX4I1 or COX4I2 isoforms (Figure 1c). As we showed previously [15], HEK293 cells (wt) do not show detectable levels COX4I2 protein. On the contrary, COX4I1 isoform was detected by both monoclonal (COX4I1) and polyclonal (dual COX4 isoform-specific) antibodies. COX4I2 protein was detected only in the positive control COX4I2 knock-in cell line (Figure 1c).

In addition to classical electrophoretic and immunodetection approach, relative levels of cIV subunits were assessed in a high-throughput manner using mass spectrometry label-free quantification (MS LFQ). Analysis of two technical replicates of two representative clones for each COX4I2 KO, COX4I1 KO, and COX4I1/4I2 KO yielded reliable data for nine cIV subunits (Figure 1d). These confirmed our previous findings of generalized cIV subunit deficiency in COX4I1 KO and COX4I1/4I2 KO cells, with the most affected subunit besides the KO-targeted COX4I1 being MT-CO2. COX4I2 KO clones were not significantly changed as cIV subunit amounts were comparable to wild-type cells (Figure 1d).

Steady-state levels of representative subunits of other OXPHOS complexes (NDUFA9 of cI, SDHA of cII, UQCRC2 (CORE2) of cIII, and ATP5F1A ($F_1\alpha$) and ATP5MC1 (F_0c) of cV) were also examined by SDS-PAGE with Western blot immunodetection (Figure 1e). Differences between model cell lines were observed in protein level of NDUFA9, which was decreased in COX4I1, COX4I1/4I2, and COX4I2 KO clones when data were normalized to actin (Figure S1b). Upon normalization to citrate synthase, NDUFA9 was at a significant level approximately twofold lower, but only in the COX4I1/4I2 KO cells (Figure 1f). UQCRC2 subunit was also decreased in COX4I1/4I2 KO, but not in COX4I1 single KO. On the other hand, cII and cV subunits' levels were similar or even moderately increased in COX4I1/4I2 and COX4I1 KO cells (Figure 1e, Figure S1b).

The Western blot-based quantification data were complemented by MS LFQ analysis of the complete cellular proteome. Overall, we observed only slightly decreased quantity

of MitoCarta-annotated proteins in COX4I1/4I2 KO (Figure S2a,b) and COX4I1 KO (Figure S2b), which indicates that in spite of the cIV impairment, proteins can still be efficiently imported into mitochondria. However, it is also obvious that levels of cI subunits show profound and uniform decrease in COX4I1/4I2 and COX4I1 KO (Figure S2c). Data for individual subunits of OXPHOS complexes are presented as heatmaps (Figure 1g), quantitating 38 cI subunits, all 4 subunits of cII, 8 subunits of cIII, and 12 subunits of cV. From the perspective of multi-subunit comparison, the decrease of NDUFA9 shown by Western blot analysis was confirmed by MS LFQ analysis. The levels of cI subunits were between 1.5- and 3-fold decreased in COX4I1 and COX4I1/4I2 KO clones, while levels of cI subunits in COX4I2 KO were comparable to wild-type. On average, levels of cIII subunits were slightly decreased in COX4I1/4I2 KO cells, but it was less apparent in COX4I1 KO (Figure 1g, Figure S2c). The remaining OXPHOS complexes, cII and cV, were not altered in the COX4I1 and COX4I1/4I2 KO clones. Consistently, the levels of mitochondrial proteins overall, as well as of individual OXPHOS complexes in COX4I2 KO clones were not significantly affected, with the exception of cIII that was slightly decreased (Figure 1g, Figure S2b,c).

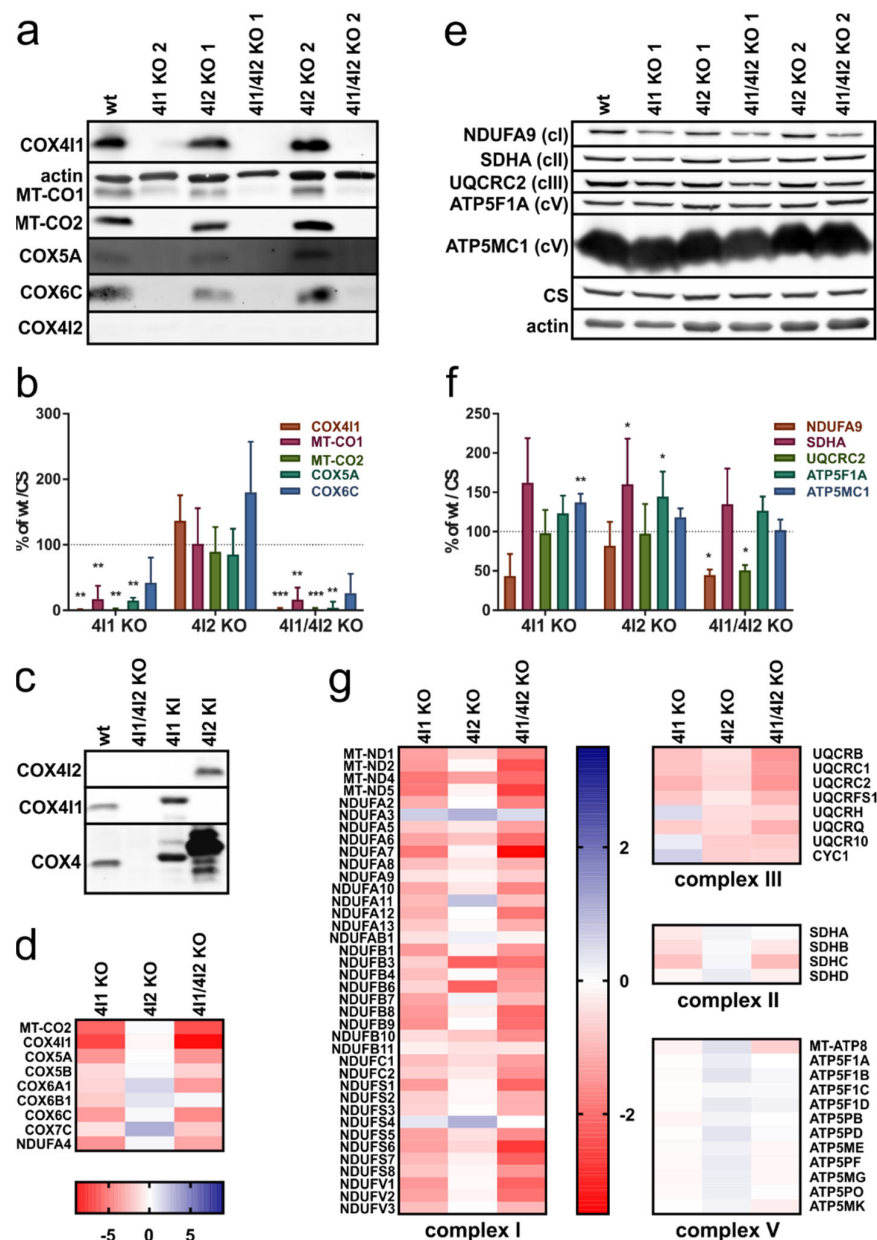


Figure 1. (a) Representative Western blot analysis of cytochrome c oxidase (cIV) subunits in KO clones. Whole cell lysates (30 μ g of protein) of HEK293 (wild-type (wt)), COX4I1 KO, COX4I2 KO, and COX4I1/4I2 KO clones were subjected to SDS-PAGE and Western blotting. Actin and citrate synthase (CS) antibodies were used as a loading control. (b) Quantification of cIV subunit content normalized to citrate synthase. Data represent mean $\pm$ SD of wt ($n = 2$), COX4I1 KO ($n = 4$), COX4I2 KO ($n = 4$), and COX4I1/4I2 KO cells ($n = 3$). Statistically significant differences between wt and KO cells were calculated using ANOVA in GraphPad Prism 8. Asterisks (*) represent p -value: * < 0.05 ; ** < 0.01 ; *** < 0.001 . (c) COX4I2 antibody testing. Whole cell lysates (30 μ g of protein) of HEK293 (wt), COX4I1/4I2 KO, and COX4I1/4I2 KO, transfected with cDNA constructs of either COX4I1 (COX4I1 KI) or COX4I2 (COX4I2 KI), were subjected to SDS-PAGE and Western blotting followed by detection with antibodies specific for COX4 isoforms (COX4I1 and COX4I2, respectively), as well as with polyclonal antibody detecting both COX4 isoforms. (d) Proteomic analysis of relative content of cIV subunits using mass spectrometry label-free quantification (MS LFQ). Figure shows decreased content of cIV subunits in COX4I1 and COX4I1/COX4I2 KO clones, with MT-CO2 being the most affected. COX4I2 gene KO does not have deleterious effect on cIV subunit level. Data in heat map represent $\log_2$ values of quantity fold changes relative to wild-type, according to color scale shown below the heat map. (e) Western blot analysis of representative oxidative

phosphorylation (OXPHOS) subunits for complexes I, II, III, and V. Whole cell lysates (30 μ g of protein) of HEK293 (wt), COX4I1 KO, COX4I2 KO, and COX4I1/4I2 KO clones were subjected to SDS-PAGE. Immunodetected actin and citrate synthase were used as loading controls. The antibodies used are listed in Table S1. (f) Quantification of OXPHOS complex subunits content normalized to citrate synthase. Data represent mean $\pm$ SD of wt ($n = 2$), COX4I1 KO ($n = 4$), COX4I2 KO ($n = 4$), and COX4I1/4I2 KO cells ($n = 3$). Statistically significant differences between wt and KO cells were calculated using ANOVA in GraphPad Prism 8. Asterisks (*) represent p -value: * < 0.05 ; ** < 0.01 ; *** < 0.001 . (g) Proteomic analysis of OXPHOS complex subunits using mass spectrometry label-free quantification (MS LFQ). Data in heat map represent $\log_2$ values of quantity fold changes relative to wild-type, according to the color scale shown at the respective heat maps.

3.2. Compromised OXPHOS Function in COX4I1-Lacking Cells Was Compensated by Increased Glycolysis

To characterize the bioenergetic phenotype of the KO clones, we examined the rates of mitochondrial respiration and glycolysis (Figure 2). After recording basal mitochondrial respiration (oxygen consumption rate (OCR); Figure 2a) and glycolysis (extracellular acidification rate (ECAR); Figure 2b), addition of oligomycin (inhibitor of cV) caused decrease of OCR in wild-type HEK293 as oxygen consumption coupled to ATP synthesis was inhibited and compensated by increased glycolytic activity represented by ECAR. Addition of chemical protonophore FCCP (which uncouples ADP phosphorylation and oxidation processes) increased OCR, allowing us to determine maximal electron transport chain (ETC) capacity. After final addition of inhibitors rotenone, antimycin A, and 2-deoxyglucose, both OXPHOS and glycolysis were inhibited, and therefore OCR and ECAR decreased to minimum and residual background rates were recorded.

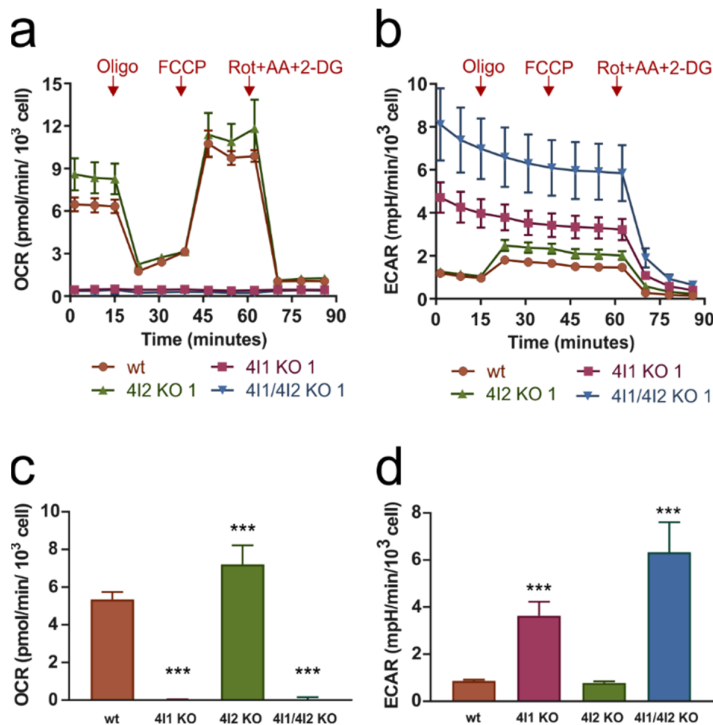


Figure 2. Parallel measurements of (a) cellular oxygen consumption rate (OCR) and (b) extracellular acidification rate (ECAR) were performed by Seahorse XF Bioenergetic Analyzer with 10 mM glucose as a substrate, and after subsequent additions of 1 μ M oligomycin (Oligo); 1 μ M FCCP ; and inhibitor cocktail of 1 μ M rotenone, 1 μ g/mL antimycin A, and 100 mM 2-deoxyglucose (Rot + AA + 2DG). Data are plotted as mean values $\pm$ SD of independent experiments (biological replicates) for control HEK293 cells (wt, $n = 5$), COX4I1 KO clone 1 ($n = 5$),

COX4I2 KO clone 1 ($n = 4$), and COX4I1/4I2 KO clone 1 ($n = 3$) and demonstrate complete absence of mitochondrial OXPHOS activity in COX4I1 and COX4I1/4I2 KO clones. (c) OCR and (d) ECAR bar graphs represent mean $\pm$ SD basal rates after subtraction of background rates in control HEK293 cells (wt, $n = 5$), COX4I1 KO clone 1 ($n = 5$), COX4I2 KO clone 1 ($n = 4$), and COX4I1/4I2 KO clone 1 ($n = 3$). Statistically significant difference of KO clones versus wild-type HEK293 were calculated using ANOVA in GraphPad Prism 8. Asterisks (*) represent p -value: *** < 0.001 .

