

Role of Complex IV in early postnatal heart development

Inaugural-Dissertation

Zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln



vorgelegt von
Milica Popovic
aus Sremska Mitrovica, Serbia

Köln 2023

Berichterstatter: Prof. Dr. Aleksandra Trifunovic
Prof. Dr. Ana Garcia Saez

Tag der mündlichen Prüfung: 19.12.2023

*"I have what I have and I am happy.
I've lost what I've lost and I am still happy."
Rupi Kaur
To my sister and brother, my biggest support.*

Table of Contents

List of Figures	6
List of Tables	7
Abbreviations.....	8
Abstract.....	11
Abstract.....	12
1 Introduction	13
1.1 Postnatal heart development	13
1.2 Mitochondria.....	16
1.3 OXPHOS	16
1.4 The integrated stress response	23
1.5 Objectives.....	26
2 Materials and Methods.....	28
2.1 Mouse experiments.....	28
2.1.1 Animal care	28
2.1.2 Experimental mouse models.....	28
2.2 DNA isolation and Genotyping.....	29
2.2.1 Agarose gel electrophoresis	29
2.3 Molecular biology.....	30
2.3.1 RNA isolation	30
2.3.2 Quantification of nucleic acid concentration	30
2.3.3 Reverse transcription PCR (RT-PCR)	30
2.3.4 Quantitative PCR (qPCR)	31
2.4 Biochemistry	32
2.4.1 Protein isolation from tissues	32
2.4.2 SDS-PAGE.....	32
2.4.3 Isolation of mitochondria from mouse tissues.....	34
2.4.4 BN-PAGE	34
2.4.5 <i>In-gel</i> activity assay.....	35
2.4.6 <i>In-organello</i> translation.....	35
2.5 Histology	36
2.5.1 Tissue Sectioning.....	36
2.5.2 COX staining	36
2.5.3 Haematoxylin and Eosin staining.....	36
2.6 Omics.....	36
2.6.1 Proteomics	36
2.6.2 Metabolomics	37
2.6.3 Complexomics.....	38
2.7 Image processing and data analyses	39

2.7.1	Image processing	39
2.7.2	Statistical analysis	39
2.7.3	Enrichment analysis	39
2.8	Chemicals	39
3	Results	42
3.1	Full body depletion of COX7C	42
3.2	Heart specific COX7C deficiency	44
3.2.1	Effect of COX7C depletion to OXPHOS	47
3.3	Stress responses of the heart upon loss of COX7C in the heart	56
3.3.1	Proteomics of COX7C ^{MKO} hearts	57
3.3.2	ISR in hearts upon loss of COX7C	59
3.3.3	Proteomics of COX10 ^{MKO} hearts	60
3.3.4	ISR activation differences between COX7C ^{MKO} and COX10 ^{MKO}	62
3.4	Heart metabolome changes upon loss of COX7C	64
3.4.1	Changes in BCAA metabolism in COX7C ^{MKO} mice	67
3.4.2	Pyrimidine synthesis in COX7C mice	67
3.4.3	Changes in COX10 ^{MKO} heart metabolism	68
3.5	OXPHOS in postnatal heart development	73
3.5.1	Changes in heart OXPHOS complexes at birth	75
3.5.2	Developmentally regulated isoforms responsible for CIV shift	77
3.6	Time-resolved metabolome of postnatal heart development	81
3.6.1	Metabolic changes at birth	82
3.6.2	Metabolic changes at P2	84
3.6.3	Metabolic changes at P10	85
3.6.4	Metabolic maturation of cardiomyocytes after P10	86
4	Discussion	88
4.1	Development-specific differences in phenotype of CIV deficiency	88
4.2	Loss of COX7C affects stability and assembly of CIV	89
4.3	Developmental state of the heart dictates severity of mitochondrial cardiomyopathy	90
4.4	ISR in COX7C and COX10	92
4.5	Complexomics of postnatal heart development	93
4.6	Metabolic maturation during postnatal heart development	96
4.7	Summary	98
5	References	100

List of Figures

Figure 1.1 Mitochondria as metabolic hubs of the cell. Adapted from (Chakrabarty and Chandel, 2022)	22
Figure 1.2 OXPHOS system, adapted from (Sazanov, 2015).....	17
Figure 1.3 OXPHOS supercomplexes: Respirasome (C1CIII ₂ CIV), C1CIII ₂ and CIII ₂ CIV. Adapted from	22
Figure 1.4 Diverse mitochondrial functions as signaling organelles (Chakrabarty and Chandel, 2022)	23
Figure 3.1. Whole body deletion of <i>Cox7c</i> leads to late embryonic lethality..	42
Figure 3.2. BN-PAGE analysis of mitochondria from whole embryos.	44
Figure 3.3. Heart-specific mouse model of COX7C develops early onset cardiomyopathy.	45
Figure 3.4. Changes to cardiac tissue structure upon loss of COX7C.	46
Figure 3.5. Loss of COX7C in the heart leads to an isolated complex IV deficiency.	47
Figure 3.6. Time-resolved complexome profiling of COX7C ^{MKO}	49
Figure 3.7. Dynamics of CIV loss are similar between COX7C ^{MKO} and COX10 ^{MKO}	50
Figure 3.8. Assembly of CIV. (Brischigliaro and Zeviani, 2021)	51
Figure 3.9. Complexome profiling for CIV during postnatal development of COX7C ^{MKO} mice.	53
Figure 3.11. Complexome profiling confirms redistribution of CI and CIII.	55
Figure 3.12. Development of heart mitochondrial phenotype in COX7C ^{MKO} mice.	57
Figure 3.13. Proteomics analysis of COX7C ^{MKO} mice.....	58
Figure 3.14. ISR and OXPHOS changes in COX7C ^{MKO} mice.....	60
Figure 3.15. COX10 ^{MKO} proteomics and ISR activation.	61
Figure 3.16. Proteome changes seen only in COX10 mice.	62
Figure 3.17. ISR activation differs between COX7C ^{MKO} and COX10 ^{MKO}	64
Figure 3.18. Changes in energetic status of COX7C mice is regulated by AMPK.	65
Figure 3.19. Metabolomics of COX7C ^{MKO} hearts.....	66
Figure 3.20. BCAA metabolism in COX7C mice.....	67
Figure 3.21. Pyrimidine synthesis in COX7C hearts.	68
Figure 3.22. Comparison of metabolic profiles of COX7C and COX10 mice	69
Figure 3.23. Fatty acid metabolism differs between COX7C ^{MKO} and COX10 ^{MKO} mice.....	71
Figure 3.25. Time resolved complexome profiling of the heart during postnatal development.....	73
Figure 3.26. Dynamics of supercomplex structures during postnatal heart development.....	74

Figure 3.27. OXPHOS changes in the heart at birth	76
Figure 3.28. Dynamic changes in CIV - containing structures of the heart at birth.	77
Figure 3.29. Developmentally regulated subunit COX6A is responsible for CIV shift in size.	78
Figure 3.30. Complex II dynamics during early postnatal heart development. ...	79
Figure 3.31. Complex V dynamics during postnatal heart development.	80
Figure 3.32. Metabolic changes during postnatal development.	81
Figure 3.33. Metabolic changes at birth.	83
Figure 3.34. Metabolic changes at p2.	84
Figure 3.35. Heart metabolic profile at p10.	86
Figure 3.36. Metabolic maturation of cardiomyocytes continues past P10.	87

List of Tables

<u>Table 2.1 Primers used for genotyping of mice</u>	29
<u>Table 2.2. qPCR primers used to determine gene expression</u>	31
<u>Table 2.3. Antibodies used for WB</u>	33
<u>Table 2.4. Secondary antibodies used for WB</u>	34

Abbreviations

°C	degree Celsius
³⁵ S-Met	radioactive methionine isotope
AAA+	ATPases Associated with diverse cellular Activities
Actb	β-actin
ADP	adenosine diphosphate
AKT	RAC-alpha serine/threonine-protein kinase
ALDH18a1	Aldehyde Dehydrogenase 18 Family Member A1
AMPK	5'-AMP-activated protein kinase
APS	ammonium persulfate
ASNS	Asparagine synthetase
ARS2s	aminoacyl-tRNA synthetases 2, mitochondrial
ARSs	aminoacyl-tRNA synthetases, cytosolic
ATP	adenosine triphosphate
BN	Blue Native
BSA	bovine serum albumin
CAA	chloroacetamide
cDNA	complementary DNA
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
CKMM	Creatine-kinase muscle mitochondrial
CoQ	Coenzyme Q
Cre	Bacteriophage P1 derived site-specific recombinase
Cytc	cytochrome c
CV	complex V
D-loop	displacement loop
(d)dH ₂ O	(double) distilled water
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMMA	European Mouse Mutant Archive
E	embryonic day (e.g., embryonic day 1 – E1)
ETC	electron transport chain
eIF2α	α subunit of the eukaryotic initiation factor 2
EtBr	Ethidiumbromide
ETC	Electron transport chain
FADH ₂	flavin adenine dinucleotide
FAO	Fatty acid oxidation
FC	Fold change

FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Fe-S (cluster)	iron-sulphur (cluster)
fl	floxed
GO	gene ontology
GOBP	gene ontology
H-strand	heavy strand of mitochondrial DNA
HCl	hydrogen chloride
fl	Floxed, loxP flanked
g	Gravitational acceleration (9.81m/s ²)
h	Hour
HIGD1A	5HIG1 hypoxia inducible domain family member 1A
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hmgu	Helmholtz Zentrum München GmbH
HRP	horseradish peroxidase
HSP	heavy strand promotor
HW/BW	heart weight to body weight ratio
HZ	heterozygous
IRES	internal ribosome entry site
IMM	inner mitochondrial membrane
IMS	intermembrane space
ISR	integrated stress response
KCl	potassium chloride
KH ₂ PO ₄	potassium dihydrogen phosphate
KO	knockout
L-strand	light strand of mitochondrial DNA
LB	lysogeny broth
LFQ	label free quantification
LSP	light strand promotor
MEF	mouse embryonic fibroblast
MgCl ₂	magnesium chloride
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
mt	mitochondrial
mtDNA	mitochondrial DNA
Na ₃ VO ₄ ·2H ₂ O	sodium orthovanadate
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NaF	sodium fluoride
NaOH	sodium hydroxide
ncDNA	nuclear DNA
NCR	non-coding region
ns	non-significant
OH	origin of the heavy strand replication
OL	origin of the light strand replication

OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline + Tween 20
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PTFE	polytetrafluoroethylene
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
rpm	rotation per minute
rRNA	ribosomal RNA
RT	room temperature
SC	supercomplex
SD	standard deviation
SDS	sodium dodecyl sulphate
SHMT2	Mitochondrial serine hydroxymethyltransferase
Surf1	Surfeit locus protein 1
SSC	saline-sodium citrate
TBE	Tris/Borate/EDTA
TCA	tricarboxylic acid cycle
TEAB	tetraethylammonium bromide
TEM	transmission electron microscopy
TEMED	tetramethylethylenediamine
TES	N-[Tris(hydroxymethyl)-methyl]-2-aminoethanesulphonic acid
tm1a	targeted trap allele 1a
TMPD	tetramethylphenylendiamin
TFAM	transcription factor A, mitochondrial
Tris	Trisaminomethane
tRNA	transfer RNA
v/v	volume/volume percentage
w/v	mass/volume percentage
WT	wild type
µg	Micro-gram

Abstract

Die oxidative Phosphorylierungsmaschinerie ist das Herzstück der mitochondrialen Energieproduktion. Ihre Funktion und Regulierung hat weitreichende Auswirkungen auf alle energieabhängigen zellulären Prozesse. Ein immer wiederkehrendes Motiv in der Mitochondrienforschung ist der Beitrag der Mitochondrien und der OXPHOS-Aktivität zum metabolischen Umbau, der die Zellproliferation, -differenzierung und -pathologie bestimmt. Die Beteiligung der OXPHOS an der postnatalen Herzentwicklung und der Differenzierung der Kardiomyozyten ist jedoch nach wie vor nicht klar. Umfangreiche Forschungsarbeiten zur Genexpression in der Herzentwicklung haben bisher eine auffällige Veränderung der Expression von Stoffwechselgenen gezeigt, die an der Glykolyse und Lipidoxidation beteiligt sind. Diese Forschungen haben dazu beigetragen, das zu beschreiben, was wir als metabolischen Wechsel in der postnatalen Herzentwicklung kennen, bei dem proliferative, glykolytische Kardiomyozyten zu differenzierten erwachsenen Kardiomyozyten wechseln, die hauptsächlich auf den oxidativen Stoffwechsel angewiesen sind. Diese Umstellung des Stoffwechsels, die durch Veränderungen der Sauerstoffverfügbarkeit, der Substratverwertung und des Energiebedarfs bedingt ist, liegt dem letzten Entwicklungsstadium des Herzens zugrunde. Ziel dieser Studie ist es, die OXPHOS-Komplexe während der postnatalen Entwicklung des Mäuseherzens zu untersuchen, mit besonderem Augenmerk auf Komplex IV (CIV). CIV ist der einzige OXPHOS-Komplex mit herzspezifischen Isoform-Untereinheiten, die während der postnatalen Reifung der Mitochondrien ausgetauscht werden.

Wir haben zwei Mausmodelle für einen CIV-Mangel im Herzen entwickelt und verglichen, wobei wir CIV in der frühen postnatalen Entwicklung durch Deletion von Cox7c oder CIV im erwachsenen Herzen durch Deletion von Cox10 angreifen. Durch den Vergleich dieser beiden Mausmodelle haben wir beschrieben, wie CIV an der Konditionierung der metabolischen Reifung von Kardiomyozyten über die metabolische Umschaltung hinaus beteiligt sein könnte, und seine Beteiligung an der Kardiomyozytenreifung festgestellt. Unsere Daten zeigen, dass der schnelle und fortschreitende Verlust von CIV in der postnatalen Entwicklung zum Teil auf eine Blockierung des Zusammenbaus von CIV in der Anfangsphase der komplexen Biogenese zurückzuführen ist. Der Verlust von CIV in diesen Mäusen führt zu metabolischen Veränderungen, die eine Neuverdrahtung des Glukosestoffwechsels, des Nukleotid-Synthesewegs und Veränderungen im BCAA-Stoffwechsel mit einer leichten Beteiligung der ISR bewirken. Im Vergleich dazu führt der Verlust von Cox10 zu einer robusten ISR-Aktivierung und einem umfassenden Umbau sowohl des Proteoms als auch des Metaboloms, während der CIV-Mangel zwischen den beiden Modellen vergleichbar ist, was darauf hinweist, dass der Differenzierungsstatus der Kardiomyozyten zu den phänotypischen Unterschieden beitragen könnte. Um eingehend zu beschreiben, wie sich die OXPHOS während der postnatalen Entwicklung verändert, haben wir ein Zeitreihen-Komplexom-Profilierung der

mitochondrialen Komplexe des Mäuseherzens von E18,5 bis P10 durchgeführt. Zusammen mit der Zeitreihen-Metabolomanalyse wollten wir beschreiben, wie die Dynamik der OXPHOS die metabolischen Veränderungen während der Reifung der Kardiomyozyten bedingt.

Abstract

Oxidative phosphorylation machinery sits at the core of mitochondrial role in energy production, its function and regulation has far-reaching implications in all energy dependent cellular processes. Recurring motif in mitochondrial research is the contribution of mitochondria and OXPHOS activity to metabolic remodelling that conditions cell proliferation, differentiation, and pathology. However, involvement in OXPHOS in postnatal heart development and differentiation of cardiomyocytes stays elusive. Extensive research conducted on gene expression in heart development so far shows a prominent change of expression for metabolic genes involved in glycolysis and lipid oxidation, describing what we know as the metabolic switch in postnatal heart development. The metabolic switch, where proliferative, glycolytic cardiomyocytes switch to differentiated adult cardiomyocytes reliant mostly on oxidative metabolism is conditioned by changes in oxygen availability, substrate utilisation and energy demands underlying the final developmental stage of the heart.

This study aims to dissect OXPHOS complexes during postnatal development of the mouse heart, with specific accent on Complex IV (CIV). CIV is the only OXPHOS complex with heart specific isoform subunits, that exchange during postnatal maturation of mitochondria. We have developed and compared two mouse models for CIV deficiency in the heart, targeting CIV in early postnatal development by deleting *Cox7c*, or adult heart CIV, by deleting *Cox10*. By comparing these two mouse models, we have described how CIV might be involved in conditioning metabolic maturation of cardiomyocytes beyond the metabolic switch, finding its involvement in cardiomyocyte maturation. Our data shows that fast and progressive loss of CIV in postnatal development is in part due to stalled assembly of CIV at the initial stage of complex biogenesis. Loss of CIV in these mice leads to metabolic changes propagating rewiring of glucose metabolism, nucleotide synthesis pathway and alterations in BCAA metabolism, with mild involvement of ISR. In comparison, loss of *Cox10* lead to a robust ISR activation and extensive remodeling of both proteome and metabolome, while the CIV deficiency between the two models is comparable, indicating that differentiation status of cardiomyocytes could contribute to phenotypical differences. To describe in-depth how OXPHOS changes during postnatal development, we resolved time-series complexome profiling of mouse heart mitochondrial complexes starting from E18.5 to P10. Together with time series metabolome analysis, our aim was to describe how dynamics in OXPHOS could condition the metabolic alterations happening during maturation of cardiomyocytes.

1 Introduction

1.1 Postnatal heart development

Heart is a highly functional muscular organ that works like a pump: enabling the distribution of nutrients, oxygen, and carbon dioxide by pumping blood throughout the body. It is the first organ to form in a developing embryo from the mesoderm, emerging from the primitive streak during gastrulation (Fishman and Chien, 1997, Buckingham et al., 2005). Heart development is a highly orchestrated process that can be roughly separated to embryonic and postnatal phase (Tan and Lewandowski, 2020), each consisting of essential events that lead to the formation of the adult mature heart. Adult mammalian heart is composed of muscle cells called cardiomyocytes, which make up most of the heart, together with smooth muscle cells, fibroblasts, and endothelial cells (Garry and Olson, 2006).

Differentiation of cardiomyocytes from stem cells is marked as the earliest event in heart formation during embryonic development. Embryonic development entails the formation of the heart organ from the primitive streak through proliferation of mononucleated cardiomyocytes (Foglia and Poss, 2016, Lunt and Vander Heiden, 2011). The myocardial progenitor cells migrate from the primitive streak to the anterior of the embryo and form the cardiac crescent (Lescroart et al., 2014, Kelly et al., 2014). As the cardiac crescent fuses down the midline, the early cardiac tube is formed. Cardiac chambers are formed by further expansion of the cardiac tube (Fishman and Chien, 1997, Kelly et al., 2014).

The maturation of cardiomyocytes from embryonic immature to adult cardiomyocytes happens mostly in the postnatal phase of heart development. Postnatal heart development starts with birth and lasts for couple of weeks in rodents (Piquereau and Ventura-Clapier, 2018). Firstly, blood oxygen levels increase dramatically as the lungs become functional and supply blood directly to the left side of the heart (Kelly et al., 2014, Rudolph, 1979, Heymann et al., 1981, Rudolph and Heyman, 1974). As the heart environment becomes normoxic and the oxygen levels increase, the heart growth pattern changes: cardiomyocytes switch from hyperplastic (cell proliferation) to hypertrophic (increase in cell size) growth (Bertagnoli et al., 2014, Bensley et al., 2010). Hypertrophy of cardiomyocytes entails cell division without cytoplasmic separation, resulting in the formation of binucleated myocardial cells which is a marker of maturation of the heart (Manasek, 1968, Polinger, 1973). In rodents, an increase in binucleated cardiomyocytes is evident in specific timepoints: from postnatal day 4 (P4) DNA synthesis shows a striking increase, and by P7 cardiomyocytes arrest cell cycle and do not proliferate anymore (Soonpaa and Field, 1998). Additionally, reactive oxygen species (ROS) levels increase significantly in cardiomyocytes from P1 to P7, contributing to the cell cycle arrest in cardiomyocytes (Puente et al., 2014). Mitochondrial ROS are generated as electrons leak from the electron transport chain, leading to damage of proteins, lipids, or DNA, leading to cell-cycle arrest (Puente et al., 2014, Hoeijmakers, 2009). Some reports suggested that increase in oxygen levels

is a one of the main causes for cell-cycle arrest of cardiomyocytes, forcing hypertrophic growth over proliferation (Puente et al., 2014, Ikenishi et al., 2012).

Other than a change in growth of the heart and cell cycle arrest, a metabolic switch occurs in postnatal development, where cardiomyocytes gradually switch substrate for OXPHOS, from glucose to fatty acid oxidation (Lopaschuk and Spafford, 1990, Itoi and Lopaschuk, 1993, Girard et al., 1992). In the mature heart, mitochondrial oxidative metabolism normally provides over 90% of the heart's energy demands, and fatty acid oxidation is normally the major source of mitochondrial oxidative metabolism (Wang et al., 2010, Stanley et al., 2005, Piquereau and Ventura-Clapier, 2018). Still, it is important to note that the mature heart is one of the most metabolically flexible tissues, capable of oxidizing several additional fuels including glucose, lactate, ketones, and amino acids when in acute stress (Wang et al., 2010). Even though it seems energetically unfavorable, maintaining glycolytic metabolism during embryonic development seems to facilitate growth of the embryonic heart through proliferation of cardiomyocytes (Chung et al., 2007, Chung et al., 2010). The low levels of fatty acids and high levels of lactate *in-utero* further contribute to the glycolytic metabolism of the fetal heart (Portman et al., 1969, Girard et al., 1992, Bartelds et al., 1998, Makinde et al., 1998). As a result, only 15% of total energy production derives from the use of fatty acids in the embryonic heart (Lopaschuk and Jaswal, 2010), as an adaptation to the low oxygen environment (Martin et al., 2014). However, to be able to differentiate from stem cells during embryonic heart formation, cardiomyocytes need OXPHOS: anaerobic metabolism of embryonic heart has to change into more efficient mitochondrial oxidative metabolism for cardiac differentiation (Chung et al., 2007). This energetic switch is programmed by rearrangement of the metabolic transcriptome that encodes components of glycolysis, fatty acid oxidation, the Krebs cycle, and the electron transport chain (Stanley et al., 2005). After birth, the cardiomyocytes further increase utilization of OXPHOS, and can transform into adult mature cardiomyocytes (Chung et al., 2010, Chung et al., 2007).

While the necessity for OXPHOS in cardiomyocyte differentiation has been explained, until recently it was unclear what triggers the switch to OXPHOS utilization during postnatal development and how it relates to maturation of the heart. Recent studies show that the substrate availability is the answer to what triggers the metabolic switch of postnatal cardiomyocytes: metabolic transition seems to be coordinated by maternally supplied γ -linolenic acid (GLA), an 18:3 omega-6 fatty acid enriched in the maternal milk (Paredes et al., 2023). GLA binds and activates retinoid X receptors (RXRs), ligand-regulated transcription factors that are expressed in cardiomyocytes from embryonic stages (Paredes et al., 2023). Lack of RXR in embryonic cardiomyocytes caused changes to the chromatin profile, thus preventing the induction of an RXR-dependent expression genes involved in homeostasis of mitochondrial fatty acid metabolism (Paredes et al., 2023). The ensuing defect included inability to produce lipid derived energy in mitochondria and an increase in glucose consumption, leading to perinatal cardiac dysfunction and death (Paredes et al., 2023).