COX4I2 KO pattern of respiratory and glycolytic fluxes was comparable to wild-type (Figure 2a,b), the OCR was even slightly increased compared to wild-type (Figure 2c), while ECAR was not significantly changed in COX4I2 KO (Figure 2d). Consistent with the profound decrease in cIV subunit levels, COX4I1 and COX4I1/4I2 KO clones showed complete absence of OXPHOS activity (OCR) or response to additions of uncoupler and inhibitors (Figure 2a). Thus, ATP production was independent of OXPHOS. Complete impairment of OXPHOS function was compensated by significantly increased glycolytic activity (ECAR) compared to wt cells (Figure 2b,d). Moreover, ECAR displayed no response to oligomycin, indicating that energy requirements of cells lacking COX4I1 are met by high steady-state glycolytic rates, likely operating near its full capacity. ECAR seemed to be higher for COX4I1/4I2 KO clone than for COX4I1 KO clone, but this probably reflected unique metabolic settings of individual KO clones.

Importantly, the respiratory OXPHOS impairment can be complemented by knock-in (KI) of either COX4I1 or COX4I2 isoforms of cIV subunit 4. Expression of either isoform on the COX4I1/4I2 KO background fully restored the respiratory pattern to match control cells, as was already shown by respirometric data in our previous study [25].

3.3. Effect of COX4 KO on OXPHOS Complexes Content and Assembly Status

To examine effect of OXPHOS subunits changes caused by COX4 isoforms knock-outs, we studied content and assembly status of OXPHOS complexes in native state by BN-PAGE followed by Western blot and immunodetection (Figure 3).

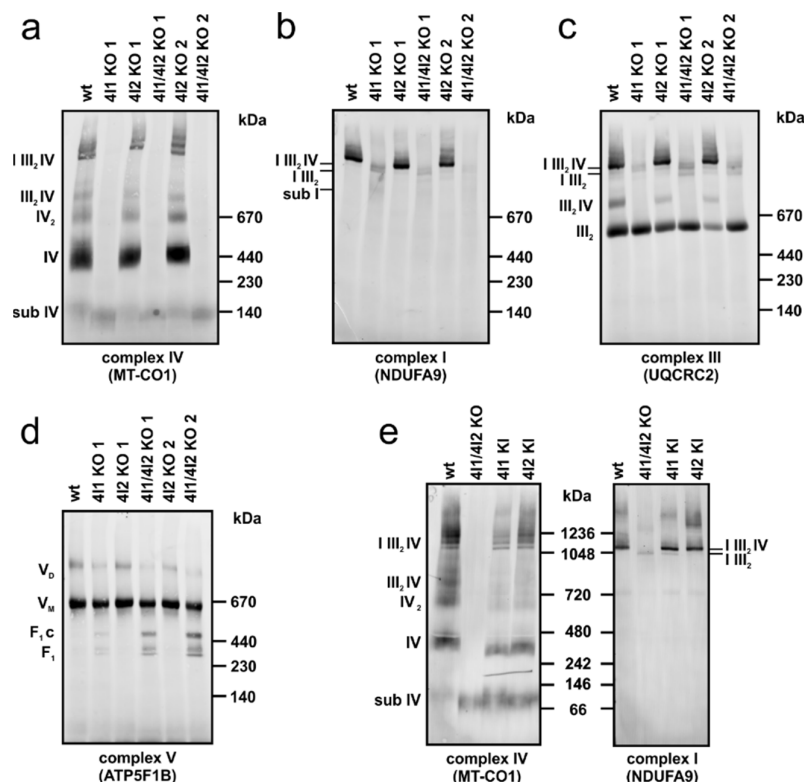


Figure 3. Western blot analysis of OXPHOS complexes and supercomplexes. Representative images of three independent analyses are shown. Digitonin solubilized mitochondria (30 µg of protein) of HEK293 (wt), COX4I1 KO, COX4I2 KO, and COX4I1/4I2 KO clones were subjected to blue-native (BN)-PAGE to detect OXPHOS complexes: (a) complex IV using MT-CO1 antibody, (b) complex I using NDUFA9 antibody, (c) complex III using UQCRC2 antibody, (d) complex V using ATP5F1B antibody. (e) Complex IV and complex I content and assembly status comparison of COX4I1/4I2 KO and COX4I1/4I2 KO cells rescued by overexpression of either COX4I1 (4I1 KI) or COX4I2 KI (4I2 KI). The antibodies used are listed in Table S1.

Immunodetection with an antibody against MT-CO1 subunit revealed the total absence of all assembled forms of cIV in COX4I1 and COX4I1/4I2 KO clones. Residual MT-CO1 subunit was detected with apparent migration around 130 kDa, likely representing the S1 (early MITRAC) assembly intermediate (Figure 3a). Early incorporating subunit COX5A, which also showed residual presence in SDS/PAGE experiments, was not detected in association with MT-CO1 in COX4I1 or COX4I1/COX4I2 KO clones (not shown). In contrast, COX4I2 KO displayed a band pattern and amount of cIV-containing complexes comparable to wild-type, including cIV monomer (IV), dimer (IV₂), and III₂IV and I III₂IV supercomplexes (Figure 3a). cI detection under native conditions using NDUFA9 antibody also confirmed the findings from SDS/PAGE and MS LFQ, showing a profound decrease in fully assembled cI in COX4I1 and COX4I1/4I2 KO clones. Moreover, while cI was detected mainly in supercomplexes (I III₂IV) in wild-type and COX4I2 KO, the remaining cI in COX4I1-lacking clones was mainly present in association with cIII (I III₂) or detected as a faint band corresponding to a cI assembly intermediate (sub I) (Figure 3b). Detection of cIII-containing complexes using an antibody against UQCRC2 subunit showed major changes in complex distribution within various assembly forms as a result of COX4I1 absence, rather than changes in total enzyme content. In wild-type and COX4I2 KO cells, cIII was detected as dimer (III₂), the III₂IV complex, and in supercomplexes. In contrast, lack of cIV following COX4I1 gene knock-out shifted almost all of cIII to non-associated dimer (III₂), and minor portion was bound to the residual cI in (I III₂) (Figure 3c). A mild effect of cIV absence was also observed on cV, detected by ATP5F1B antibody, which showed a lower amount of cV dimer, and also minor accumulation of F₁ and F₁-c subassemblies in COX4I1 and COX4I1/4I2 KO clones (Figure 3d).

WB analysis of BN-PAGE revealed that content of complexes I and IV, as well as their ability to form supercomplexes was restored in cells expressing either COX4 isoforms on the background of COX4I1/4I2 KO (Figure 3e). Comparable patterns of the bands detected by both MT-CO1 and specific COX4I2 antibody in the COX4I2 KI cells confirmed the efficiency of isoform 2 to act in de novo complex assembly and its incorporation into supercomplexes (Figure S3).

The content and assembly status of respiratory chain complexes was further examined in COX4I1/4I2 KO (clone 2) by SILAC/complexome profiling analysis, comparing the amounts and assembly status to those of the control HEK293 (wt) (Figure 4). Due to the profound cIV deficiency in these cells, only two cIV subunits, COX5B and NDUFA4, were detected in the KO sample. Both subunits migrated in the low molecular weight region, thus representing unassembled subunits or early assembly intermediates. cIV-associated proteins HIGD1A and HIGD2A retained robust signal in KO cells, where a portion of their signal comigrated with the COA3 protein. The shift of COA3 migration in KO clones to the region around 130 kDa in COX4I1/4I2 KO likely represents the cIV assembly intermediate that was detected with MT-CO1 antibody in WB experiments (Figure 3a), as COA3 is a MT-CO1-interacting protein [20]. In accordance with BN-PAGE WB analysis and proteomic LFQ studies, SILAC-based complexome profiling confirmed the profound decrease in complex I displayed by the KO cells, being decreased approximately fivefold relative to control HEK293. In control cells, complex I was distributed between respirasome (I III₂IV) and I III₂ supercomplexes. Interestingly, the low amounts of fully assembled cI in the KO were found to either be associated to cIII in the I III₂ supercomplex, or as cI assembly intermediates migrating at ≈750 kDa. These species

impairment, or rather caused by a defect in earlier steps of protein expression. Labeled proteins were separated by SDS-PAGE and detected by phosphor storage screen fluorescence (Figure 5a). These pulse-chase experiments revealed that apart from the MT-ATP6 and MT-ATP8 components of cV, the majority of mtDNA-encoded OXPHOS subunits were synthesized less in COX4I1 KO and COX4I1/4I2 KO clones than in the wt HEK293 cells in the “pulse” samples. Again, COX4I2 KO clones were not affected, having synthesis rates and protein patterns comparable to the wild-type. Detection of labeled proteins following the 24 h chase in non-labeling medium allowed for the estimation of protein turnover. In general, levels of labeled proteins were decreasing compared to pulse in all analyzed cell lines (Figure 5a). Strikingly, the MT-CO2/MT-CO3 double band completely disappeared during the chase period in COX4I1 and COX4I1/4I2 KO clones (Figure 5a, Figure S5), reflecting their degradation due to an inability to assemble, as suggested by previous data. In contrast, residual levels of MT-CO1 were still detectable after the 24h chase. Concerning other OXPHOS complexes, levels of cIII and cV components displayed higher stability than cI (MT-ND1, MT-ND2) and cIV (MT-CO1-3) subunits in cells lacking COX4I1 (Figure 5a, Figure S5), in agreement with data on steady-state levels of these complexes. Therefore, cIV deficiency caused by COX4I1 gene KO may have deleterious effect on mitochondrial proteostasis at multiple levels, affecting the biogenesis of the OXPHOS complexes, especially cIV and cI, from synthesis to assembly.

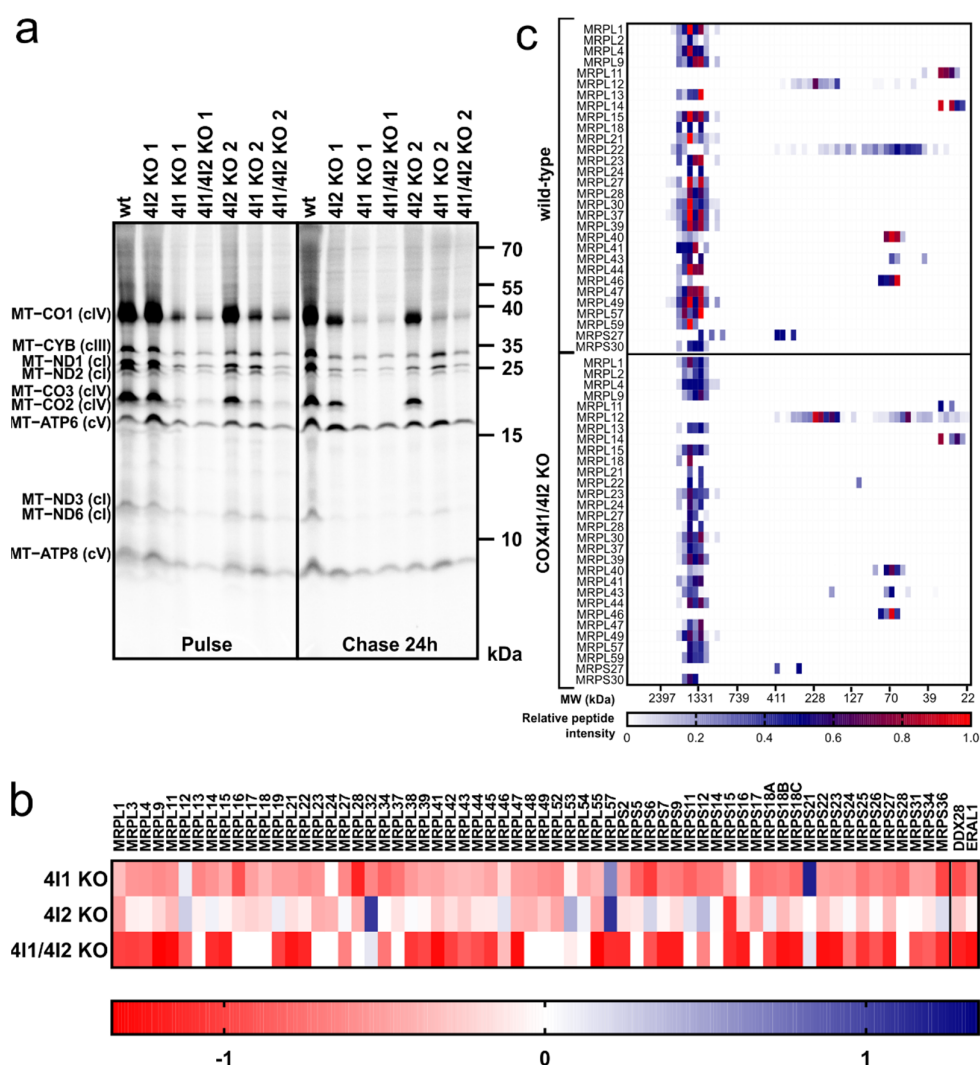


Figure 5. Mitochondrial protein synthesis. (a) ^{35}S in vivo labeling of mtDNA-encoded OXPHOS subunits. Representative image of two independent experiments showing autoradiographic detection of labeled proteins in 60 μg protein of whole cell lysates analyzed by SDS-PAGE uncovers lower “pulse” level of most mtDNA-encoded proteins due to COX4I1 gene KO. (b) MS LFQ analysis revealed lower quantity of mitochondrial ribosomal proteins in COX4I1 and COX4I1/4I2 KO clones. COX4I2 KO clones were comparable to wild-type HEK293 cell line. Data in heat map represent $\log_2$ values of quantity fold changes relative to wild-type. (c) SILAC/complexome profiling of mitochondrial ribosomal proteins. Heatmap displays relative content and migration of proteins constituting large and small subunits of mitochondrial ribosome. Data represent relative peptide intensity profile of each protein normalized to the slice with the highest intensity, averaged from duplicate experiments.