A component of postnatal metabolic switch is the formation of a functional mitochondrial compartment in which fatty acids are efficiently oxidized by β -oxidation (FAO) (Piquereau and Ventura-Clapier, 2018), indicating the importance of mitochondrial dynamics in the process. Postnatal development is actually marked by an increase in the cardiac mitochondrial mass and the expression of mitochondrial proteins (Piquereau et al., 2010, Hallman, 1971, Glatz and Veerkamp, 1982, Lai et al., 2014, Martin et al., 2014). Further on, deletion of mitofusin 1 and 2 (Mfn1 and Mfn2), which are involved in mitochondrial fusion, postnatally leads to ultrastructural disorganization and heart failure (Papanicolaou et al., 2012). In general, many genes involved in mitochondrial biogenesis show increase in expression during the first weeks of life (Pohjoismaki et al., 2012), which lead to a hypothesis that mitochondria might be the drivers behind maturation of cardiomyocytes (Taegtmeyer et al., 2010). Reports supporting this claim include a study where Parkin-mediated mitophagy was shown to be essential for postnatal metabolic switch by mediating removal of immature mitochondria so they can be substituted by new, mature mitochondria (Gong et al., 2015, Wai et al., 2015).

Mitochondrial maturation is essential for energy distribution in the differentiated cardiomyocytes (St John et al., 2005). Expansion of the mitochondrial network leads to maturation of the energetic system in cardiomyocytes, supplying nascent sarcomeres and providing resource for excitation-contraction coupling (Chung et al., 2007, Cho et al., 2006, St John et al., 2005). Further on, maturation of sarcomeres sits at the core of energy handling and excitation-contraction coupling of adult cardiomyocytes. While in the embryo, ATP can freely diffuse through the cell to its consumption (Brooks and Storey, 1991, Ventura-Clapier et al., 2011), in adult cardiomyocytes, cellular structure is too packed to allow for diffusion (Saks et al., 2008). In the complex architecture of adult cardiomyocytes, where the main energy consumers are sarcoplasmic reticulum ATPases (SERCA) and ATPases of myosin myofilaments (myosin-ATPases), the energy needs to be transferred from the mitochondria (Ventura-Clapier et al., 1998). For this reason, the heart employs a more sophisticated cellular energy transfer system based on phosphorylation of creatine (Cr) by creatine kinase enzyme (CK) (Bessman and Geiger, 1981). In the adult heart, nearly 90% of energy leaves mitochondria in the form of P-Cr, not ATP. The high-energy phosphate moiety of PCr is shuttled near ATPases where it can be used to re-phosphorylate the locally produced ADP (Saks et al., 2006). This mechanism is the main pathway by which oxygen consumption adjusts to cardiac work (Saks et al., 2006). Other than delivering energy, this shuttle system enables information transfer to the mitochondria by sensing ADP/ATP ratios (Saks et al., 1991, Ventura-Clapier et al., 1998). While the very low CK activity of the fetal heart prohibits any transfer by the CK shuttle (Hoerter et al., 1991), CK activity increases dramatically after birth, indicating energetic maturation of the heart (Hoerter et al., 1991, Fischer et al., 2010). Progressively with the increase in CK activity, the complexification of the intracellular structure and the increase in energy demand happens, the CK system is now able to

ensure an effective energy production (Hoerter et al., 1991, Fischer et al., 2010, Piquereau et al., 2010).

1.2 Mitochondria

Mitochondria seem to play a key role in enabling postnatal heart maturation. The canonical roles of mitochondria established decades ago include production of adenosine triphosphate (ATP), which is the energy currency of the cell. Through the reaction of oxidative phosphorylation, mitochondria produce ATP, enabling the cell to harness the energy contained in carbon fuels.

Mitochondria are cellular organelles that consist of outer and inner membrane, contain their own DNA, and serve as a host of the cellular respiration and energy production. They are dynamic organelles that form a network within the cell, and their function and maintenance is regulated through processes of mitochondrial dynamics, such as biogenesis, mito-fusion, and mito-fission. Even though they possess their own DNA, and the nucleus encodes for a full set of genes needed to maintain mitochondrial function and gene expression. Mitochondrial DNA is significantly different from nuclear DNA. The mammalian mitochondrial DNA (mtDNA) is localized within the mitochondrial matrix. It is a circular molecule of a 16 kb length (Anderson et al., 1981). mtDNA encodes 13 core proteins of OXPHOS, and to maintain its independent protein synthesis machinery, it also expresses its own 22 tRNAs, and 2 rRNAs (Mercer et al., 2011). mtDNA consists of a heavy (H) strand and a light (L) strand. It also contains a non-coding control region, where promoters for each strand can be found (HSP or LSP, respectively) strand (Montoya et al., 1982). An additional LSP (LSP2) has been recently discovered (Tan et al., 2022). Mitochondrial genes encode 12 messenger RNAs (mRNA), 14 transfer RNAs (tRNA), and 2 ribosomal RNAs (rRNA) are localized on the H-strand. The genes on the L-strand then encode the remaining 1 mRNA and 8 tRNAs (Mercer et al., 2011). Mitochondrial transcription factor A (TFAM) has been shown to pack mtDNA into nucleoid structures (Kukat et al., 2015).

1.3 OXPHOS

Oxidative phosphorylation (OXPHOS) is a set of oxidation-reduction reactions within the inner mitochondrial membrane, carried out by four complexes: Complex I (CI, or NADH: ubiquinone oxidoreductase); Complex II (CII, SDH or Succinate Dehydrogenase), Complex III (CIII, or cytochrome bc1 oxidoreductase), Complex IV (CIV or cytochrome C Oxidase) and two mobile electron carriers: ubiquinol and cytochrome c, that comprise the electron transport chain (ETC), and finally Complex V (CV or ATP synthase) (Figure 1.3). Components of the ETC are responsible for accepting and transferring electrons from the tricarboxylic acid cycle (TCA) and fatty acid oxidation (FAO) to final electron acceptor – oxygen. Electrons from TCA and FAO in the form of NADH and FADH are transferred to CI and CII, respectively. Further on from CI and CII, electrons are transferred to the first electron carrier in the ETC, coenzyme Q. Complex III oxidizes coenzyme Q and shunts electrons to the second electron carrier, cytochrome C. In the final reaction of ETC, cytochrome C is oxidized by CIV, which is coupled to reduction of O₂ to H₂O. CIII is then responsible for

accepting electrons from both CI and CII, acting as a funnel for electrons originating from different metabolic pathways. Other than CI and CII, there are other enzyme complexes that contribute to electron flow on the level of CIII. Other dehydrogenases that also donate electrons to ubiquinone are dihydroorotate dehydrogenase, glycerol 3-phosphate dehydrogenase, choline dehydrogenase, proline dehydrogenase, sulfide: quinone oxidoreductase (Alcázar-Fabra et al., 2021, Wang and Hekimi, 2016). Out of four complexes of the ETC, three complexes also have a proton pumping function. Complexes I, III and IV are responsible for pumping protons across the inner mitochondrial membrane (IMM), from the matrix to the intermembrane space (IMS), thus creating a proton gradient. Proton gradient generated through proton pumping of ETC components is then utilized by ATP synthase. Protons accumulated in the IMS can then enter mitochondrial matrix only through ATP synthase, causing it to turn. This mechanical system of turning the components of ATP synthase is necessary for phosphorylation reaction of ADP to ATP (Leone and Faraldo-Gomez, 2016, Liu et al., 2016).

The three proton pumping complexes employ distinct mechanisms for proton translocation through the IMM. Complex I utilizes antiporter-like subunits for proton transfer (Jones et al., 2017, Sazanov, 2015) and Complex III uses the Q cycle (Mitchell, 1976, Trumpower, 1990). Proton pumping mechanism for CIV is yet unconfirmed. Firstly, two proton-pathways have been described within CIV. One is going from matrix to the heme a3 site for oxygen reduction. Another one is going from matrix to IMS for proton translocation. Iwata et al. proposed two proton pathways, called K-pathway and D-pathway, according to the structure of prokaryotic cytochrome c oxidase (Iwata et al., 1995). According to the crystal structures of CIV, the third proton pathway, H-pathway, containing His413 at the matrix side and Asp51 at the IMS side, was proposed (Kannt et al., 1998, Rottenberg, 2022). In this model, the low-spin heme a takes the role for proton pumping across the membrane (Kannt et al., 1998). While Complex II does not have proton pumping capacity, it contributes by shuffling electrons to the Q pool. Through these mechanisms, OXPHOS provides energy to all living systems.

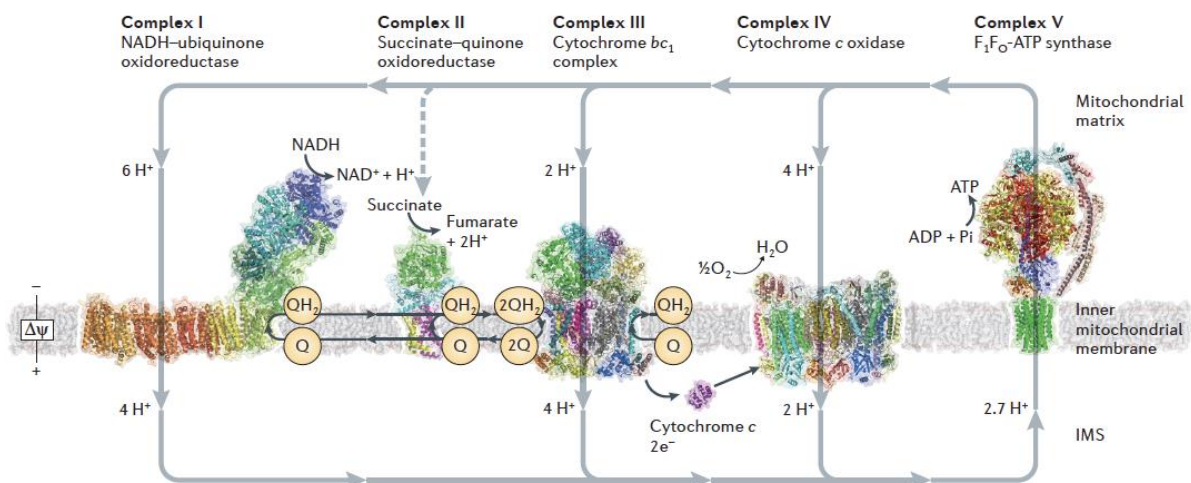


Figure 1.1 OXPHOS system, adapted from (Sazanov, 2015).