Interestingly, the MS LFQ dataset provided further support for the hypothesis of disturbed mitochondrial translation, as content of mitochondrial ribosomal proteins showed a generalized decrease of about 1.5-fold in COX4I1 and COX4I1/4I2 KO clones (Figure 5b, Figure S2). Moreover, content of ribosomal assembly factors DDX28 and ERAL1 was decreased twofold in COX4I1-lacking cells (Figure 5b). MS LFQ data were confirmed in the SILAC/complexome profiling experiment, which also showed 1.5- to 2-fold reduction of both large and small mitoribosome subunits in COX4I1/4I2 KO (clone 2) in comparison with control HEK293 (Figure 5c).

4. Discussion

Previously, loss of function (LOF) models of OXPHOS genes in mammalian cells were relying on RNA interference (RNAi) technologies or deriving embryonic fibroblasts from mouse knockout animals (MEFs). The arrival of the relatively straightforward CRISPR-Cas9 gene editing technology [41] conveniently broadened experimental repertoire for construction of LOF models in human cell lines, avoiding the problems with residual content of the targeted protein encountered in the case of RNAi.

A high-throughput study employing CRISPR death screen searching for OXPHOS essential genes in human myelogenous leukemia K562 cells identified COX4I1 as KO-lethal under conditions when galactose was the sole carbohydrate source [50]. Using a targeted approach, we prepared single COX4I1 and COX4I2 KO cells, as well as double COX4I1/4I2 KO of both isoforms in HEK293 cells. Even under standard culture conditions, cultivation of COX4I1-lacking clones represented a significant challenge due to their highly glycolytic phenotype. The proliferation of KO cells was improved using richer DMEM/F-12 medium supplemented with uridine. Therefore, careful selection of a metabolically flexible cell line and optimization of culturing conditions is necessary for successful experimentation with knock-outs of essential OXPHOS genes.

As COX4 is an early incorporating subunit [4,13,16,51], impaired assembly of cIV enzyme was expected in COX4I1 and COX4I1/4I2 KO clones. Indeed, native electrophoresis confirmed presence of MT-CO1-containing cIV assembly intermediate, most likely representing a MITRAC complex [20]. Fully assembled cIV monomer and dimer were undetected. Moreover, the complete absence of fully assembled cIV was coupled with loss of supercomplexes containing cIV - III₂IV and I III₂IV (respirasome). Thus, our model cell lines unambiguously confirmed that COX4I1 subunit is essential for enzyme assembly process.

The possible compensation of COX4I1 isoform loss by expression of the second isoform, COX4I2, was tested, as isoform replacement mechanism was described while lost COX7A1 was substituted by its partner, COX7A2 isoform, in COX7A1 KO mice heart [52]. However, the COX4I2 isoform was not detected even after COX4I1 gene KO, and thus substitution of missing COX4 isoform 1 by COX4I2 isoform was not activated in HEK293 cells. Therefore, switch of COX4 isoforms seems to depend strictly on oxygen-regulated expression [53,54] and was not triggered by energy crisis in the studied cell line. A COX4 isoform switch in HEK293 cells is also improbable since COX4I2 isoform is not expressed in this cell line under normoxia. However, when the expression was forced by transfecting

COX4I2 on the COX4I1 KO background, the second isoform was able to complement the cIV defect both at the structural and functional levels, as also seen in our previous study [15].

The catalytic function of cIV enzyme in COX4I1 KO clones was expected to be completely impaired due to loss of catalytic core mtDNA-encoded subunit MT-CO2, indispensable for cIV catalytic function [55]. The results of Seahorse measurement confirmed our hypothesis, as COX4I1 KO and COX4I1/4I2 KO clones demonstrated complete absence of oxygen consumption (OCR) and thus OXPHOS activity. Impaired OXPHOS function was compensated in COX4I1 KO and COX4I1/4I2 KO clones by increased glycolytic activity (ECAR). Therefore, ATP production is quite independent on OXPHOS and energy demands of COX4I1 KO cells are covered by ATP production on substrate level in glycolysis.

Since COX4I2 is not normally expressed in HEK293 cells cultured in normoxic conditions, the assembly processes of cIV and other OXPHOS complexes (cI, cII, cIII, cV) were not affected by COX4I2 gene KO. Moreover, cIV-containing supercomplexes were detected in COX4I2 KO clones comparably to wild-type. Furthermore, COX4I2 gene KO did not have any impact on mitochondrial respiration, as metabolic phenotype determined by Seahorse measurements was comparable to wild-type. Thus, no significant and functionally relevant changes were observed within COX4I2 KO clones in comparison with wild-type HEK293.

The most affected OXPHOS component apart from cIV in COX4I1 KO and COX4I1/4I2 KO clones was cI, as shown by NDUFA9 immunodetection in BN-PAGE WB and more comprehensively by both label-free and SILAC-based quantitative proteomics. Previous studies reporting similar cases of cIV–cI biogenesis interdependency in mouse cells caused by knock-out of COX10 [36], or by knock-down of COX4 expression [37], concluded that cIV absence caused defect in cI assembly. These findings were in line with the hypotheses that complexes I, III, and IV need to associate into supercomplexes to successfully proceed through their interdependent assembly pathways [34,35]. Recently, this hypothesis was confirmed by study showing that loss of cIII mtDNA-encoded subunit MT-CYB impairs assembly of not only cIII but also of cI and cIV. Specifically, cI assembly was stalled in late assembly intermediate before addition of the matrix-facing N-module, which occurs already in pre-respirasome association harboring cIII and also part of cIV [35]. The accumulation of cI assembly intermediates and the fact that residual mature cI was only found in association with III₂ was also evidenced in our COX4I1/4I2 KO cell line using complexome profiling. This indicated that impaired cI assembly was involved in this case of cI-deficiency due to the lack of cIV assembly. In contrast to recent findings [40], in our BN-PAGE or complexome profiling experiments, we did not observe association of the residual cIV subunits or their assemblies with the I III₂ supercomplex. In that study, Δ MT-CO2 cybrids harbored non-canonical subassembly of MT-CO1, COX5B, and HIGD2A binding to III₂ and preventing its turnover [40]. Even though these subunits were not completely absent in COX4I1 lacking HEK293, their content was decreased in comparison with the Δ MT-CO2 cybrids, perhaps to a level unable to mediate the I III₂ stabilization. It is also possible that COX4I1 absence prevents assembly of this non-canonical subcomplex. Nevertheless, impairment of cI assembly or stability might not be the only components of the observed cI deficiency, as our data further indicate that the interdependency started already at earlier levels of OXPHOS complex biogenesis. Indeed, the rate of mitochondrial protein synthesis was severely reduced both in COX4I1 single or COX4I1/4I2 knock-out cells. Notably, the synthesis of cIV and cI subunits was more affected than cIII MT-CYB and especially the mtDNA-encoded subunits of cV. This might reflect the recently reported spatial organization of mitochondrial translation [56], where translation of cV subunits occurs at separate mitoribosomes to facilitate their insertion directly into cristae membranes. Nevertheless, attenuation of mitochondrial translation was not observed in previously published cases of cIV–cI interdependency [35–38], indicating that complete absence of COX4 subunit affects cIV biogenesis at an

earlier step than other cIV defects. This might be due to assembly-controlled translational plasticity realized by MITRAC complex, where absence of early assembly nuclear-encoded subunits such as COX4 leads to arrest of translation of MT-CO1 subunit that remains stalled as a ribosome-nascent chain complex containing MITRAC component COX14 [57]. Therefore, cIV assembly cannot proceed. Moreover, a portion of mitochondrial ribosomes might stay occupied by MT-CO1 transcripts, which could explain decreased synthesis of other cIV and cI subunits. In cases of interdependency on the basis of COX10 and MT-CO1 mutations, COX4 subunit was detectable and MT-CO1 synthesis was proceeding without translation stalling [36,38]. The cIV defect was instead associated with MT-CO1 destabilization due to defective hemylation or C-terminus truncation. Therefore, another possible link between early phases of cI and cIV biogenesis may be represented by COA1 protein. This mammalian homologue of yeast COX1 translational regulator COA1 was initially identified as a cIV biogenesis factor [20]. However, it was recently shown that it also serves as a translational factor of cI ND2 subunit in a similar manner to COX14 function during MT-CO1 synthesis [58]. COA1 may coordinate biogenesis of cIV and cI as a bifunctional protein to ensure production of corresponding amount of these respiratory complexes. One could speculate that stalling of MT-CO1 within MITRAC complex may negatively influence the availability of COA1 for MT-ND2 synthesis. The ND2 assembly module of complex I serves as a core for addition of other assembly modules [4], and therefore its deficiency would have a significant effect on cI content. Nevertheless, our data indicate that attenuation of cIV and cI mtDNA-encoded subunits is generalized. This could be explained by the decreased content of mitochondrial ribosomal proteins identified by MS LFQ analysis in COX4I1 and COX4I1/4I2 KO cells, as well as by complexome profiling in COX4I1/4I2 KO. The putative mitoribosome insufficiency could be associated with decreased content of ribosomal assembly factors DDX28 and ERAL1 detected in MS LFQ dataset. Contribution of other secondary mechanisms negatively influencing mitoribosome levels should also be considered. Energetic insufficiency or changes in redox status caused by mitochondrial defects can trigger integrated stress response controlled by the ATF4 transcription factor, which was shown to involve reduction of the content of mitoribosome protein components and subsequent downregulation of mitochondrial protein synthesis [59]. However, residual cIV amounts are sufficient to prevent an associated defect in cI, as indicated previously by study using knock-down of COX4 in HEK293 cells, which displayed unchanged levels of complex I [60]. Complete clarification of the observed interdependency warrants further studies.

In summary, CRISPR-Cas9-mediated knock-out of COX4 isoforms confirmed its position as the initiator subunit of cIV assembly. In normoxia, the COX4I2 isoform is not significantly expressed in HEK293, nor is it induced upon deletion of isoform COX4I1, and therefore COX4I1 single KO and COX4I1/4I2 KO cells displayed the same phenotype. Importantly, knock-in of either isoform 1 or 2 into COX4I1/4I2 KO was able to complement cIV defect, indicating that even COX4I2 is able to initiate de novo enzyme assembly. COX4I1-lacking cells presented with cIV–cI biogenesis interdependency associated with yet a novel mechanism involving the decrease in the amounts of mitochondrial ribosomes and the downregulation of mitochondrial protein synthesis. This illustrates the complicated and intertwined mechanisms governing the biogenesis of mitochondrial respiratory chain complexes and opens the ground for further elucidation of such mechanisms.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4409/10/2/369/s1: Figure S1: Densitometric quantification of WB experiments. Figure S2: MS LFQ proteomic analysis. Figure S3: Complementation of COX4I1/4I2 KO by COX4I2 isoform. Figure S4: Quantitative analysis of pulse-chase experiments. Table S1: List of antibodies used for Western blot detection.

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Article

Cytochrome *c* Oxidase Subunit 4 Isoform Exchange Results in Modulation of Oxygen Affinity

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Abstract: Cytochrome *c* oxidase (COX) is regulated through tissue-, development- or environment-controlled expression of subunit isoforms. The COX4 subunit is thought to optimize respiratory chain function according to oxygen-controlled expression of its isoforms COX4i1 and COX4i2. However, biochemical mechanisms of regulation by the two variants are only partly understood. We created an HEK293-based knock-out cellular model devoid of both isoforms (COX4i1/2 KO). Subsequent knock-in of COX4i1 or COX4i2 generated cells with exclusive expression of respective isoform. Both isoforms complemented the respiratory defect of COX4i1/2 KO. The content, composition, and incorporation of COX into supercomplexes were comparable in COX4i1- and COX4i2-expressing cells. Also, COX activity, cytochrome *c* affinity, and respiratory rates were undistinguishable in cells expressing either isoform. Analysis of energy metabolism and the redox state in intact cells uncovered modestly increased preference for mitochondrial ATP production, consistent with the increased NADH pool oxidation and lower ROS in COX4i2-expressing cells in normoxia. Most remarkable changes were uncovered in COX oxygen kinetics. The p_{50} (partial pressure of oxygen at half-maximal respiration) was increased twofold in COX4i2 versus COX4i1 cells, indicating decreased oxygen affinity of the COX4i2-containing enzyme. Our finding supports the key role of the COX4i2-containing enzyme in hypoxia-sensing pathways of energy metabolism.