Complex I is responsible for coupling the oxidation of NADH and reduction of ubiquinone to the pumping of four protons across the inner mitochondrial membrane (IMM) into the intermembrane space (IMS). Complex I is the largest among OXPHOS complexes, with 44 subunits and a mass of almost 1MDa (Zhu et al., 2016, Fiedorczuk et al., 2016). There are 14 core subunits that form the inner part of the complex, surrounded by 31 supernumerary subunits (Fiedorczuk et al., 2016, Zhu et al., 2016). Out of 14 core subunits, seven subunits (ND1 – ND6 and ND4L) are encoded by mitochondrial DNA and membrane embedded (Chomyn et al., 1985, Chomyn et al., 1986). Rest of the core subunits form the hydrophilic or matrix arm of CI and are responsible for NADH oxidation (Birrell et al., 2011), since it contains the primary electron acceptor flavin mononucleotide (FMN) and eight Fe-S clusters (Baradaran et al., 2013). The enzymatic core is encompassed by supernumerary subunits, whose functions are either in stability of assembly of CI (Stroud et al., 2016). Assembly of CI is a highly regulated process with over 20 assembly factors identified so far (Guerrero-Castillo et al., 2017, Formosa et al., 2020). Assembly starts from the independent formation of modules, that eventually come together: N module or dehydrogenase module, which is towards the matrix side, Q module or hydrogenase module, and P module or proton translocation module. During assembly process of CI, P module is assembled by combining 4 modules: P_P-a and P_P-b; P_D-a and P_D-b, while N-module is added last (Guerrero-Castillo et al., 2017).

Complex II is the only enzyme of OXPHOS that is completely nuclear encoded. It is composed of four subunits and exists as a functional monomer (Rasheed and Tarjan, 2018). Membrane embedded subunits are SDHC and SDHD, and their assembly and embedding are facilitated by assembly factors SDHAF1-F4 (Moosavi et al., 2019). The two soluble matrix subunits SDHA and SDHB contain the primary electron acceptor FAD and three iron-sulfur clusters and are linked to the IMM by the membrane bound subunits (Sun et al., 2005).

Complex II is a crucial player in cellular metabolism, given its position at the intersection between two key pathways, the Krebs cycle and the OXPHOS system: it couples the oxidation of succinate to fumarate in the Krebs cycle (Blaut et al., 1989) with the reduction of ubiquinone to ubiquinol, the substrate of complex III₂ of the ETC (Ruprecht et al., 2011).

Complex III₂ couples ubiquinol oxidation to reduction of the soluble electron carrier cytochrome c, while taking up two protons from the matrix and releasing four into the IMS. It has an obligate homodimer structure related to its function, with three catalytic subunits per monomer (or protomer) that are highly conserved from bacteria to eukaryotes: cytochrome b (also known as MT-CYB), the only mitochondrial DNA-encoded subunit, cytochrome c1 (also known as CYC1) and the Rieske iron–sulfur protein (ISP; also known as UQCRFS1) (Xia et al., 2013). Cytochrome b contains two haem groups, named haem bL and haem bH, constituents of the low-potential redox chain, whereas cytochrome c1 harbours haem c1, a constituent of the high-potential redox chain along with the Fe–S cluster in the Rieske protein (Gao et al., 2003, Xia et al., 2013).

The mechanism of action of complex III₂ is referred to as the Q cycle (Mitchell, 1976, Trumpower, 1990) as the ISP subunit transfers electrons from quinone to cytochrome c₁, and can be performed only in the dimeric form (Xia et al., 1997).

The Q cycle begins with the binding of one quinol at the haem b_L site. From there, the first electron and the first proton are donated to the Fe–S cluster and a conserved histidine of ISP, respectively (rate-limiting step of the reaction). This generates a highly reactive quinone radical, which eventually is fully oxidized by haem b_L. From haem b_L, the electron is passed to haem b_H and then to an oxidized quinone at the haem b_H site. The partially reduced quinone at the haem b_H site is then fully reduced to quinol (Xia et al., 2013). In addition to the catalytic subunits, complex III₂ contains supernumerary subunits, whose composition differs across species, with the mammalian enzyme containing eight such subunits: the so-called core 1 and core 2 proteins (UQCRC1 and UQCRC2) in the matrix and six additional supernumerary subunits, five encapsulate the catalytic core (UQCRH, UQCRB, UQCRQ, UQCR10 and UQCR11)(Xia et al., 2013, Zong et al., 2018a) and one (subunit 9) between the core proteins (Zong et al., 2018a). Correctly assembled complex III₂ seems to be included in stability and maintenance of other OXPHOS complexes, as it was shown to be necessary for the maturation of complex I and complex IV, pointing towards a functional crosstalk among the ETC complexes (Protasoni et al., 2020).

Complex IV is the last enzyme of the ETC. It catalyzes the transfer of electrons from cytochrome c to oxygen to produce water in the process of cellular respiration. Further on, it contributes to the proton gradient by pumping a proton for each cytochrome c molecule (Yoshikawa et al., 2012). Complex IV appeared as 13 subunits-containing functional dimer in crystal structures (Yoshikawa et al., 1998). More recently monomeric structure of CIV has been resolved with detection of a 14th subunit NDUFA4, shown to be loosely bound to monomer of CIV, thereby preventing dimer formation (Zong et al., 2018b). Out of 14 subunits that comprise CIV, three are encoded by mitochondrial genome and form the enzymatic core, and 11 are nuclear encoded supernumerary subunits, that have been implicated in modulation of CIV enzymatic function (Timon-Gomez et al., 2018). Three core subunits are COX1 (mtCO1), COX2 (mtCO2), COX3 (mtCO3). They are encoded by mitochondrial DNA and make up the enzymatic core of Complex IV. COX1 contains heme a and heme a₃, and a copper center Cu_B. COX2 contains the second copper center Cu_A, which acts as the primary electron acceptor. COX3 contains three tightly bound phospholipids implicated in oxygen uptake (Shinzawa-Itoh et al., 2007). The Cu_A site is responsible for the initial step of the catalytic cycle by accepting the electrons transferred from cytochrome c, which then reduces the heme site in COX1. These electrons are then transferred to the Cu_B-heme a₃ site, where molecular oxygen binds and gets reduced to water (Babcock and Wikstrom, 1992). Rest of the subunits are nuclear encoded, and imported into the mitochondria for assembly into CIV. They are regarded as supernumerary subunits, and considered to have a role in either modulating activity of CIV, or contributing to assembly and stability of CIV (Cunatova et al., 2020). Most of

the subunits contain a transmembrane region, except for subunits 5A and 5B, which are bound to the matrix side, and subunit 6B, which faces the intermembrane space.

Cytochrome c oxidase is the only enzyme of OXPHOS which has isoforms for nuclear encoded subunits. In early studies on CIV it has been shown that some nuclear subunits have tissue specific isoforms (Taanman et al., 1993). While role of these isoforms was not clear, very soon the regulation for switch of the isoforms was determined. Isoform switch can be hypoxia-induced, with isoforms for COX4, NDUF4A, or be or developmentally induced, COX6A, COX7A and COX8 (Taanman et al., 1993, Sinkler et al., 2017).

Under hypoxic conditions the hypoxia-inducible factor 1 (HIF-1) is activated to regulate the oxygen homeostasis by regulating expression of multiple genes responsive to oxygen levels. Two of HIF-1 targets in the lung are LONP1 and COX4I2, and upon HIF-1 activation, LONP1 selectively degrades COX4I1 to allow the switch to COX4I2 isoform (Fukuda et al., 2007). COX5B and COX4I1 must be phosphorylated to be recognized by LONP1 as mutations in the respective phosphorylation sites protected the substrates against LONP1. The phosphorylation is mediated by the protein kinase A (PKA) (Sepuri et al., 2017).

Isoform switch for COX6A and COX7A subunits was investigated in muscle development (Ewart et al., 1991, Van Beeumen et al., 1990) and postnatal heart development (Bonne et al., 1993), where steady state transcript levels were measured for both isoforms during fetal development and in adult human tissues. While the respective contributions of these isoforms in pathological conditions have been investigated in context of pathological and benign infantile respiratory deficiencies, no connection to the pathology could be found (Boczonadi et al., 2015).

Isoforms for COX4I and COX6B are present in the lung and testes, respectively (Huttemann et al., 2003, Huttemann et al., 2001). The subunits COX6A, COX7A and COX8 have muscle-specific isoforms and non-muscle or liver isoforms (Grossman and Lomax, 1997). Liver specific isoform is the ubiquitously expressed form, while skeletal muscle and heart express the muscle-specific form (Bonne et al., 1993, Taanman et al., 1993). The isoforms have been shown to modulate the activity of CIV to adapt to change in environment, as with COX4I isoforms, or to adapt to the energy demand of the cell (Ye et al., 2023). Other than being modulated by isoform specificity, posttranslational modifications such as acetylation, methylation, nitrosylation were shown (Kalpage et al., 2019).

De-novo synthesis of CIV was seen as a sequential process, in which assembly of CIV happens in steps, and intermediates are formed by addition of subunits on top of the enzymatic core (Nijtmans et al., 1998). Currently, assembly of CIV is considered as a modular process, in which three modules containing each of the core subunits are formed independently and combine to form fully assembled CIV (Vidoni et al., 2017). COX1 module is formed first, where a seed containing COX5A and COX4, associated via HIGD1a, attaches COX1 subunit (Vidoni et al., 2017). COX1 expression is regulated in a post-transcriptional fashion, involving TACO1 and LRPPRC as components of mitochondrial gene expression machinery (Weraarpachai et al., 2009).

Additionally, synthesis and stability of COX1 is regulated by MITRAC complex (Mick et al., 2012). Second module contains COX2 and the following nuclear encoded subunits: COX76C, 7B, 7C, 8A, 5B (Timon-Gomez et al., 2018). COX3 module is the final stage of assembly of CIV, with the association of 6A, 6B and 7A making a holoenzyme (Vidoni et al., 2017, Lobo-Jarne et al., 2020, Tang et al., 2020, Timon-Gomez et al., 2018).

Complex V exploits the proton gradient generated by complexes I, III₂ and IV to phosphorylate ADP and generate ATP (Mitchell, 1961). ATP synthases can also function in the opposite direction, to generate proton-motive force via ATP hydrolysis (Abbas et al., 2020, Kühlbrandt, 2019). Complex V features two main components: the Fo domain with the c-ring, which is membrane embedded and responsible for proton translocation, and the soluble F1 domain — composed of three α -subunits and three β -subunits forming a globular assembly — which comprises the catalytic head responsible for the phosphorylation of ADP to ATP (Kühlbrandt, 2019). The assembly of ATP synthase is a modular process featuring the independent formation of five subcomplexes, aided by the assembly factors ATPAF1, ATPAF2, TMEM70, C7orf55 and TMEM242 (Carroll et al., 2021).

Other than existing as free individual complexes or in oligomeric structures, OXPHOS complexes have been shown to interact with each other and form so-called supercomplexes (Figure 1.3). These supercomplex structures, containing more than one OXPHOS complex within their structure, have primarily been observed on blue native polyacrylamide gel electrophoresis (BN-PAGE), by solubilizing mitochondria with a mild detergent called digitonin. Their existence has further been proven and described by cryoelectron microscopy (Schägger and Pfeiffer, 2000, Chavez et al., 2018, Liu et al., 2018, Rieger et al., 2017, Gu et al., 2016, Letts et al., 2016). Supercomplex structure that has been described the best so far is the respirasome, containing CI, CIII₂ and CIV. The respirasome structure has been solved as well (Letts et al., 2016, Gu et al., 2016), and it is described as the simplest supercomplex that contains a complete electron transfer chain: CI accepts electrons from NADH, transfers them to coenzyme Q (ubiquinol) which is oxidised by CIII₂, from where cytochrome c finally transfers electrons to CIV, which is then responsible for reducing O₂. Additionally, isolated respirasome is a functioning bioenergetic unit, capable of respiring (Acin-Perez et al., 2008). While it is undeniable they exist, their supposed function is highly debated, from contributing to bioenergetic plasticity and metabolic flux, to substrate channeling theories and stabilisation of complex assemblies (Balsa et al., 2019, Lapuente-Brun et al., 2013). Their role in substrate channeling has been investigated thoroughly, with reports showing no sustainable evidence of sequestering pools of cytochrome c or coenzyme Q (Sousa et al., 2016, Molinie et al., 2022, Fedor and Hirst, 2018). Similarly, contribution of the respirasome to cellular bioenergetics has very recently been elucidated. By engineering a mouse model incapable of forming the respirasome structure, group of Nils-Göran Larsson from Karolinska Institute in Sweden, elegantly showed that these mice are not bioenergetically dependent on respirasome, both in steady state or upon exercise (Milenkovic et al., 2023). They also

do not show any increase in ROS production or complex stability or assembly (Milenkovic et al., 2023). In plants, another supercomplex structure showed similar lack of effect to plant physiology, where CIII₂-CIV containing supercomplex was disrupted (Rohricht et al., 2023). Therefore, supercomplexes remain a mystery in the field, and their role in mitochondrial function elusive.

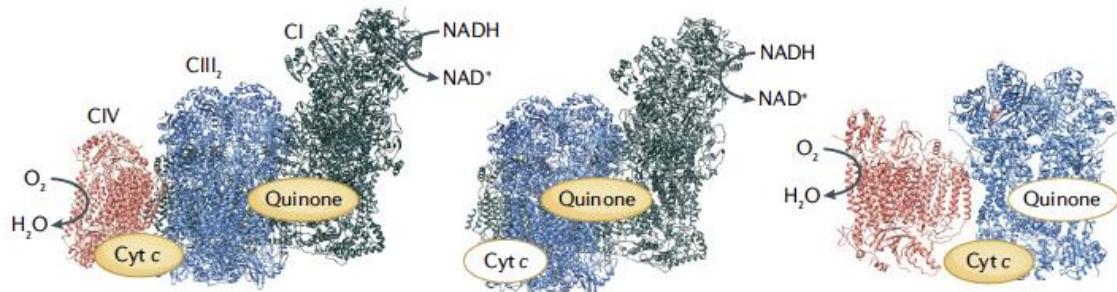


Figure 1.2 OXPHOS supercomplexes: Respirasome (CI-CIII₂-CIV), CIII₂-CIV and CIII₂-CI. Adapted from

As mentioned, OXPHOS acts as a funnel for electrons from many metabolic pathways, needed to build a proton-motive force for ATP production. Other than providing energy, mitochondria provide important biosynthetic intermediates. TCA cycle resides within mitochondrial matrix of eucaryotic cells, and oxidizes pyruvate from glycolysis, but also fatty acids and amino acids, generating metabolic intermediates and reducing equivalents like NADH and FADH₂. Metabolic intermediates created in mitochondria further contribute to anabolic pathways, such as lipid, carbohydrate and protein synthesis, and nucleotide synthesis pathways.

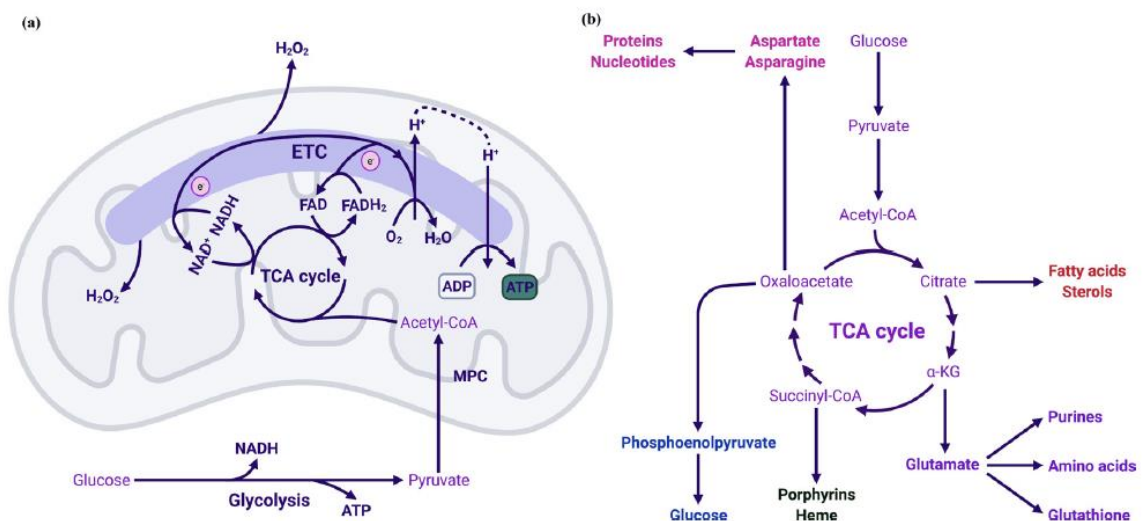


Figure 1.3 Mitochondria as metabolic hubs of the cell. Adapted from (Chakrabarty and Chandel, 2022)

In mammalian systems, different cell types have different bioenergetic demands. For example, heart tissue demands much more energy production than liver, as a muscular organ with high energy demand and inability to store energy. Bioenergetic demands of the cell also vary depending on the developmental status of the cell. Thus,

understanding of mitochondria as cellular organelles responsible for ATP production has been replaced by a more updated, modern definition of mitochondria as metabolic, signaling and energetic hub of the cell, responsible for receiving, processing, and responding to signals from internal and external stimuli, thus regulating cellular responses and maintaining homeostasis. Other than being a bioenergetic and metabolic hub of the cell, mitochondria are also involved in regulating cell death, innate immunity, and cell fate (Figure 1.4).

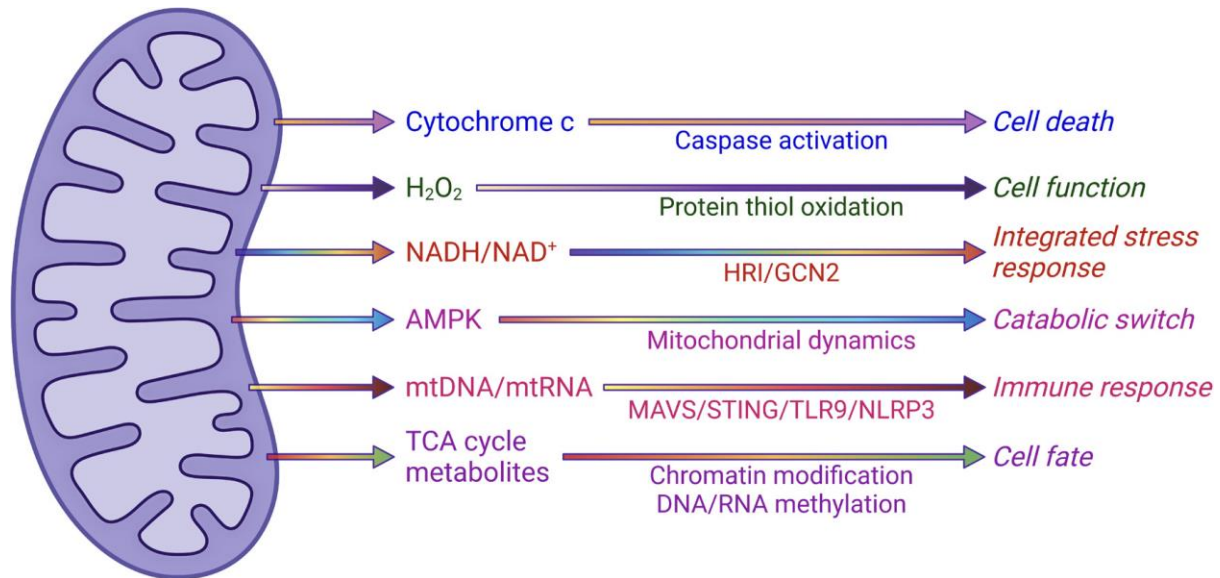


Figure 1.4 Diverse mitochondrial functions as signaling organelles (Chakrabarty and Chandel, 2022)

1.4 The integrated stress response

One of the outcomes of mitochondrial dysfunction is the metabolic remodeling caused by activation of the mitochondrial branch of the integrated stress response (ISR). ISR acts as a junction of stress signals coming from different cellular compartments and different insults such as redox stress, proteostasis stress, nutrient starvation, or viral infection, for one of two avails: cell death or pro-survival metabolic compensation (Costa-Mattioli and Walter, 2020). Main mechanism of action is a reduction in global cellular translation rate, while maintaining translation of a specific set of transcription factors, that act as effectors of ISR changing the transcriptome to support metabolic remodeling (Neill and Masson, 2023).

Suppression of global translation is achieved by modulating levels of translation initiation factor eIF2 through phosphorylation of its α subunit. eIF2 α , together with β and γ subunits, comprises a heterotrimeric eukaryotic translation initiation factor eIF2 (Kashiwagi et al., 2019). Additionally, a guanosine nucleotide (GDP or GTP), and methionine bound initiator tRNA are needed to form the eIF2a ternary complex (TC) which is considered active and functional translation initiation factor when loaded with GTP (Kashiwagi et al., 2019, Kenner et al., 2019). Binding to the start codon on the mRNA initiates hydrolysis of GTP, after which eIF2-GDP dissociates, enabling ribosomal assembly and elongation phase of translation (Kenner et al., 2019). Exchange of GDP to GTP is essential for proper activation of eIF2, and it is catalyzed

by eIF2B, a designated guanine nucleotide exchange factor (GEF) for eIF2 (Algire et al., 2005, Kenner et al., 2019). In eukaryotes phosphorylation of eIF2 α is mediated by four stress-sensing kinases that respond to different stressors: general amino acid control nonderepressible 2 – GCN2, that senses amino acid depletion (Harding et al., 2019), dsRNA-dependent protein kinase - PKR senses viral infection (Dey et al., 2005), PKR-like ER kinase - PERK mediates response to protein misfolding and ER stress (Harding et al., 1999), and heme-regulated inhibitor - HRI, canonically known to respond to heme depletion (Wek, 2018, Han et al., 2001). They all contain a conserved kinase domain able to phosphorylate Ser51 residue of eIF2 α , and a variable regulatory domain that senses different stressors (Donnelly et al., 2013).

Upon stress, eIF2 α is phosphorylated at Ser51, turning it into an inhibitor of eIF2B and blocking its GEF activity and global translation (Kenner et al., 2019). Although global translation is blocked due to inability of the TC containing eIF2 to assemble, a subset of transcription factors known to act as the effectors of ISR are being synthesized. They are a group of transcription factors that contain another open reading frame (ORF) upstream of their canonical AUG, in their 5'- untranslated region. In normal conditions when translation is ongoing in homeostasis conditions, translation starts both at this upstream reading frame and the canonical AUG. In unstressed cells, low levels of eIF2 α phosphorylation favor early capacitation of such reinitiating ribosomes directing them to the inhibitory uORF2, which precludes subsequent translation. With activation of ISR and reduction in formation of ternary complex, translation from the canonical ORF is favored and the ORF is translated in full (Lu et al., 2004, Vatter and Wek, 2004). Although the reason for preferential translation is unknown, it is proposed that upon phosphorylation of eIF2 α , there is stalling of ribosomes at the uORF, leaving the canonical ORF available for remaining TC to translate from (Morris and Geballe, 2000). Additionally, in a recent screen it has been shown that over 50% of all mRNA have a uORF, yet they are not all translated. This mechanism of translational control of a cellular response is conserved from yeasts, where yeast GCN4 transcription factor is regulated in exactly this manner (Hinnebusch, 1997). The most investigated effector of ISR is ATF4, which is the mammalian homologue of GCN4. ATF4 belongs to ATF/CEBP family of transcriptional factors together with ATF5 (Zhou et al., 2008), CHOP (Palam et al., 2011) GADD34 (Lee et al., 2009). Once made, these transcription factors lead to transcription of their target genes, which are usually metabolic and antioxidant enzymes leading to protective metabolic remodeling of the cell.