Keywords: mitochondria, OXPHOS, respiratory chain, cytochrome *c* oxidase, COX, COX4 isoforms, COX4i2, oxygen affinity, p_{50} , oxygen sensing

1. Introduction

Cytochrome *c* oxidase, the terminal enzyme of the electron transport chain, is an indispensable part of mitochondrial machinery needed for ATP production in mammalian cells (OXPHOS). In addition to three mitochondria-encoded subunits, which are necessary for COX catalytic function, eleven nuclear-encoded subunits build up the COX enzyme and participate in the regulation of COX enzyme activity, as well as in the regulation of the whole OXPHOS system [1]. The crucial position in the respiratory chain pathway, a large drop of Gibbs free energy during enzyme turnover, and relatively low in vivo reserve capacity [2] predispose COX to serve as a mitochondrial OXPHOS regulator. This is indeed reflected by the emergence of numerous regulatory subunits during the evolution of individual eukaryotic lineages [3], as well as by the discovery of numerous post-translational modifications signifying that COX has become a target of signaling pathways [4]. In mammals, six COX subunits have isoforms with expression regulated in developmental, tissue-specific (COX6a, 7a, 8, and 6b), and environmental manners (COX4, NDUFA4). Subunits 6a, 7a, and 8 all exist in two variants, the L (liver) isoform is expressed ubiquitously, and the H (heart) isoform

is expressed postnatally in heart and skeletal muscle [5]. In contrast to the L isoforms, the triplet of H isoforms functions as an ATP/ADP sensor and modulates COX turnover and its H^+/e^- efficiency [5]. An additional 7a variant, COX7a2L, is responsible for the association of complexes III and IV into supercomplexes [6]. A second isoform of subunit 6b was discovered in mammalian testis (6b-2). Subunit 6b forms part of the cytochrome *c* binding site, so the testis isoform was hypothesized to evolve in association with the testis isoform of cytochrome *c* [7].

The largest of the nuclear encoded subunits, COX4, is ideally predisposed to serve as a regulatory factor thanks to its interactions with other subunits throughout multiple parts of the COX enzyme. Its C-terminal part protrudes into the intermembrane space where it interacts with COX2 and helps shape the docking site for cytochrome *c*. The COX4 transmembrane helix is tightly associated with catalytic subunit COX1 and runs parallel alongside helices of nuclear-encoded subunits COX7b, COX8a, and COX7c. Moreover, the large membrane extrinsic domain of COX4 is located in the mitochondrial matrix and may function as a metabolic sensor. Indeed, COX4 has long been recognized as a target of allosteric ATP binding that decreases enzyme turnover, and this mechanism has been denoted as a second mechanism of respiratory control [8]. Furthermore, it was reported that ATP binding is abolished upon phosphorylation of serine 58 in COX4-1 by the protein kinase A pathway, with all components located in the mitochondrial matrix, and is positively regulated by CO_2 and thus may serve to match the rates of the Krebs cycle with the respiratory chain [9]. In yeast, two isoforms of the COX4 homolog COXV were discovered (COXVa and COXVb). Their ratio is exclusively regulated by oxygen concentration, with COXVb being expressed under hypoxia [10]. The second isoform of mammalian COX4, COX4i2, discovered in 2001, was found to be predominantly expressed in the lung, and to a minor extent in the heart and brain [11]. COX4i1 and COX4i2 are highly homologous in the C-terminal region; however, significant differences exist in the N-terminal part comprising the matrix domain. COX4i2 lacks the regulatory S58 residue, and in contrast to COX4i1, contains three cysteine residues that may form disulphide bridges under oxidative conditions and thus function as redox sensors [11]. Importantly, and similarly to yeast, the mammalian COX4 isoform expression is regulated by oxygen availability [12–14]. Interestingly, the gene duplication event giving rise to the isoforms occurred independently in both eukaryotic lineages. This suggests that the COX4 position in the enzyme enables the isoform switch to serve as a homeostatic response to optimize respiratory chain function according to oxygen availability. In support of that, studies on the mouse COX4i2 knockout model revealed that this protein is essential for acute hypoxic pulmonary vasoconstriction [15,16]. Later it was shown that COX4i2 is also likely involved in oxygen sensing by carotid bodies [17,18]. In human cells, the function of COX4 isoforms has been partially characterized in HeLa and HEK293 cells where COX4i2 expression under hypoxia increased enzyme activity and decreased ROS production from mitochondria [13]. Increased activity of the COX4i2-containing enzyme was also found in astrocytes, associated with decreased sensitivity to allosteric inhibition by ATP [19]. In contrast, a recent study in glioblastoma cells reported increased activity of the COX containing COX4i1 isoform [20]. Some of these contradictory findings may be attributed to cellular models used in these studies, which were largely based on shRNA knock-down technology. Such an approach cannot completely abolish the expression of targeted proteins, and therefore, at least a minor expression of both isoforms is always present.

To unambiguously characterize the functional differences between COX4 isoforms, we constructed cellular models with exclusive knock-in of respective isoforms in the background of double CRISPR-Cas9-mediated COX4i1/2 knockout in HEK293 cells. We report that COX4 isoforms 1 and 2 are able to modulate COX oxygen affinity and can affect mitochondrial OXPHOS and the redox state even under normoxic conditions.

2. Materials and Methods

2.1. Cell Culture

HEK293 (human embryonic kidney) cells (ATCC® CRL-1573™) were cultivated under standard conditions (37 °C, 5% CO_2 atmosphere) in nutrient-rich DMEM/F-12 (31331-028, Thermo Fischer

Scientific, Waltham, MA, USA) supplemented with 10% (*v/v*) FBS (Thermo Fischer Scientific, 10270-106), 40 mM HEPES, 50 μ M uridine, and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, Thermo Fischer Scientific, 15140-122) to facilitate growth of highly glycolytic knock-out cells.

2.2. Preparation of HEK293 Cellular Knock-Out Models

Double COX4i1 and COX4i2 gene knock-out (KO) was introduced into HEK293 cells by CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) knock-out technology employing Cas9-D10A paired nickase with two chimeric RNA duplexes [21]. First, the COX4i2 KO HEK293 model was established and was subsequently used to introduce COX4i1 KO to obtain COX4i1/2 KO cells.

gRNAs for COX4i1 and COX4i2 obtained from a commercially available library (Merck, Darmstadt, Germany) were used for knock-out preparation. The HSR0002687616/HSR0002687619 gRNA pair was chosen to generate COX4i2 KO and the HSL0001377215/HSR0001377224 gRNA pair for COX4i1 KO. gRNAs were received in the form of the expression plasmid (U6-gRNA, expressed by the U6 promoter), along with the expression plasmid for Cas9-D10A nickase fused with GFP (Merck). The efficiency of different combinations of gRNA pairs for the Cas9-D10A nickase system was determined by SURVEYOR assay (Integrated DNA Technologies, Coralville, IA, USA). Cells were transfected with relevant plasmids using Metafectene Pro (Biontex Laboratories GmbH, München, Germany), incubated for 72 h, trypsinized, and then diluted to a concentration of 20 cells/mL. This suspension was aliquoted (100 μ L) into 96-well plates. Wells containing single-cell colonies were identified and further cultivated. Confluent cells in 6-well plates were harvested and used for selection of a knock-out clone by sequencing of CRISPR-targeted sites to identify nonsense mutations resulting in premature stop codons and by SDS-PAGE WB to confirm the complete absence of COX4 isoforms at the protein level.

2.3. COX4 Isoform Overexpression

COX4i1 (CCDS10955.1) and COX4i2 (CCDS13187.1) isoforms of the human COX4 subunit full-length cDNAs including the 3' end sequence encoding the FLAG tag (DYKDDDDK) were obtained as synthesized DNA strands with 5' and 3' overhangs containing restriction sites used for insertion into the pcDNA3.1⁺ mammalian expression vector (Thermo Fischer Scientific). COX4i1-FLAG or COX4i2-FLAG constructs were transfected into COX4i1/2 KO cells using Metafectene Pro (Biontex Laboratories GmbH); the selection of stably-transfected cells was started 48 h post-transfection with 1 mg/mL G418. Single-cell clones were later established from G418-resistant cells, and three clones of each COX4i1 and COX4i2 knock-in cells were used for the following experiments.

2.4. COX4i2 Site-Directed Mutagenesis

The COX4i2-FLAG pcDNA3.1⁺ construct was used to introduce mutations leading to single amino acid (AA) substitutions of three individual cysteine residues at positions 40, 54, and 108. Each cysteine was replaced by three AA variants, serine, representing a sterically similar amino acid where only sulphur is replaced by oxygen, or glycine and valine, which represent amino acids present at homologous positions in COX4i1. Mutations were introduced using the QuikChange Lightning kit (Agilent, Santa Clara, CA, USA), using primers suggested by the manufacturer's on-line primer design tool. Mutagenesis was confirmed by sequencing, and mutated constructs were used for knock-in into COX4i1/2 KO cells as was the wild-type COX4i2-FLAG vector.

2.5. Electrophoretic Analyses

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), specifically tricine SDS-PAGE, was used for separation of proteins according to their molecular weight under denaturing conditions [22]. Frozen aliquots of harvested cellular pellets were suspended in SLB buffer (sample lysis buffer; 2% (*v/v*) 2-mercaptoethanol, 4% (*w/v*) SDS, 50 mM Tris (pH 7.0), 10% (*v/v*) glycerol, 0.02% Coomassie Brilliant Blue R-250) to a final concentration of 2–5 mg protein/mL. Samples were sonicated and then incubated for 20 min at 40 °C. Samples (10–50 μ g of protein as

indicated) were separated on 12% gel using a Mini-PROTEAN III apparatus (Bio-Rad, Hercules, CA, USA).

Blue-native (BN-PAGE) electrophoresis [23] was used for analysis of native protein complexes. For protein complex solubilization, mild non-ionic detergent digitonin was used, as it maintains OXPHOS complexes and their supercomplexes and enables their detection.

Mitochondria from HEK293 cells were released by hypotonic shock and isolated using differential centrifugation as described [24] and then stored as dry pellets at -80°C . Mitochondrial pellets were resuspended in solubilization buffer (50 mM NaCl, 50 mM imidazole/HCl, 2 mM 6-aminocaproic acid, 1 mM EDTA; pH 7.0) at a protein concentration 10 mg/mL. Digitonin (20% (*w/v*) stock solution) was added at 6 g/g protein, and solubilization proceeded on ice for 10 min. Samples were centrifuged at $30,000\times g$ (20 min, 4°C), and supernatant containing released membrane proteins was collected. Then, glycerol was added to a final 5% (*v/v*) concentration, and Coomassie Brilliant Blue G-250 (CBB) was supplemented according to a detergent/dye ratio of 8:1 (*w/w*). Samples were separated on 5–16 % gradient polyacrylamide gel using a Mini-PROTEAN III apparatus (Bio-Rad).

2.6. Western Blot and Immunodetection

Proteins separated by electrophoresis were transferred onto PVDF (polyvinylidene difluoride) membrane (Immobilon FL 0.45 μm , Merck) by semi-dry electroblotting using a Transblot SD apparatus (Bio-Rad). After blotting, the PVDF membrane was washed for 5 min in TBS (150 mM Tris-HCl, 10 mM NaCl; pH 7.5) and blocked in 5% (*w/v*) fat-free dry milk diluted in TBS for 1 h. Then, the membrane was washed 2×10 min in TBST (TBS with 0.1% (*v/v*) detergent Tween-20). For immunodetection, the membrane was incubated for 2 h in primary antibody (diluted in TBST) at room temperature. The following primary antibodies were used in the study: FLAG (Merck F1804), complex IV (COX1: Abcam 14705, Cambridge, UK, COX2: Abcam 110258, COX4i1: Abcam 14744, COX4i2: Abnova H00084701-M01, Taipei City, Taiwan, COX5a: Abcam 110262, COX6c: Abcam 110267), complex I (NDUFA9: Abcam, 14713), complex II (SDHA: Abcam 14715), complex III (Core 2: Abcam 14745), and citrate synthase (Abcam 129095). For quantitative detection, the corresponding infra-red fluorescent secondary antibodies (Alexa Fluor 680, Thermo Fisher Scientific; IRDye 800, LI-COR Biosciences, Lincoln, NE, USA) were used. Detection was performed using the fluorescence scanner Odyssey (LI-COR Biosciences) and signals were quantified by ImageLab software (Bio-Rad).

2.7. Immunoprecipitation and Mass Spectrometry Analysis

Whole-cell digitonin solubilizates (2 g/g of protein) from COX4i1-FLAG KI, COX4i2-FLAG KI, and COX4i1/2 KO (background control not containing FLAG-tagged protein) cells were immunoprecipitated with anti-FLAG M2 magnetic beads (Merck M8823). Washed beads with captured immunocomplexes were digested “on beads” with trypsin using the sodium deoxycholate procedure as described in [25]. Desalted peptide digests were separated on a 50 cm C18 column using a 1 h elution gradient and were analyzed in an Orbitrap Fusion (Thermo Fisher Scientific) mass spectrometer. Resulting raw files were processed in MaxQuant (v. 1.6.6.0, maxquant.org) with the label-free relative quantification (LFQ) algorithm MaxLFQ [26]. Downstream analysis and visualization were performed in Perseus (maxquant.org/perseus/) [27].

2.8. Cytochrome *c* Oxidase Activity

COX activity was analyzed using Oxygraph-2k (Oroboros, Innsbruck, Austria) in mitochondrial extracts essentially as described [28]. Mitochondria isolated from cells were suspended in buffer composed of 10 mM K-HEPES (pH 7.4), 40 mM KCl, 2 mM EGTA, 1% Tween 20, and protease inhibitor cocktail (Merck), sonicated, and centrifuged at $30,000\times g$ for 2 min. Then, 200 μg protein of supernatants was added to 2 mL oxygraph chambers. COX activity was followed as the rate of oxygen consumption in the presence of 20 mM ascorbate serving as a reductant for cytochrome *c* (bovine, Merck) titrated sequentially to reach final concentrations of 2.5–30 μM . Parallel measurement with COX activity fully inhibited by 0.5 mM KCN was always performed to subtract non-specific oxygen

consumption due to autoxidation of ascorbate. A hyperbolic Michaelis-Menten function was fitted to experimental data using Prism 8 software (GraphPad, San Diego, CA, USA).