In general, ISR is considered as a response that can be activated by different stressors and maintains homeostasis of the cell regardless of where the stress comes from. This is evidenced by the four canonical kinases that lead to its activation. PKR is activated by dsRNA, and secondary structures of mRNA resembling dsRNA (Dey et al., 2005, Garcia et al., 2007). PERK is a transmembrane kinase, embedded in the ER and responsible for sensing misfolded proteins in the lumen of ER (Wang et al., 2018), and is the connecting branch of unfolded protein response (UPR) and ISR.

GCN senses deacylated His-tRNA when amino acids are low, thus monitoring the levels of available charged tRNAs and controlling protein synthesis pathways (Misra et al., 2021, Xiao and Guo, 2022). Other than monitoring aminoacyl t-RNA levels, it has been shown to activate upon interaction with a component of large ribosomal subunit protein uL10, indicating a direct role in mRNA translation (Harding et al., 2019, Inglis et al., 2019).

HRI kinase canonically senses heme depletion, through its N-terminal domain that has a heme binding site. It was primarily investigated in erythrocytes following heme deficiency (Han et al., 2001), but more recently has been shown to respond to cytosolic protein aggregation, mitochondrial dysfunction, and ROS (Guo et al., 2020, Fessler et al., 2020, Han et al., 2001, Neill and Masson, 2023). How mitochondrial dysfunction is sensed to trigger the ISR is a major open question. Defects in mtDNA maintenance and expression *in-vivo* mostly lead to and are associated with ISR (Kuhl et al., 2017). In a study to determine how different mitochondrial insults activate ISR and how cell status affects the plasticity of ISR, C2C12 cells were treated with different small molecule inhibitors of OXPHOS (Mick et al., 2020). C2C12 cells are useful to investigate cell status differences, since they exist as myoblasts, which can be induced to exit cell cycle and then terminally differentiate into myotubes (Yaffe and Saxel, 1977). By treating either myoblasts, which are highly proliferative, or differentiated myotubes with different OXPHOS inhibitors, they disentangled the cell status differences of ISR profile. In myoblasts, primary trigger of ISR related gene expression was impaired NADH oxidation, and correction of NADH imbalance in the cytosol was enough to prevent ISR activation (Mick et al., 2020). More specifically, block of CI lead to a 16-fold decrease in aspartate and asparagine as its derivative, and it was shown that supplementing cells with asparagine was enough to attenuate GCN dependent ISR activation (Mick et al., 2020). Collectively, these experiments indicate that in myoblasts, the rise in the NADH/NAD⁺ ratio is a major driver of ISR-related gene expression following complex I inhibition (Mick et al., 2020). As aspartate is being utilized through malate-aspartate shuttle to maintain the redox balance of the cell, it is being depleted and consequently leads to activation of GCN2 (Mick et al., 2020). Surprisingly, differentiated myotubes activate ISR in response to membrane hyperpolarization, but not by ETC inhibition (Mick et al., 2020), indicating that there is a difference in response of the cells in different maturation state to the same stressors depending on their metabolic demands (Clissold and Cole, 1973, Larson et al., 1993, Frangini et al., 2013, Talvas et al., 2006)

Although it is not clear which aspects of mitochondrial dysfunction led to activation of HRI, the signaling pathway and mechanism of mito-nuclear communication in ISR activation has been recently elucidated *in-vivo*. Mitochondrial protease OMA1 cleaves an inner mitochondrial membrane protein DELE1, leading to its accumulation in cytosol. In cytosol DELE1 interacts and activates HRI even when there is no evident effect on heme levels (Fessler et al., 2020, Guo et al., 2020). Mitochondrial stress induced in these studies relates to membrane potential perturbations, which caused activation of OMA1 protease and consequent DELE1 processing.

The outcome of ISR seems to depend on variables such as effector transcription factors and their targets, cell cycle and differentiation status, and triggers that induce ISR. Still, many studies have described a specific gene set program associated with ISR activation as a signature of mitochondrial dysfunction (Mick et al., 2020), showing it mostly includes changes to amino acid transport and biosynthesis, cytosolic tRNA synthetases and translation factors, antioxidant defense, proteostasis and pro-apoptotic factors (Han et al., 2013, Harding et al., 2003). Most noted and investigated change is to mitochondrial one carbon metabolic pathways (1C metabolism) (Kuhl et al., 2017, Tynismaa et al., 2010, Nikkanen et al., 2016). It remains unclear whether the ISR plays a protective role in mitochondrial disease or rather contributes to pathology (Khan et al., 2017, Suomalainen and Battersby, 2018).

Resolution of ISR activation is dependent on couple phosphatases that can dephosphorylate eIF2a upon restoration of homeostasis, such as GADD34 and CReP (Novoa et al., 2001, Jousse et al., 2003). If homeostasis isn't reached and ISR activation turns chronic, ATF4 association with CHOP and Trib3 leads towards cell death (Ohoka et al., 2005, Hu et al., 2018). Duration and intensity of the stress also contribute to the signalling outcome, as shown recently in a mitochondrial dysfunction model caused by mutation in mitochondrial aspartyl tRNA synthase DARS2, where CHOP inhibition is beneficial in early stages of disease progression, but detrimental in late stages. In chronic ISR activation, CHOP competitively binds to C/EBPB and restoring global protein synthesis. This likely results in ER stress, loss of Ca^{2+} homeostasis, and death (Kaspar et al., 2021). Regulation of the outcome might lay in the severity of ISR activation, where the acute, low grade ISR activation leads to short term metabolic remodeling to cope with stress, and the prolonged intense activation of ISR, without resolution of initial stressor, leads to cell death.

Unfortunately, the simplification of the outcomes rarely coincides with scientific reports on the topic. Despite the simplicity of the system described above, the transcriptional program induced by ISR varies significantly across species and cell types, and even developmental stages. Sources of variability for the outcome of ISR are multiple, starting from the fact that ATF4 interaction partners condition ATF4 binding site between CRE and CARE elements on a promoter (Hai and Curran, 1991, Huggins et al., 2016). Further on, ATF4 can actually act as both an activator and inhibitor of transcription (Ameri and Harris, 2008). The exact targets of ATF4 in each cell seem to depend on posttranslational modifications as well as binding partners and histone modifications surrounding target genes (Pakos-Zebrucka et al., 2016), accounting for most variability in ATF4 - dependent response.

1.5 Objectives

Complex CIV has been shown to be dynamically changing during differentiation of cardiomyocytes. Its function and activity depend on isoform switch for its nuclear encoded subunits to facilitate mitochondrial maturation. This study aims to dissect the role of metabolic rewiring upon changes to CIV, and how it reflects a highly coordinated process of maturation of cardiomyocytes. By targeting CIV in different stages of heart development, we aimed to dissect how cellular differentiation status drives phenotype

progression upon mitochondrial dysfunction and elucidate the role of CIV in the process. Additionally, we set out to describe how metabolic changes upon mitochondrial dysfunction might be regulated depending on the differentiation status of the heart.

Further on, a pressing matter in the field is the role of OXPHOS in cell fate and development. To shed light on dynamics in OXPHOS during heart development and cardiomyocyte differentiation we set out analyse in depth how these complexes might fascilitate metabolic maturation of the heart. In summary, these are the aims of this study:

1. Generate a mouse model targeting CIV function in postnatal heart development
2. Describe mitochondrial dysfunction upon loss of CIV in postnatal heart development
3. Describe in detail OXPHOS changes during postnatal heart development
4. Identify metabolic pathways that are crucial for cardiomyocyte maturation

2 Materials and Methods

2.1 Mouse experiments

2.1.1 Animal care

The mice with the C57BL/6 background were bred and cared for in the CECAD *in-vivo* Research facility. They were kept in the groups of 2-5 mice in individually ventilated cages at an ambient temperature (22–24 °C) in 12h light/dark cycles starting at 6 am. The mice had an *ad libitum* access to drinking water and a normal chow diet (R/M-H-V1554, sniff Spezialdi.ten GmbH, Germany). The animals were sacrificed at 2, 5, 10 days of age. All animal experiments were approved by the local governmental authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, LANUV). All animal handling was performed according to the guidelines and recommendations of the Federation of European Laboratory Animal Science Associations (FELASA).

2.1.2 Experimental mouse models

2.1.2.1 COX7C KO mice

The *Cox7c^{tm1a(KOMP)Mbp}* mouse line was purchased from the KOMP repository. The L1L2_Bact_P cassette was inserted at position 86046136 of Chromosome 13 upstream of the critical exon. The cassette is composed of an FRT site followed by lacZ sequence and a loxP site. This first loxP site is followed by neomycin under the control of the human beta-actin promoter, SV40 polyA, a second FRT site and a second loxP site. A third loxP site is inserted downstream of the targeted exon at position 86045505. The critical exon were thus flanked by loxP sites and subsequent cre expression then excised this critical sequence resulting in a reporter knockout allele.

2.1.2.2 COX7C^{MKO}

COX7C heart specific knock out mice were obtained by crossing *Cox7c^{fl/fl}* mice with *CkmmCre^{tg/+}* mice. Those animals harbour Cre-recombinase driven by the muscle creatine kinase promoter, which is active at embryonic day 13.0 (E13.0) (Lyons et al., 1991). Due to their phenotype, the animals die before they are ready to be weaned, with an average of 11 days, which is a heavy burden. At the time of birth up to an age of about 8 days, the KOs are just as well off as their litter siblings and are completely inconspicuous. With an age of about 9 days the development of the phenotype begins. Within the first hours (approx. 12-24 h) the animals are conspicuous by a 'clumsy' gait and a lower willingness to move. Afterwards, the animals rapidly deteriorate. This is evident from a curved posture, shaggy fur and self-isolation. Once the animals have reached this stage, they die within the next few hours. For this reason, we have set the humane endpoint at the point in time when the very first abnormalities ('clumsy' gait, reduced willingness to move) occur (with 10 days at the latest). The analyses

performed so far have shown that these animals develop a severe cardiomyopathy, which in the final stage most likely leads to death by heart failure.

2.1.2.3 COX10^{MKO}

Heart and skeletal muscle-specific *Cox10* deficient mice (*CkmmCre^{tg/+}*, *Cox10^{fl/fl}*, from now on referred to as COX10^{MKO}) were generated from inter-crossing of *Cox10^{fl/fl}* mice with *CkmmCre^{tg/+}* mice. Those animals harbour Cre-recombinase driven by the muscle creatine kinase promoter, which is active at embryonic day 13.0 (E13.0) (Lyons et al., 1991)

In addition to the COX10^{MKO}, wild-type littermates (*CkmmCre^{+/+}*, *Cox10^{fl/fl}* or *Cox10^{fl/wt}*) resulting from this mating were used as controls (WT mice). Mice were sacrificed at 10 days and 3-3.5 weeks of age (later on referred to as 3 weeks). Sacrifice was performed by either decapitation or cervical dislocation.

2.2 DNA isolation and Genotyping

A piece of tissue was incubated with 500 µL DNA lysis buffer (100 mM Tris-HCl, pH 8.5; 5 mM EDTA, pH 8.0; 0.2% w/v SDS; 200 mM NaCl; 0.1 mg/mL Proteinase K) at 55 °C while shaking (1400 rpm) for 1 hour or until dissolved. One volume of isopropanol was added, samples were vortexed and centrifuged at full speed at 4 °C for 20 min. After discarding the supernatant the pellets were washed with 500 µL 70% ethanol and centrifuged at full speed at 4 °C for 15 min. Supernatants were discarded and pellets were air-dried at room temperature. Finally, pellets were resuspended in 50–200 µL H₂O.