2.9. High-Resolution Respirometry

Mitochondrial respiration was measured at 30 °C as described in [29] using Oxygraph-2k (Oroboros, Innsbruck, Austria). Freshly harvested cells (0.6 mg protein) were suspended in 2 mL of KCl medium (80 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 5 mM K-Pi, 10 mM Tris-HCl; pH 7.4), and digitonin (0.05 g/g) protein was used to permeabilize the plasma membrane. For measurements, the following substrates and inhibitors were used: 2 mM malate, 10 mM pyruvate, 10 mM glutamate, 10 mM succinate, 10 mM glycerol 3-phosphate, 1 mM ADP, 0.5 µM oligomycin, 100–300 nM FCCP, 0.5 µM rotenone, 10 mM malonate, 0.25 µM antimycin A, 2 mM ascorbate, 1 mM TMPD, 0.5 mM KCN. The oxygen consumption was expressed in pmol O₂/s/mg protein.

Affinity to oxygen was determined by the p_{50} value, the partial oxygen pressure (pO₂), at which the cellular respiratory rate is half-maximal, as described before [30]. Briefly, the volume-specific rate of oxygen consumption (oxygen flux) was calculated as the negative slope of the oxygen concentration recorded at 1 s time intervals. The signal was deconvoluted with the exponential time constant of the oxygen sensor (3 to 5 s) and corrected for the instrumental background, which is a linear function of experimental pO₂ and results from oxygen consumption by the sensor and oxygen back-diffusion from low-capacity oxygen reservoirs. The p_{50} parameter was obtained from a hyperbolic function, $J_{O_2} = (J_{MAX} \cdot pO_2)/(p_{50} + pO_2)$, fitted over the low oxygen range of 0 to 1.1 kPa. All calculations were performed using routine functions of Datlab 2 software (Oroboros) [31].

2.10. XF Seahorse Bioenergetics Analyser Measurement

Parallel measurement of mitochondrial respiration and the glycolytic rate was performed using a Seahorse XFe24 analyzer (Agilent). One day prior to measurement, 3×10^4 cells were seeded in poly-L-lysine coated wells of the measuring plate and were cultivated in DMEM/F12 medium (see above). The next day, the microplate was washed with 1 mL of XF assay medium (modified DMEM, pH 7.4, 37 °C); 500 µL of the same medium with 0.2 % (w/w) BSA was pipetted, and the microplate was incubated at 37 °C for 30 min. Meanwhile, an XFe24 sensor cartridge was prepared by injection of substrates and inhibitors according to the described protocol [32] to record metabolic rates with endogenous substrates before any addition (basal), and after subsequent additions reaching final concentrations of 10 mM glucose (Glu), 1 µM oligomycin (Oligo), 1 µM FCCP (FCCP), and inhibitor cocktail of 1 µM rotenone, 1 µg/mL antimycin A, and 100 mM 2-deoxyglucose (Rot+AA+2DG). For precise normalization of rates according to cell counts, immediately after the Seahorse run, cell nuclei were stained by Hoechst 33342 (final concentration 5 µg/mL). Images of whole wells were acquired by a Cytation 3 Cell Imaging Reader (BioTek, Winooski, VT, USA) and were analyzed using Gen5 software (BioTek) to obtain cell counts for each well.

2.11. ROS Production

ROS production was estimated as 1 µM CM-H₂DCFDA (chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate, Thermo Fisher Scientific) fluorescence as in [33]. Cells (3×10^5) were seeded in poly-L-lysine coated 24-well plates, and the next day, cells were incubated for 2 h at 37 °C with CM-H₂DCFDA. The increase in the fluorescence was recorded with an Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 495/525 nm.

2.11. NAD⁺/NADH Ratio

Redox balance in terms of NAD⁺/NADH was measured in cell lines using the NAD⁺/NADH-Glo assay (Promega) essentially as described [34]. Briefly, 5×10^3 cells were seeded into 96-well plates one day prior to measurement. NAD⁺ and NADH concentrations in cell extracts were determined according to the manufacturer's protocol, and the luminescence signal was recorded with an Infinite M200 plate reader (Tecan Group Ltd.).

3. Results

3.1. Comparable COX Content and Composition in COX4i1 and COX4i2 KI Cells

To obtain an optimal research model for unbiased comparison of functional features of COX isozymes distinguished solely by the presence of either COX4i1 or COX4i2, we constructed HEK293-based cell lines with CRISPR/Cas9 mediated knock-out of both COX4 isoforms (COX4i1/2 KO), followed by stable knock-in of either isoform. Recombinant COX4 isoforms were expressed with the C-terminal FLAG tag to facilitate isoform content comparison as well as their immunoprecipitation using the same antibody.

For this study, three representative single-cell clones of both COX4i1 and COX4i2 knock-in cell lines (4i1 KI and 4i2 KI, respectively) were chosen. Exclusive expression of respective COX4 isoforms was verified in whole cell lysates by SDS-PAGE WB followed by immunodetection (Figure 1A). Of note here is that COX4i2 was not detected in parental HEK293 cells, suggesting that under normoxic conditions, HEK293 cells express exclusively the COX4i1 isoform. The content of the mtDNA-encoded subunit COX2 was identical in 4i1 and 4i2 KI, in both cases reaching approximately 50% of the COX2 level in parental HEK293 cells when normalized to the citrate synthase content (Figure 1A,C). Similarly, levels of other tested COX subunits, COX5a and COX6c, were comparable in 4i1 and 4i2 KI cell lines (Figure 1B,C). These data indicate a similar COX content in 4i1 and 4i2 KI, even though the quantification of the FLAG tag signal present on recombinant COX4 isoforms showed an approximately 2.5-fold higher COX4i2 steady-state level compared to COX4i1 in the respective KI cell lines (Figure 1B,D). To compare the content of other OXPHOS complexes in whole cell lysates, SDS-PAGE WB immunodetection was also performed with representative subunits of complexes I (NDUFA9), II (SDHA), and III (core 2). Their amount was not significantly different in 4i1 and 4i2 KI, corresponding to 85, 65, and 80% of the control levels of complexes I, II, and III, respectively (Figure 1B,C).

To analyze the assembly status of COX complexes and their interactions in OXPHOS supercomplexes, isolated mitochondria from 4i1 and 4i2 KI cells were solubilized with the mild detergent digitonin and were analyzed by BN-PAGE. Immunodetection of COX1, the core catalytic subunit, revealed that the COX content and its distribution between enzyme monomeric and dimeric forms, as well as its association into respiratory supercomplexes is identical for 4i1 and 4i2 KI (Figure 1E). In accordance with the quantification of the COX subunit contents (Figure 1C), the amount of the native COX complexes in the 4i1 and 4i2 KI cell lines corresponded to 54% and 61% of the COX level in parental HEK293, respectively.

Since crystal structure data are only available for the enzyme containing the COX4i1 isoform, we further focused on detailed characterization of native COX complexes containing either COX4i1 or COX4i2 isoforms. This was performed by mass spectrometric (MS) analysis of proteins immunoprecipitated from whole cell digitonin solubilizates using antibody directed against the FLAG tag present in recombinant COX4 isoforms. Comparison of COX4 interacting proteins between 4i1 and 4i2 KI revealed that the majority of COX subunits as well as COX assembly factors was associated with both COX isozymes (Figure 1F). Except for obvious hits of COX4i1 and COX4i2 in their respective KI cell lines, only two other mitochondrial proteins showed a significantly different association with either of the COX isozymes enriched in the COX4i1 KI cell line—assembly factor COX11 and m-AAA protease AFG3L2. These proteins associate with COX complexes during enzyme biogenesis to either facilitate copper insertion into COX1 in the case of COX11 or to impose protein quality control in the case of AFG3L2, and both have not been previously reported as components of mature COX complexes. Therefore, with the exception of COX4 isoforms, the fully assembled COX in both 4i1 and 4i2 KI cell lines had identical composition. In fact, increased association of COX4i1 with AFG3L2 protease could explain the differential content of the FLAG-tagged isoforms. While unassembled isoform 1 was efficiently degraded, a portion of isoform 2 accumulated even outside the COX complex.

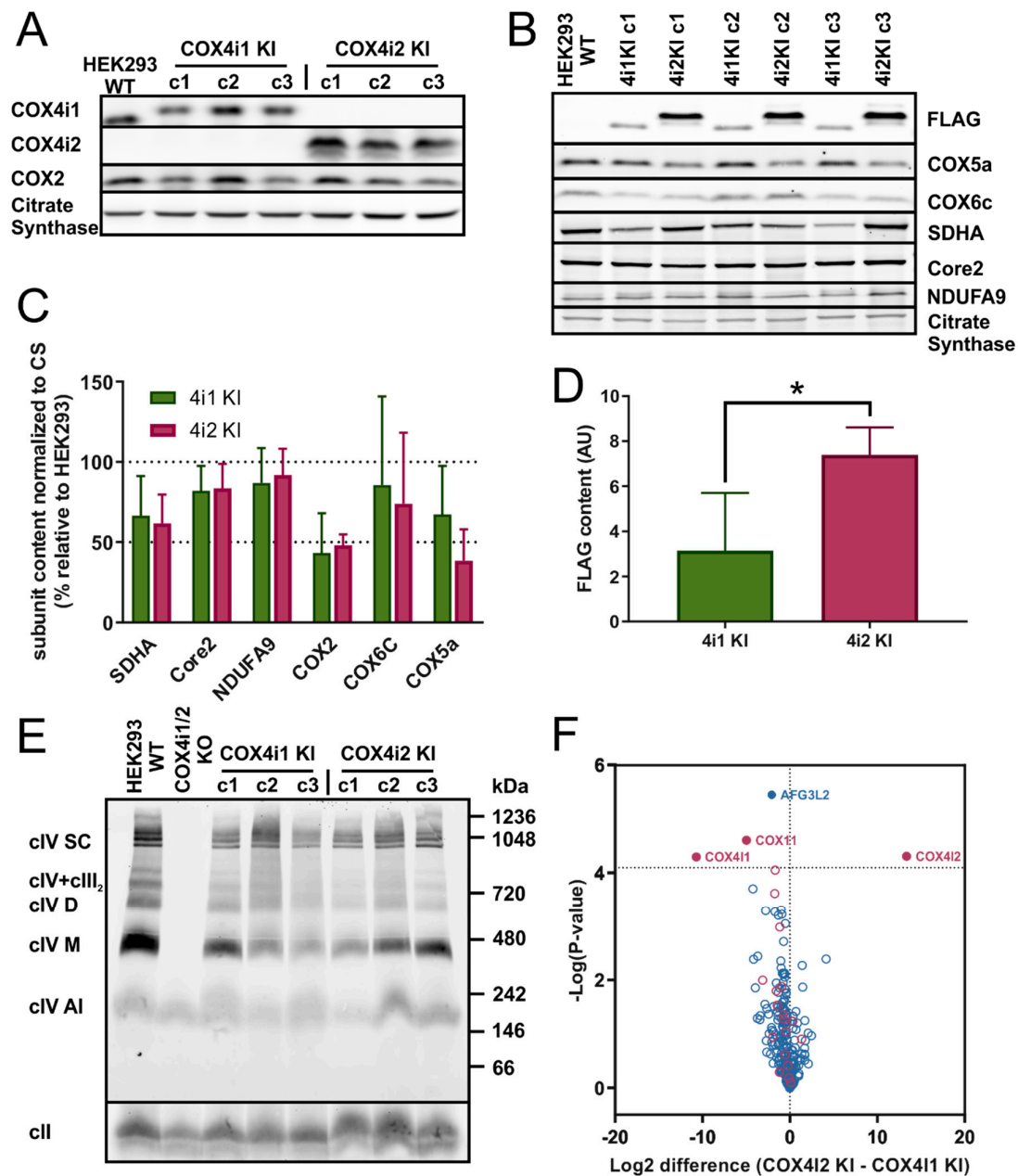


Figure 1. (A) Western blot analysis of COX4 isoforms in knock-in cell lines. Whole cell lysates (30 μ g of protein) of control HEK293 and three clones of each COX4i1 KI and COX4i2 KI were subjected to SDS-PAGE and Western blotting. COX4i1 and COX4i2 isoforms were detected with specific antibodies (see details in Methods), along with the COX2 subunit. Citrate synthase was used as a loading control. (B) Western blot analysis of COX subunits and OXPHOS proteins. Whole cell lysates (30 μ g of protein) of control HEK293 and three clones of each COX4i1 KI and COX4i2 KI were subjected to SDS-PAGE and Western blotting. Representative image of one of three sets of samples of the HEK293 control and three clones of each KI cells are shown. (C) Quantity of OXPHOS proteins. Antibody signals from WB images were quantified densitometrically using Image Lab software (Bio Rad). Data are plotted as the mean $\pm$ S.D. of COX4i1 and COX4i2 KI values expressed in percent relative to control HEK293 cells ($n = 9$, three independent samples of three clones of each KI cell line). (D) Quantity of FLAG-tagged COX4 isoforms. Relative content of FLAG-tagged COX4i1 and COX4i2 in respective KI cell lines determined by densitometry quantification of the FLAG antibody signal. Data are plotted as the mean $\pm$ S.D. of COX4i1 and COX4i2 KI cells ($n = 9$, three independent samples

of three clones of each KI cell line). (E) BN-PAGE analysis of native forms of COX. Isolated mitochondria from HEK293, COX4i1/2 KO, and KI cells were solubilized with digitonin (6 g/g protein); sample aliquots of 30 μ g protein were separated on 5%–16% native gel. WB analysis with the COX1 antibody revealed multiple forms of the COX complex as indicated on the left of the image: COX1-containing assembly intermediate (cIV AI), monomer (cIV M), dimer (cIV D), assemblies with complex III (cIV + cIII), and respiratory supercomplexes (cIV SC). Molecular weight marker migration is indicated on the right. Complex II (cII) detected by the SDHA antibody was used as a loading control. (F) Mass spectrometry analysis of immunoprecipitated COX. Volcano plot depicting differential contents of Mitocarta-annotated proteins immunoprecipitated with the anti-FLAG antibody (blue circles) from 4i1 or 4i2 KI cells. Red circles mark COX subunits and assembly factors. Relative quantities (4i2 KI/4i1 KI) plotted on the X-axis are expressed in log₂ scale. Significantly different hits are shown above the probability cut off (dashed horizontal line) with protein names annotated.