The polymerase chain reaction (PCR) was used to determine the genotypes of the animals. The reactions were performed using the GoTaq G2 Flexi DNA polymerase (Promega, USA). The PCR reaction mix was as follows:

DNA 0.5 µL
5x GoTaq Reaction Buffer 4 µL
MgCl₂ (25 mM) 1.2 µL
dNTP mix (1.25 mM each) 1 µL
Primers (10 mM) 0.8 µL each
GoTaq polymerase 0.05 µL
ddH₂O Up to 20 µL

Table 2.1 Primers used for genotyping of mice

Gene	Forward primer	Reverse primer
COX7C KO		
COX7C ^{MKO}	GAAACAAAAGAAGAGGGCC	CGTTCAAAGCATCTGAACTTG
COX10 ^{MKO}	GAGAGGAGTCAAGGGGACC	GGCCTGCAGCTCAAAGTGTA

2.2.1 Agarose gel electrophoresis

The PCR products were analysed by agarose gel electrophoresis. The agarose (Carl Roth, Germany) was boiled in 0.5x TBE buffer (AppliChem, Germany) to form a

1.5-2% gel. After boiling, ethidium bromide (Sigma-Aldrich, USA) was added (4 μ L per 100 mL of agarose suspension) and the gel was poured into a gel cast with slot combs. After cooling down and polymerizing the gel, electrophoresis was performed in chambers filled with 0.5x TBE at 135 V. The GeneRuler Ladder Mix (Thermo 31 Fisher Scientific, USA) was used as a size marker for the separated DNA fragments. The gels were analysed using the GelDoc system (BioRad, USA).

2.3 Molecular biology

2.3.1 RNA isolation

For total RNA isolation, approximately 20 mg of the frozen heart was homogenized in 1 mL of TRIzol reagent (Invitrogen, USA) using 1.4 mm ceramic beads (Omni International, USA) and Precellys tissue homogenizer (Bertin Technologies, France) 32 (5000 rpm, 30 s). After incubating the samples at room temperature (RT) for 5 min, 200 μ L of chloroform was added and the samples were vigorously mixed. To separate protein, DNA, and RNA the samples were centrifuged at 12 000 g for 15 min at 4 °C. The top aqueous layer with RNA was carefully collected and precipitated with 500 μ L of isopropanol. 10 min incubation at RT followed, and subsequent centrifugation at 12 000 g for 15 min at 4 °C. The RNA pellet was washed with 1 mL of ice-cold 75% ethanol by centrifugation at 7 500 g for 5 min at 4 °C. After air-drying, the pellet was resuspended in 50 μ L of UltraPure water (Gibco, USA). Soluble RNA was stored in -80 °C.

2.3.2 Quantification of nucleic acid concentration

Nucleic acid concentration was measured with NanoDrop 8000 Spectrophotometer was utilised (Thermo Fisher Scientific, USA). The purity of nucleic acids was estimated by absorbances ratios. Pure DNA has the 260/280 ratio ~1.8, whereas pure RNA has the 260/280 ratio ~2. And the 260/230 ratio in the range of 1.8 – 2.2 is accepted as pure for both RNA and DNA. 260 nm is the absorbance maximum of nucleic acids, 280 nm is the absorbance maximum of proteins, and 230 nm absorbance signifies a presence of other contaminants.

2.3.3 Reverse transcription PCR (RT-PCR)

Utilizing the High-capacity reverse transcription kit (Applied Biosystems's, Life Technologies), 2 μ g RNA were used for cDNA synthesis. The reaction mix contained:

2 μ L	RT buffer (10 $\times$)
2 μ L	random Primer (10 $\times$)
0.8 μ L	dNTP mix (25 $\times$)
1 μ L	Reverse transcriptase
16.2 μ L	ddH ₂ O

PCR programme:

Annealing	10 min at 25 °C
Elongation	120 min at 37 °C
Denaturation	5 sec at 85 °C
Hold	4 °C

The cDNA was adjusted to a final concentration of 25 ng/μL and stored at –80 °C.

2.3.4 Quantitative PCR (qPCR)

The qPCR experiments were performed on 96-well plates. 50 ng of DNA was mixed with the Brilliant III Ultra-Fast SYBR Green qPCR Master Mix kit (Agilent Technologies, USA). The reactions were performed in the QuantStudio 7 Flex Real Time PCR System (Thermo Fisher Scientific, USA) with the following PCR program:

Denaturation 95 °C 3 min

For 40 cycles:

Denaturation 95 °C 5 s

Annealing and elongation 60 °C 15 s

Dissociation curve

Denaturation 95 °C 15 s

Annealing 60 °C 1 min

Temperature increase until 95 °C 15 s

The relative gene expression was normalised to the endogenous housekeeping gene *Hprt* and was quantified using the $2^{-\Delta\Delta C_t}$ method.

Table 2.2. qPCR primers used to determine gene expression

Gene	Forward primer	Reverse primer
<i>Hprt</i>	GCCCCAAATGGTTAAGGTT	TTGCGCTCATCTTAGGCTTT
<i>Ppar-α</i>	AACATCGAGTGTGCAATATGTGG	CCGAATAGTTTCGCCGAAAGAA
<i>Ppar-γ</i>	CCTGAAGCTCCAAGAATACCA	GCCTGATGCTTTATCCCCACA
<i>Pgc1α</i>	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
<i>Gdf15</i>	GAGCTACGGGGTCGCTTC	GGGACCCCAATCTCACCT
<i>Aldh18a1</i>	AATCAGGGCCGAGAGATGATG	GGCCTCTAAGACCGGAATTGC
<i>Fgf21</i>	GTGTCAAAGCCTCTAGGTTTCTT	GGTACACATTGTAACCGTCCTC
<i>Atf4</i>	GCAAGGAGGATGCCTTTTC	GTTTCCAGGTCATCCATTGC
<i>Psat1</i>	AGTGGAGCGCCAGAATAGAA	CTTCGGTTGTGACAGCGTTA
<i>Mthfd2</i>	CTGAAGTGGAATCAACAGTGAG	GTCAGGAGAAACGGCATTGC
<i>Nppa</i>	ATGGGCTCCTTCTCCATCA	CCTGCTTCCTCAGTCTGCTC
<i>Nppb</i>	GGATCTCCTGAAGGTGCTGT	TTCTTTTGTGAGGCCTTGGT
<i>Tfam</i>	AACCTTTGACACTCAGTTCAATTTTCT	CAAGGCTCAAAAGGTCAACAGA
<i>Glut1</i>	TCAGGCGGAAGCTAAC	GGAGGAAACATGCAGTCATC
<i>Glut4</i>	GAGCCTGAATGCTAATGGAG	GAGAGAGAGCGTCCAATGTC

<i>Acads</i>	TCGGCTGTTTTGCCCTCAGT	GGAAGGCACTGATACCCTTGT
<i>Acadl</i>	TCTTTTCCTCGGAGCATGACA	GACCTCTCTACTCACTTCTCCAG
<i>Acadvl</i>	CTACTGTGCTTCAGGGACAAC	CAAAGGACTTCGATTCTGCCC
<i>Pdk4</i>	AGGGAGGTCGAGCTGTTCTC	GGAGTGTTCACTAAGCGGTCA
<i>C-myc</i>	AGCCCCTAGTGCTGCATGA	TCCACAGACACCACATCAATTTC
<i>Gadd45a</i>	CCGAAAGGATGGACACGGTG	TTATCGGGGTCTACGTTGAGC
<i>Cdkn1a</i>	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
<i>Eif4ebp1</i>	GGGGACTACAGCACCCTC	CTCATCGCTGGTAGGGCTA
<i>Fbxo32</i>	CAGCTTCGTGAGCGACCTC	GGCAGTCGAGAAGTCCAGTC
<i>Trim63</i>	GTGTGAGGTGCCTACTTGCTC	GCTCAGTCTTCTGTCCTTGA

2.4 Biochemistry

2.4.1 Protein isolation from tissues

A piece of frozen tissue was cut and placed in organ lysis buffer (50 mM HEPES, 1% v/v Triton X-100, 50 mM NaCl, 0.1 M NaF, 10 mM Na₃VO₄·2H₂O, 10 mM EDTA, 0.1% v/v SDS, 1x protease inhibitor cocktail, 2 mM PMSF, pH 7.4) and homogenized using 1.4 mm ceramic beads (Omni International, USA) and Precellys tissue homogenizer (Bertin Technologies, France) (5500 rpm, 2x20", pause 30"). Homogenates were centrifuged at full speed/4 °C /45 min, the supernatant was collected and stored at -80 °C.

2.4.2 SDS-PAGE

Concentration of the protein samples was quantified using the Bradford reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. The samples were subsequently mixed with the Laemmli buffer (50 mM Tris-HCl, pH 6.8; 2% w/v SDS; 10% v/v glycerol; 1% β-mercaptoethanol; 12.5 mM EDTA; 0.02% Bromophenol Blue) and denatured at 95 °C for 5 min.

SDS-PAGE was performed using the Mini-PROTEAN Tetra Cell system (BioRad, USA). Samples were loaded on a 8–12% polyacrylamide gel (stacking gel: 5% acrylamide/bis-acrylamide (29:1), 12.5 mM Tris-HCl, 0.1% w/v SDS, 0.25% APS, 0.25% TEMED, pH 6.8; separating gel: 8–12% acrylamide/bis-acrylamide (29:1), 37.5 mM Tris-HCl, 0.1% w/v SDS, 0.1% APS, 0.1% TEMED, pH 8.8; the percentage was dependent on the desired protein size separation) in a chamber containing a running buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% w/v SDS, pH 8.3). PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, USA) was used as a marker for protein size. First, the electrophoresis was performed at a constant 75 V for 15 min to allow the protein to enter the stacking gel. Afterwards, the constant voltage was raised to 150 V and the electrophoresis continued until the loading dye reached the end of the gel.

For western blotting, the Criterion Blotter (BioRad, USA) was utilised according to the manufacturer's instructions. The Amersham Protran 0.45 nitrocellulose membrane

(Cytiva, USA) was used for the transfer of proteins. The transfer of proteins was performed at constant 400 mA/4 °C /2 h in a transfer buffer (30 mM Tris-HCl, 240 mM glycine, 0.037% SDS, 20% methanol). Afterwards, the membrane was washed shortly in dH₂O and stained by Ponceau S solution to assess the quality of the loading and the transfer of proteins. The Ponceau S staining was later removed by a wash in PBST (1x PBS, 0.1% w/v Tween-20) and the membrane was blocked in 5% milk-PBST. Primary and secondary antibodies were prepared according to the manufacturers. The membranes were incubated in the primary antibodies overnight at 4 °C, which was followed by 3 rounds of a wash in PBST for 5 min. Afterwards, an incubation in the HRP-conjugated secondary antibodies was performed at RT for 1 hour, which was followed again by 3x washing in PBST for 5 min. To activate the chemiluminescence of the secondary antibodies, Pierce ECL Western Blotting substrate (Thermo Fisher Scientific, USA) was applied to the membrane according to the manufacturer's instructions. Afterwards, the membranes were exposed to Super RX-N films (Fujifilm, Japan) and the films were developed in an automatic X-ray film processor (Kodak, USA).