In summary, electrophoretic and MS analyses demonstrated that cell lines with exclusive expression of either COX4i1 or COX4i2 isoforms of the COX4 subunit had a comparable enzyme content and composition, and thus represent a convenient model to study functional differences conferred by these isoforms.

3.2. Identical Cytochrome *c* Oxidase Activity in KI Cell Lines

Respirometric assay of COX activity during titration of increasing concentrations of cytochrome *c* in mitochondrial solubilized extracts was employed to obtain maximal activity rates as well as data on the cytochrome *c* binding kinetics of the COX complex in 4i1 and 4i2 KI cells.

In both knock-in cell lines, COX activity displayed a hyperbolic response to increasing substrate concentrations. In terms of maximal activity rates at 30 μ M concentration of cytochrome *c*, 4i1 and 4i2 KI cell lines displayed equal values, corresponding to 55% and 52% of control HEK293 values, respectively (Figure 2A). When the data were normalized to the content of the COX2 subunit in the solubilized extracts, maximal rates in KI cell lines matched the controls (Figure 2B). These findings indicate that specific activities of cytochrome *c* oxidation of COX4i1- and COX4i2-containing COX isozymes in KI cell lines cannot be distinguished from each other and are perfectly analogous to the activity of the wild-type enzyme in parental HEK293 cells. Furthermore, COX affinity to cytochrome *c* was assessed by fitting a hyperbolic Michaelis–Menten function through experimental data (Figure 2B). The obtained K_m values of 9.9 ± 2.26 and 9.0 ± 2.39 μ M for 4i1 and 4i2 KI cells, respectively, were not significantly different. Overall, the isoform switch between COX4i1 and COX4i2 isoforms did not result in alterations of affinity and maximal velocity of cytochrome *c* oxidizing activity of the COX complex.

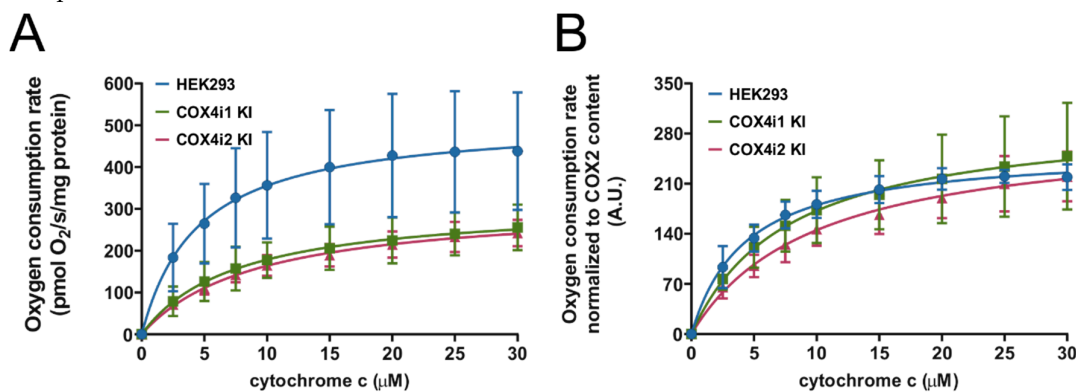


Figure 2. COX activities normalized to the protein concentration (A) or COX2 subunit content (B). COX activities in Tween-20 extracts of isolated mitochondria measured as oxygen consumption using Oxygraph-2k (Oroboros) at increasing concentrations of cytochrome *c* (0–30 μ M) are plotted as the mean $\pm$ S.D. value of three (HEK293, blue) and nine (three replicates of three clones each) COX4i1

(green) and COX4i2 KI (magenta) cells each. Hyperbolic fit of the Michaelis-Menten function is shown as full lines in respective colors.

3.3. Decreased COX Oxygen Affinity in COX4i2 KI cells

To analyze the COX function in the context of a fully operating respiratory chain, mitochondrial respiration was measured in situ in cells permeabilised with digitonin. The complex substrate/inhibitor titration protocol (Figure 3A), including parallel electron input to both major dehydrogenases in the HEK293 respiratory chain, complexes I and II, as well as to glycerol-3-phosphate dehydrogenase, was employed to obtain the capacities of oxidative phosphorylation (OXPHOS, i.e., coupled mitochondrial respiration in the presence of ADP and saturating substrate concentrations), electron-transport chain capacity (ETC, i.e., mitochondrial respiration uncoupled by FCCP under saturating substrate concentrations), and COX capacity (COX, i.e., oxygen consumption with artificial substrates ascorbate and TMPD donating electrons to cytochrome c). Furthermore, two aerobic/anoxic transitions were included, in OXPHOS and ETC states, to obtain data for analysis of oxygen kinetics.

In the OXPHOS state, 4i1 and 4i2 KI cells displayed comparable respiratory rates, 140.9 ± 7.04 and 140.7 ± 10.22 pmol O₂/s/mg protein, respectively, that were not significantly different from the OXPHOS capacity in control HEK293 cells (161.4 ± 18.65 pmol O₂/s/mg protein) (Figure 3B). Likewise, 4i1 and 4i2 KI and HEK293 cells could not be distinguished by respiratory rates in the uncoupled state (ETC, 284.4 ± 16.11 , 270.8 ± 23.72 , and 327.3 ± 51.64 pmol O₂/s/mg protein, respectively) (Figure 3B). These data indicate that selective knock-in of either isoform into COX4 dKO cells fully complemented the capacities of electron transfer in the respiratory chain and, importantly, the wild-type level of mitochondrial ATP production of HEK293 cells. The lower COX content in KI cells relative to the control HEK293 was only manifested during analysis of the COX capacity with artificial substrates. While the COX capacity rates in 4i1 and 4i2 KI cells were not significantly increased above the value of the respective ETC capacities, a 1.5-fold increase in parental HEK293 cells was indicative of the COX reserve capacity (Figure 3B).

Oxygen kinetics were analyzed in OXPHOS and ETC states in terms of the p_{50} parameter, the partial pressure of oxygen at the half-maximal respiratory rate, thus analogous to K_M from classical enzyme kinetics. The p_{50} values in 4i1 KI cells, 0.0637 ± 0.01291 and 0.0486 ± 0.01436 kPa in OXPHOS and ETC states, respectively, were not significantly changed compared to control HEK293 cells, which do indeed contain the 4i1 isoform (Figure 3C). In contrast, p_{50} in 4i2 KI cells in the OXPHOS state (0.12015 ± 0.01793 kPa) and ETC states (0.0705 ± 0.01215 kPa) was increased 2-fold compared to 4i1 KI and parental HEK293 cells (Figure 3C). These data represent strong indication that the COX4i2 isoform confers lower oxygen affinity of the COX complex, thus decreasing the respiratory rate under limiting oxygen supply. Simulated values of the relative respiratory rate in the oxygen-sensitive range for 4i1 and 4i2 KI cells are shown in Figure 3D.

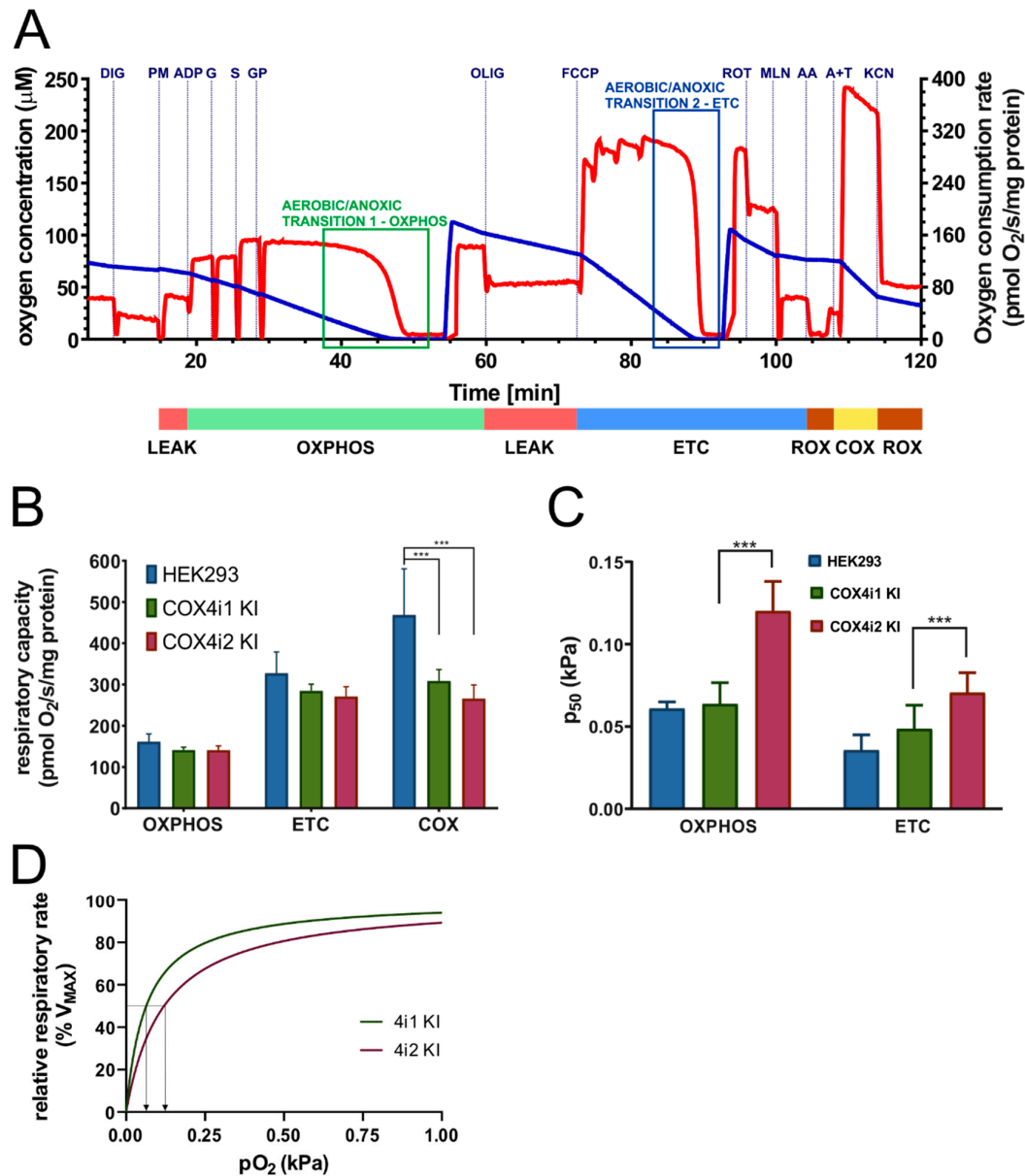


Figure 3. Mitochondrial respiration in permeabilised cells. **(A)** Representative trace of respirometric measurement. Experimental trace recorded by Oxygraph-2k (Oroboros) with 0.3 mg protein/mL of HEK293 cells from acquisition software DatLab5 showing the actual O_2 concentration (blue, left Y axis in μM) and rate of oxygen consumption (red, right Y axis in $\text{pmol O}_2/\text{s/mg protein}$). Additions of substrates and inhibitors are marked by vertical dashed lines and abbreviations above the trace (digitonin 0.05 g/g protein—DIG, pyruvate 10 mM + malate 2 mM—PM, ADP 1 mM—ADP, glutamate 10 mM—G, succinate 10 mM—S, glycerol-3 phosphate 10 mM—GP, oligomycin 0.5 μM —OLIG, FCCP 100–300 nM—FCCP, rotenone 0.5 μM —ROT, malonate 10 mM—MLN, antimycin A 0.25 μM —AA, ascorbate 2 mM + TMPD 1 mM—A+T, and KCN 0.5 mM—KCN). The diagram below is time-aligned with the experimental trace and denotes the actual respiratory state (driven by proton leak—LEAK, red; coupled to ATP synthesis—OXPHOS, green; uncoupled by protonophore FCCP—ETC, blue; residual oxygen consumption after inhibitor addition—ROX, brown; respiration with artificial substrates ascorbate and TMPD—COX, yellow). **(B)** Respiratory capacities in OXPHOS, ETC, and COX states are plotted as the mean $\pm$ S.D. value of seven (HEK293, blue) and twelve (four replicates of three clones each) COX4i1 (green) and COX4i2 KI (magenta) cells. $***p < 0.001$. **(C)** Oxygen affinity expressed as p_{50} —partial pressure of oxygen at half-maximal respiration (kPa) in OXPHOS and ETC

states—is plotted as the mean $\pm$ S.D. value of seven (HEK293, blue) and twelve (four replicates of three clones each) COX4i1 (green) and COX4i2 KI (magenta) cells. *** $p < 0.001$. (D) Simulated hyperbolic response of relative respiratory rates (as % of maximal rates) to oxygen partial pressure (0–1 kPa) was calculated using the Michaelis-Menten function, feeding mean values of experimentally analyzed p_{50} values for COX4i1 (green) and COX4i2 (magenta) KI cells. Arrows projecting from respective traces towards the X-axis mark p_{50} values of COX4i1 and COX4i2 KI.

The most remarkable structural difference between isoforms 1 and 2 of COX4 may be the exclusive occurrence of three cysteine residues in COX4i2 (C48, C54, C108). To test whether the observed changes in oxygen affinity could be assigned to the presence or modification of individual cysteine residues, site-directed mutagenesis was employed to prepare three variants, glycine, valine or serine, of each of the cysteines (C48G, C48V, C48S, C54G, C54V, C54S, C108G, C108V, C108S). These mutant KI cells displayed varying ability to complement the content of COX (Figure 4A) and mitochondrial respiration (Figure 4B). In general, serine substitutions at each of three cysteine positions were able to maintain COX levels and respiratory rates comparable to knock-in with WT COX4i2. Nevertheless, the oxygen affinity did not respond to the site-specific substitutions, as C48S, C54S, and C108S KI cell lines displayed unchanged values of p_{50} compared to 4i2 KI (Figure 4C). These findings indicate that diminished oxygen affinity of COX4i2-containing oxidase is not due to intra- or intermolecular crosslink or posttranslational modification involving either of its three cysteine residues.

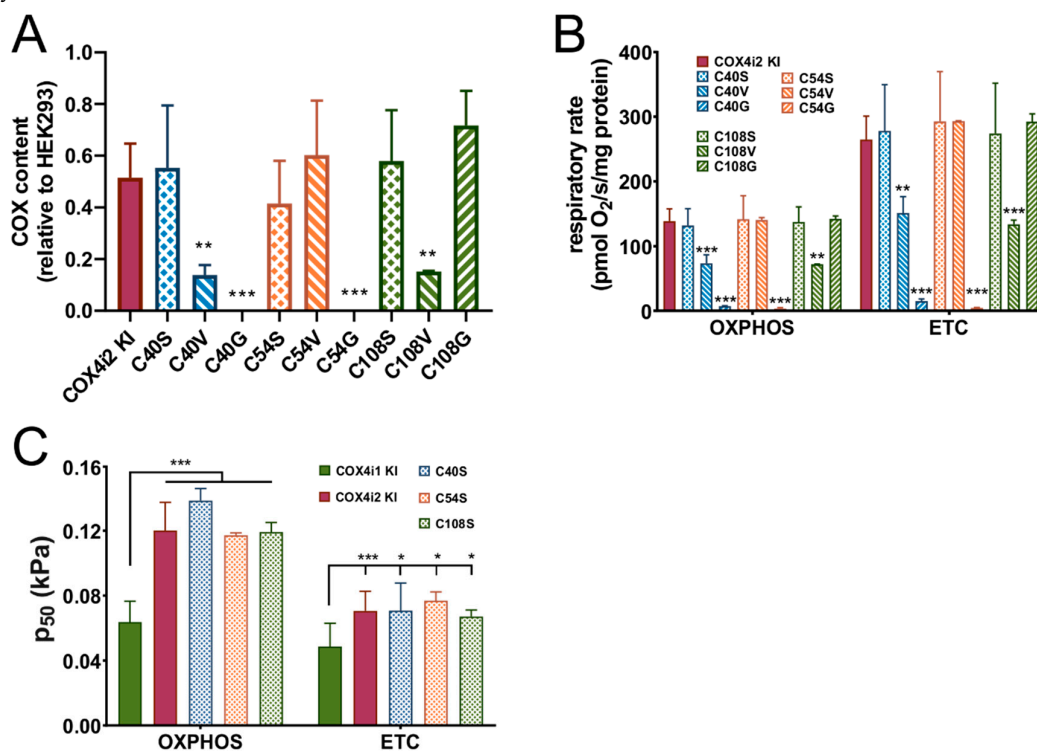


Figure 4. The COX content and respiration of COX4i2 cysteine substitution mutant KI cell lines. (A) The COX content calculated as the COX2 subunit quantified in the WB immunodetection experiment, normalized to citrate synthase, and expressed in % relative to the COX2 content in parental HEK293 cells, is plotted as the mean $\pm$ S.D. value of two measurements in each COX4i2 cysteine-substitution mutant KI cell line as indicated below the graph compared to wild-type COX4i2 KI (magenta, $n = 9$). ** $p < 0.01$, and *** $p < 0.001$ mark significant differences between individual mutant cysteine KIs and wild-type COX4i2 KI. Respiratory rates (B) and p_{50} (C) in OXPHOS and ETC states are plotted as the mean $\pm$ S.D. value of two measurements in each COX4i2 cysteine-substitution mutant KI cell line as indicated below the graph compared to wild-type COX4i2 KI (magenta, $n = 12$). * $p < 0.05$, ** $p < 0.02$,

*** $p < 0.001$ mark significant differences between individual mutant cysteine KIs and wild-type COX4i2 KI in (B) or between COX4i1 KI and wild-type or mutant variants of COX4i2 KI cells (C).

3.4. Energy Metabolism, ROS Production, and Redox Status in Intact Cells

To compare intact cell energy metabolism in 4i1 and 4i2 KI cell lines, mitochondrial respiration (oxygen consumption rate, OCR, Figure 5A) and glycolytic activity (extracellular acidification rate, ECAR, Figure 5B) were analyzed simultaneously using a Seahorse XFe24 Bioenergetic Analyzer. Metabolic rates were monitored in cells catabolizing only endogenous substrates in the beginning, followed by subsequent additions of 10 mM glucose and 1 μ M oligomycin to inhibit mitochondrial ATP synthase, 1 μ M FCCP to uncouple oxidative phosphorylation and stimulate maximal electron flux of the respiratory chain, and a combination of rotenone (1 μ M), antimycin A (1 μ M), and 2-deoxyglucose (100 mM) to inhibit both the respiratory chain and glycolytic pathway.

4i1 and 4i2 KI cells showed comparable values of basal OCR with glucose as a substrate (Figure 5A). The residual leak-driven respiration (oligomycin state) was similar in both KI cell lines, as well as the respiratory capacity assessed after FCCP addition (Figure 5A). Basal ECAR with glucose as a substrate showed a 1.5-fold higher value in 4i1 KI, but glycolytic capacity tested after oligomycin addition and the glycolytic reserve capacity were similar between 4i1 and 4i2 KI (Figure 5B). Finally, the OCR/ECAR ratio was calculated from rate values in the glucose state to assess cell preference for either glycolytic or mitochondrial ATP provision. This parameter was 1.4-fold higher in COX4i2 KI cells, indicating increased preference for ATP production by OXPHOS compared to COX4i1 KI cell lines (Figure 5C).

The mitochondrial respiratory chain is one of the major cellular producers of reactive oxygen species (ROS). To analyze whether the switch from COX4i1 to COX4i2 was associated with changes in oxidative stress, mitochondrial ROS production was measured in intact cells using the fluorescent probe H₂-DCFDA that is sensitive to broad-spectrum ROS. Basal ROS production in intact 4i1 and 4i2 KI cell lines was compared with parental HEK293 cells and the COX4 dKO cell line. While 4i1 KI displayed similar values as HEK293 cells, 4i2 KI cells showed a 1.5-fold decreased rate of ROS production. (Figure 6A). Upon inhibition of complex III by antimycin A, which leads to reduction of the mitochondrial NADH pool, rates of ROS production increased approximately twofold (Figure 6A), but no significant differences were detected between the cell lines. These data indicate that the alterations recorded under basal conditions result from the current metabolic and redox status of mitochondria rather than increased disposition for ROS production in particular cell lines. Indeed, the ROS production rate difference between 4i1 and 4i2 KI cells correlated with values of the NAD⁺/NADH ratio, which was increased by 20% in 4i2 KI, indicating a less reduced NADH pool (Figure 6B). As expected, NAD⁺/NADH was extremely low in COX4i1/2 KO cells due to an inactive respiratory chain (Figure 6B). Unfavorable redox status correlated with increased ROS production in knockout cells (Figure 6A).

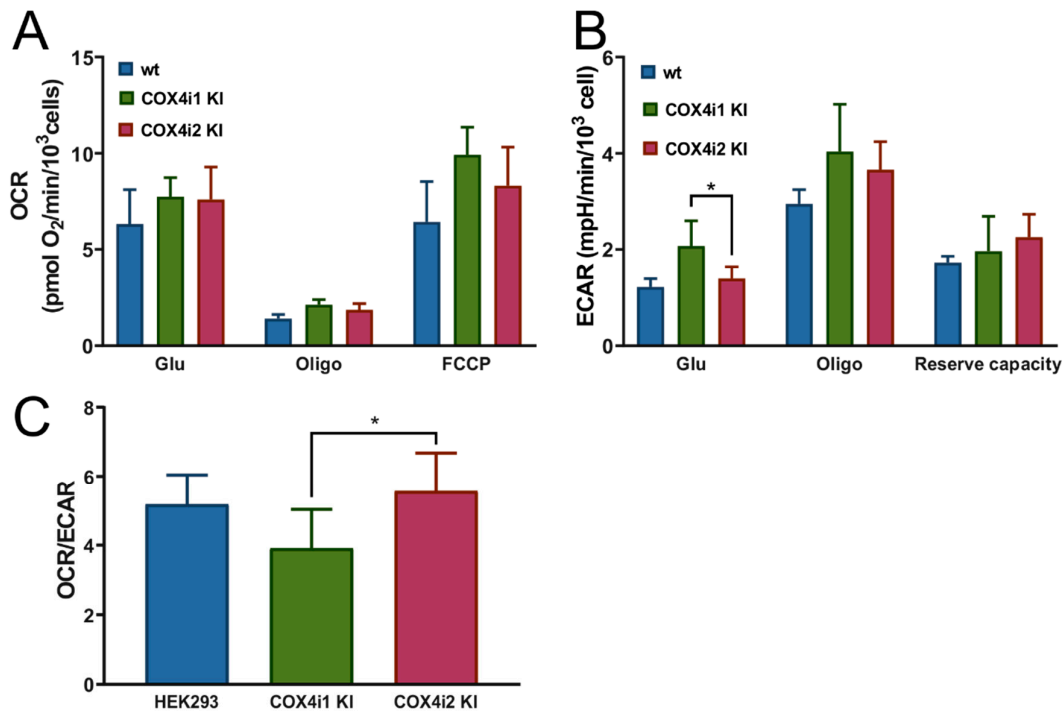


Figure 5. Energy metabolism in intact KI cells. (A) Cellular oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR) were recorded by an XFe Bioenergetics Analyzer (Seahorse) with endogenous substrates (basal) and after subsequent additions of 10 mM glucose (Glu), 1 μ M oligomycin (Oligo), 1 μ M FCCP (FCCP) in HEK293 cells (blue, $n = 5$), COX4i1 KI (green, $n = 7$, at least two replicates of each of three clones), and COX4i2 KI cells (magenta, $n = 8$, at least two replicates of each of three clones), plotted as mean values $\pm$ S.D. Glycolytic reserve capacity displayed in B was calculated as the difference between the ECAR values in Oligo and Glu states. (C) OCR/ECAR ratio (mean $\pm$ S.D.) was calculated from recorded rates in the coupled state in the presence of 10 mM glucose (Glu). * $p < 0.05$ marks a significant difference between COX4i1 and COX4i2 KI cells.

In summary, despite performing the studies under normoxic conditions, analysis of energy metabolism and the redox state in intact cells uncovered modestly increased preference for mitochondrial ATP production, consistent with increased NADH pool oxidation and lower ROS in 4i2 KI compared to 4i1 KI cells.

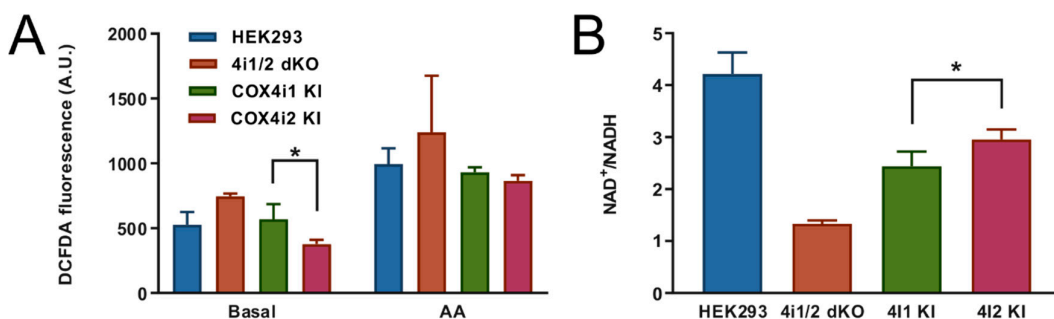


Figure 6. ROS production and redox status in intact KI cells. (A) Mitochondrial ROS production was monitored as oxidation of the fluorescent H₂-DCFDA probe in intact HEK293 (blue, $n = 3$), COX4i1/2 KO (orange, $n = 3$), COX4i1 KI (green, $n = 9$, three replicates of three clones each), and COX4i2 KI cells (magenta, $n = 9$, three replicates of three clones each). Data are plotted the mean $\pm$ S.D. (B) The NAD⁺/NADH ratio was calculated from parallel measurements of NAD⁺ and NADH contents in intact cells using NAD/NADH-Glo™ Assay (Promega). Data are plotted as the mean $\pm$ S.D. of HEK293

(blue, $n = 3$), COX4i1/2 KO (orange, $n = 3$), COX4i1 KI (green, $n = 9$, three replicates of three clones each), and COX4i2 KI cells (magenta, $n = 9$, three replicates of three clones each). * $p < 0.05$ marks a significant difference between COX4i1 and COX4i2 KI cells.

4. Discussion

Energetic metabolism of mammalian organisms is vitally dependent on aerobic respiration, with 80–90% of inhaled oxygen being utilized by cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain. The apparent K_m for oxygen ranges between 0.01 and 0.1 kPa, meaning that mitochondrial respiration is usually not limited by oxygen availability [35]. However, even with sophisticated oxygen delivery by the cardiovascular system, local pO_2 can drop to levels as low as 0.3 kPa in some tissues. Therefore, oxygen reduction to water by cytochrome *c* oxidase occurs under conditions of oxygen availability varying by two orders of magnitude [36], which under extreme cases may result in incomplete COX saturation. Oxygen affinity of COX can be further diminished by allosteric factors such as NO [37], as part of mechanisms that can redirect oxygen utilization from respiration to other oxygen-handling enzymes or non-enzymatic reactions. The significance of oxygen availability for COX function was strongly supported by the discovery of oxygen-regulated expression of COX4i2, the second isoform of regulatory, nuclear-encoded subunit COX4. Oxygen regulation of COX4i2 expression is exerted by several mechanisms, either through classical HIF-1 regulation [13] or a novel oxygen response element (ORE) [14], where transcription is regulated by the interplay of three proteins [12]. Environmental conditions thus add yet another mode of COX regulation to complement nuclear-encoded subunit isoforms that are expressed in tissue or a developmental stage-dependent manner [38]. Induction of COX4i1 expression under hypoxia was reported in multiple cell lines, and it was proposed that the COX4 isoform switch represents adaptation to decreased oxygen availability by improving efficiency of mitochondrial respiration [13]. However, huge variation of *Cox4i2* expression in mouse tissues *in vivo* [14,15] suggested that regulation of its content is complicated beyond just oxygen concentration, and the presence of the second isoform of COX4 may imply its specialized function. Indeed, high expression of *Cox4i2* in pulmonary artery smooth muscle cells (PASMCs) of the mouse lung was associated with regulation of pulmonary hypoxic vasoconstriction (HPV) [16]. More recently, increased expression of *Cox4i2*, along with two atypical COX subunits—COX8b and NDUFA4i2 triggered by HIF-2 α —was observed in glomus cells of carotid bodies in mice [17]. Such atypical COX complex present in these specialized cells was suggested as a hypothetical sensor involved in acute oxygen sensing [18].

In the present study, we aimed to identify how the switch between COX4i1 and COX4i2 isoforms is able to modulate COX function. In order to achieve this in a well-defined model, we generated knock-in cell lines of these isoforms independently in the background of COX4i1/2 double knock-out in HEK293 cells to obtain cells with exclusive expression of either isoform. Electrophoretic and MS analyses revealed that COX4i1 and COX4i2 knock-in cell lines were able to restore comparable levels of the COX complex with otherwise identical subunit composition, representing an ideal tool for unbiased characterization of isoform effects. Previously, contradicting results regarding the effect of the COX4 isoform on enzyme activity were reported. COX isolated from mouse lung containing COX4i2 had a higher turnover number than liver COX containing COX4i1 [15]; similarly, transient overexpression of COX4i2 in HEK293T cells mildly augmented COX activity and cellular respiration [13]. In contrast, the isoform switch from COX4i2 to COX4i1 in glioma cells was associated with increased COX activity and respiration [20]. In our KI cell lines, no differences between COX4i1 and COX4i2 KI were observed in the case of COX activity, affinity to cytochrome *c* or respiratory capacities, which suggests that previously published functional modulations may have been associated with regulatory mechanisms dependent on COX4 isoforms but emanating from levels of complexity beyond isoform exchange itself. Despite identical COX activities and respiratory capacities in our KI cell lines, analysis of energetic metabolism in intact cells revealed increased OCR/ECAR ratios in COX4i2 KI cell lines, indicative of the preference for mitochondrial ATP production rather than by anaerobic glycolysis. Both modes of energy provision are utilized by the

majority of cultured cell lines, and under conditions of non-limiting substrate (glucose) supply and routine energy demand, mitochondrial OXPHOS activity may be downregulated.

COX has been identified as a target for multiple regulatory mechanisms, including posttranslational modifications and allosteric binding of adenine nucleotides enabling regulation based on the ATP/ADP ratio [4,5]. Indeed, the adenine nucleotide binding site on the matrix-facing domain as well as *in vivo* phosphorylation sites was identified in the COX4 subunit [4]. In this regard, differential responses of COX4 isoforms to such regulations have been previously described. In astrocytes exposed to hypoxia, robust expression of COX4i2 was identified in contrast to neuronal cells, which retain mostly the COX4i1 isoform [19]. In this study, activity measurements of COX solubilized from astrocytes displayed significantly attenuated allosteric inhibition by ATP, suggesting that COX4i2 is less prone to such modulation than COX4i1 [19]. Further, it has been reported that allosteric ATP inhibition is modulated by the phosphorylation status of Ser58 in COX4i1 [9]. Under conditions of increased flux of the TCA cycle, an increased level of carbonate activates the intramitochondrial PKA pathway, which leads to reversible phosphorylation of Ser58 and impairment of ATP binding through charge repulsion, thus enabling robust flux of electrons through the respiratory chain to match TCA cycle activity. In COX4i2, the corresponding phosphorylation site is missing; instead, negatively charged residues are present in the incriminated region. This suggests that COX4i2-harboring COX resides are permanently in an activated state [9], matching our observation of the increased OCR/ECAR ratio. Furthermore, this finding corresponds to an increased NAD⁺/NADH ratio and decreased mitochondrial ROS production measured under the normoxic condition in COX4i2 KI cells in comparison with COX4i1 KI.

To enable functional alteration of COX under conditions of limited oxygen availability or to possess the ability to function as an oxygen sensor, COX4 isoforms should confer distinctive oxygen affinity to the enzyme. To the best of our knowledge, this aspect of the COX4 isoform switch has not been previously addressed before in sufficient detail. We analyzed the COX oxygen kinetics by high-resolution respirometry [31]. This method yields the p_{50} parameter, the “apparent K_m ”, a measure of the sensitivity of cellular respiration to oxygen availability assayed in permeabilised cells with intact mitochondrial membranes, where the COX function is integrated in the respiratory chain. Our results consistently showed that oxygen affinity of the COX4i2-containing enzyme was significantly decreased compared to the COX4i1 counterpart. In our opinion, this represents the major finding of this study. The measured values of p_{50} in parental HEK293 cells match previously reported values in isolated mitochondria and small cells [35]. It should also be noted that the two-fold p_{50} increase in COX4i2 versus COX4i1 KI can have significant *in vivo* implications. For example, similar alteration of COX oxygen affinity can be observed in cells with pathological mutations in the SURF1 protein, which lead to pathologic COX deficiency and is clinically associated with Leigh’s syndrome [29,30]. The mechanism of how COX4 isoform exchange modulates kinetics of oxygen binding is unclear, but up to three dioxygen diffusion areas with open channels relying on rapid and reversible conformational changes were proposed to operate simultaneously to sustain remarkably high affinity of COX to oxygen [39]. Thus, any perturbation in the hydrophobic core may affect oxygen diffusion to the binuclear site within the COX1 subunit. This may be conferred by the transmembrane region of COX4 that runs parallel to the transmembrane helix 12 of COX1, but all available structural data were obtained with the COX4i1-containing enzyme, preventing evaluation of structural differences between isoforms. However, the COX4 subunit capability to modulate enzyme oxygen handling seems to be conserved in eukaryotes, as the expression of the isoform pair of the yeast COX4 homolog is also regulated by oxygen [10]. COX isozymes containing the normoxic isoform COXVa (analogous to COX4i1) or hypoxia-induced COXVb (analogous to COX4i2) were analyzed by infrared spectroscopy in the CO-bound state (CO-IR) as a sensitive way to assess the effects of COXV isoforms on the binuclear reaction center environment [40]. Absorption properties of COXVa-containing COX were similar to those of bovine enzyme and indicated the presence of multiple conformers capable of rapid interconversions. In contrast, narrower CO-IR band of the COXVb isozyme suggested a less dynamic enzyme with only one conformer [40]. If oxygen diffusion to the binuclear center is indeed dependent on dynamic conformational changes, it would mean that the COXVa presence would

facilitate it, while rigid conformation of the COXVb-containing enzyme may impose a diffusion barrier. If we speculate that an analogous situation occurs with mammalian COX4 isoforms, this could translate as an increase in p_{50} . This issue should, however, be resolved by direct structural comparison of mammalian COX isozymes. In fact, our cell models with the exclusive expression of individual COX4 isoforms may in future prove useful for such studies.

The role of COX4i2 in HPV was associated with mitochondrial membrane hyperpolarization inducing increased ROS production at upstream respiratory complexes. Mitochondrial ROS serve as signaling molecules leading to inhibition of ion channels at the plasma membrane of PSMCs leading to its depolarization and Ca^{2+} influx-triggering contraction [16]. Alternatively, according to our findings in HEK293 KI cell lines, an increase in hypoxic ROS production at complex III that is critical for HPV [41] could be caused by premature inhibition of COX4i2-containing COX due to its decreased oxygen affinity, while sufficient oxygen concentration would be available at ROS-producing sites which are generally more sensitive to oxygen availability than COX [42]. Indeed, in the COX4 knock-out cell line mimicking enzyme inhibition, we observed robustly increased ROS production. Interestingly, p_{50} in COX4i2 KI cells was not modulated by mutations of either of three cysteine residues, which arguably represent the most significant structural difference between COX4 isoforms. In contrast, increased hypoxic ROS production in COX4i2-overexpressing CMT cells was attenuated by cysteine replacement [16]. This suggests that additional modification of COX4i2 may be necessary for affecting proper function as oxygen sensor. Indeed, in glomus cells of carotid bodies, HIF-2 α induced expression of COX4i2, but also two other non-canonical COX subunits, namely, COX8b and NDUFA4i2 [17,18]. The latter harbors an extra cysteine residue compared to its ubiquitous isoform, which might hypothetically form a disulphide link with COX4i2. In our study, the immunoprecipitation analysis of HEK293 KI cell lines (Figure 1F) showed COX 4i1 and 4i2 isozymes with otherwise identical isoform composition of other COX subunits. Thus, we can demonstrate that a change in oxygen affinity is solely associated with COX4 isoforms and does not involve modulation via cysteine residues. On the other hand, modulation of hypoxic ROS production may be a more complex phenomenon involving a switch in yet other subunits, present only in physiologically specialized cells that assemble the dedicated oxygen-sensing enzyme.

Overall, our study extends current knowledge on the regulatory role of COX4 isoforms and shows that besides affecting the ability of COX to respond to ATP levels [9,19], the COX4i2 subunit also facilitates a decrease in cytochrome *c* oxidase affinity to oxygen. These findings thus further characterize unique properties of the COX4i2 isoform, in accordance with the view that the COX4i2 subunit represents an important effector in regulation of COX activity and respiratory chain energetic function under hypoxic conditions. According to previously published information and novel findings of the present study, the COX4 isoform exchange regulated by oxygen availability has consequences for cellular respiration as well as mitochondrial ROS production. Activity of the energy status-regulated COX4i1 isozyme is less sensitive to hypoxia; therefore, the enzyme can operate at extremely low oxygen concentrations, limiting non-respiratory oxygen consumption. In contrast, the hypoxia-sensitive COX4i2 isozyme becomes prematurely inhibited under oxygen-limiting conditions, leading to a reduction of upstream components of the respiratory chain, promoting increased ROS production (Figure 7). Further studies are necessary to dissect the interplay of oxygen utilization, regulation of ROS production, and putative redox modification of COX4i2, as well as the role of other non-canonical COX subunits that may be involved in this remarkable physiological phenomenon of oxygen sensing.

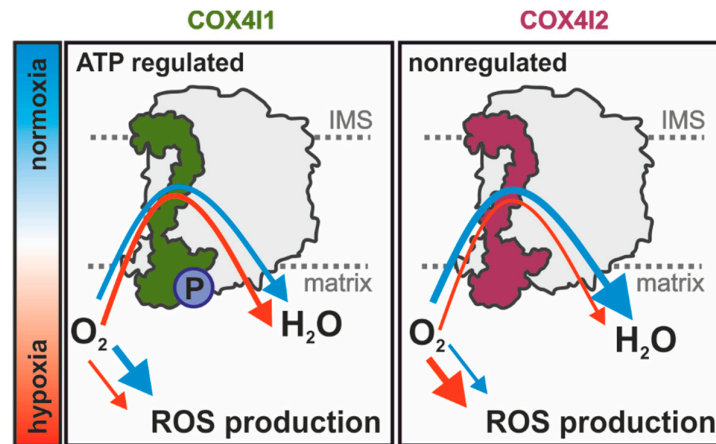


Figure 7. Model of COX functional alterations mediated by COX4 isoform exchange: Rates of oxygen consumption of COX4i1- (left) or COX4i2-containing COX (right) are depicted by variable arrow thickness in normoxia (blue arrow) and hypoxia (red arrow). P, serine58 phosphorylation site.

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