Table 2.3. Antibodies used for WB

Name	Supplier	Cat. Nr.
4EBP1	CST	9644
p-4EBP1	CST	2855
AKT	CST	9272
p-AKT	CST	4060
ALDH18A1	Sigma	HPA01260
AMPK	CST	2532
p-AMPK	CST	2535
ASNS	Santa Cruz	Sc-365809
ATP5A1	Abcam	Ab14748
CALNEXIN	Calbiochem	208880
CLPP	Sigma	WH0008192M1
CLPX	Sigma	HPA040262
COX1	Molecular Probes	459600
COX4	Molecular Probes	A21348
eIF2A	Abcam	Ab26197
p-eIF2a	Abcam	Ab32157
GLUT4	Millipore	07-1404
HSC70	Santa Cruz	Sc-7298
HSP60	ATCC	HSP60
LONP1	Proteintech	15440-1-AP
MTHFD2	Abcam	Ab56772
mTOR	CST	2972
p-mTOR	CST	2971
NDUFA9	Molecular Probes	459100
PYCR1	Abcam	Ab94780
RIESKE	MitoSciences	MS305
SDHA	Invitrogen	459200
SHMT2	Sigma Aldrich	AV46129

TOM20	Santa Cruz	Sc-17764
UQCRC1	Molecular Probes	459140
VDAC1	CST	4661

Table 2.4. Secondary antibodies used for WB

Antibody	Company	Cat Nr.
HRP linked anti-mouse IgG	Sigma	A 4416
HRP linked anti-rabbit IgG	Sigma	A 6154

2.4.3 Isolation of mitochondria from mouse tissues

Whole embryos or neonatal hearts were dissected and placed in ice-cold mitochondria isolation buffer with BSA (MIB+BSA; 100 mM sucrose, 50 mM KCl, 1 mM EDTA, 20 mM TES, 0,2% w/v BSA free from fatty acids) in 15 mL Falcon tube. Prior to homogenization, subtilisin A was added (1 mg protease per 1 g tissue) to the tissue and everything was subsequently transferred to a glass-on-glass Dounce homogeniser. The tissue was further homogenised by hand with 15 strokes using the loose pestle, followed by 10 strokes with the tight pestle. The homogenate was transferred to a 15 mL Falcon tube and centrifuged at 8 500 g at 4 °C for 5 min. Afterwards, supernatant was discarded, the pellet was resuspended in 5 mL MIB+BSA and centrifuged at 800 g at 4 °C for 10 min. The supernatant was collected to a new 15mL tube and kept on ice. The pellet was resuspended in another 5 mL MIB+BSA and re-centrifuged at 800 g at 4 °C for 10 min. The supernatants were combined and centrifuged at 8 500 g at 4 °C for 5 min. The mitochondrial pellet was resuspended in 600–800 µL MIB without BSA, transferred to a 1,5 mL tube and centrifuged at 8 500 g at 4 °C for 5 min. The supernatant was discarded, and the pellet was resuspended in 50–200 µL MIB without BSA. Samples were frozen in liquid nitrogen and stored at –80 °C.

2.4.4 BN-PAGE

To prepare samples for BN-PAGE, the NativePAGE Sample Prep Kit (Invitrogen, USA) was used according to the manufacturer. 10 – 15 µg freshly isolated or frozen mitochondria were solubilised in 4% digitonin or 1% dodecyl maltozide (DDM) on ice for 15 min. Following incubation, the samples were centrifuged at 20 000 g at 4 °C for 30 minutes to pellet the non-solubilised debris. The supernatant was loaded on a 4–16% Bis-Tris gradient gel. The electrophoresis was conducted in X-Cell SureLock Mini-Cell Electrophoresis system (Life Technologies, USA) according to the manufacturer. The proteins were transferred to the Amersham Hybond P 0.45 PVDF membrane (Cytiva, USA) following the western blot protocol in the section 2.4.2.

2.4.5 *In-gel* activity assay

For the *in-gel* activity assay for CI, the BN-PAGE gel was incubated in the CI activity buffer (0.1 mg/mL NADH; 2.5 mg/mL nitrotetrazolium blue; 5 mM Tris-HCl, pH 7.4) for 1 hour at 37 °C.

For the CIV in gel activity, the gel was incubated in the CIV activity buffer (1 mg/mL cytochrome c; 1 mg/mL 3,3'-diaminobenzidine; 0.4 mg/mL catalase; 50 mM Tris-HCl, pH 7.4) overnight at 37 °C. The gels were destained in a destaining solution (50% v/v methanol; 10% v/v acetic acid) for 2 h at RT and then rehydrated in dH₂O.

2.4.6 *In-organello* translation

Fresh mitochondria were isolated according to the protocol in the section 2.6.3 and the protein concentration was determined with the Bradford reagent (Sigma-Aldrich, USA) according to the manufacturer. 1 mg of mitochondria per condition was pelleted at 8500 g/4 C/2 min. The pellet was resuspended in 1 mL translation buffer + 35S-methionine [1x translation mix, pH 7.4 (100 mM mannitol; 10 mM sodium succinate; 80 mM KCl; 5 mM MgCl₂; 1 mM KH₂PO₄, pH 7.4; 25 mM HEPES, pH 7.4); 60 µg/mL amino acids (Ala, Arg, Asp, Asn, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, Val); 5 mM ATP; 20 µM GTP; 6 mM creatine phosphate; 60 µg/mL creatine kinase; 7 µL 35S-methionine] and incubated at 37 °C for 1 hour on a rotating wheel. Mitochondria were pelleted at 8500 g/2 min and resuspended with 1 mL translation buffer without 35S-methionine. Half of the sample was re-pelleted, resuspended in 50 µL SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8; 2% w/v SDS; 10% v/v glycerol; 1% v/v β-mercaptoethanol; 12.5 mM EDTA; 0.02% bromophenol blue), lysed for 30 min at room temperature and kept as “pulse” fraction. The rest was re-pelleted, resuspended in 500 µL translation buffer with non-radioactive methionine and incubated for 3 hours at 37 °C on a rotating wheel. Subsequently, the sample was pelleted, resuspended in 50 µL SDS-PAGE loading buffer, lysed for 30 min at room temperature and kept as “chase” fraction.

The proteins from pulse- and chase fractions were separated using SDS-PAGE – 10 µL of the sample was loaded in a 15% polyacrylamide gel placed in SE600X Chroma Deluxe Dual Cooled Vertical Protein Electrophoresis Unit (Hoefer Inc, USA) filled with a running buffer (25mM Tris-HCl, 250mM Glycine, 0.1% w/v SDS, pH 8.3) and the electrophoresis was performed at constant 75 V overnight. The gel was stained with InstantBlue Coomassie Protein Stain (Thermo Fisher Scientific) for 30 min and placed in the Model 583 Gel Dryer (Bio-Rad Laboratories, Hercules, USA) for 2 hours at 80 °C. The dried gel was exposed to the Amersham Hyperfilms MP (Cytiva, USA) to detect the radioactivity and the films were developed in an automatic X-ray film processor (Kodak, USA).

2.5 Histology

2.5.1 Tissue Sectioning

Cryosections were obtained with a cryostat (Leica CM1850). Following mouse sacrifice whole embryos were immediately isolated and fresh-frozen in Tissue-Tek O.C.T (Sakura) embedding medium on dry ice and stored until further use at -80 °C. Thickness of cryosections was 10 µm.

2.5.2 COX staining

COX enzymatic staining was performed on 10 µm cryosections. Cryosections were first incubated 37 °C with COX solution (0.8 mL of DAB, 0.2 mL of 500 µM cytochrome c, a few grains of catalase) for 45 min at 37 °C. Then, cryosections were washed in ddH₂O, and dehydrated through graded alcohols for 2 mins per immersion (70 % ethanol, 96 % ethanol, and 100 % ethanol), cleared in xylol and mounted with Entellan®.

2.5.3 Haematoxylin and Eosin staining

10 µm cryosections were incubated in Mayer's haematoxylin solution for 4 min, washed in tepid water for 30 sec, colour was fully developed during 15 min incubation in cold tap water. Afterwards, cryosections were briefly immersed into ddH₂O, and placed into eosin solution (Sigma-Aldrich) for 1 min. Subsequently, cryosections were washed in cold tap running water, dehydrated through graded alcohols, each immersion 2 min (70 % ethanol, 96 % ethanol, and 100 % ethanol), cleared in xylol and mounted with Entellan®.

2.6 Omics

2.6.1 Proteomics

A small piece of the frozen heart was ground to powder in liquid nitrogen and the powder was resuspended in 100 µL of the urea lysis buffer (8 M Urea; 50 mM triethylammonium bicarbonate (TEAB); 1x protease inhibitor). The sample was sonicated with the Bioruptor Pico sonication device (Diagenode, Belgium) – cycle 30/30 s for 10 min. Afterwards, the sample was centrifuged at 20 000 g for 15 min at 4 °C to discard the debris. The protein concentration was determined with Bradford reagent (Sigma-Aldrich, USA) according to the manufacturer. 50 µg of the sample was transferred to a new tube and treated with dithiothreitol (DTT, final conc. 5 mM) and incubated at 25 °C for 1 h. Chloroacetamide (CAA, final conc. 40 mM) was added and samples incubated for 30 min in the dark. Afterwards, lysyl endopeptidase (Lys-C) was added at the protease:sample 42 protein ratio 1:75 and the incubation lasted 4 h at 25 °C. The sample was diluted with 50 mM TEAB buffer to reach the final concentration of 2 M Urea. For digestion of peptides, trypsin was added in the 1:75 ratio and the trypsinisation occurred overnight at 25 °C. The following day, the sample was acidified

with formic acid (final conc. 1%) to stop the digestion. The lysates were loaded on SDB RP StageTips supplied by the CECAD proteomics core facility and submitted to the facility for the mass spectrometry analysis according to the standard procedures of the facility. The raw data were analysed with the MaxQuant proteomics software.

2.6.2 Metabolomics

Chromatographic separation of metabolites was achieved using a Millipore Sequant ZIC-pHILIC analytical column (5 μ m, 2.1 $\times$ 150 mm) equipped with a 2.1 $\times$ 20 mm guard column (both 5 mm particle size) with a binary solvent system. Solvent A was 20 mM ammonium carbonate, 0.05% ammonium hydroxide; Solvent B was acetonitrile. The column oven and autosampler tray were held at 40 °C and 4 °C, respectively. The chromatographic gradient was run at a flow rate of 0.200 mL/min as follows: 0–2 min: 80% B; 2–17 min: linear gradient from 80% B to 20% B; 17–17.1 min: linear gradient from 20% B to 80% B; 17.1–23 min: hold at 80% B. Samples were randomized and the injection volume was 5 μ l. A pooled quality control (QC) sample was generated from an equal mixture of all individual samples and analysed interspersed at regular intervals.

Metabolites were measured with Vanquish Horizon UHPLC coupled to an Orbitrap Exploris 240 mass spectrometer (both Thermo Fisher Scientific) via a heated electrospray ionization source. The spray voltages were set to +3.5kV/-2.8 kV, RF lens value at 70, the heated capillary held at 320 °C, and the auxiliary gas heater held at 280 °C. The flow rate for sheath gas, aux gas and sweep gas were set to 40, 15 and 0, respectively. For MS1 scans, mass range was set to m/z =70–900, AGC target set to standard and maximum injection time (IT) set to auto. Data acquisition for experimental samples used full scan mode with polarity switching at an Orbitrap resolution of 120000. Data acquisition for untargeted metabolite identification was performed using the AcquireX Deep Scan workflow, an iterative data-dependent acquisition (DDA) strategy using multiple injections of the pooled sample. In brief, sample was first injected in full scan-only mode in single polarity to create an automated inclusion list. MS2 acquisition was then carried out in triplicate, where ions on the inclusion list were prioritized for fragmentation in each run, after which both the exclusion and inclusion lists were updated in a manner where fragmented ions from the inclusion list were moved to exclusion list for the next run. DDA full scan-ddMS2 method for AcquireX workflow used the following parameters: full scan resolution was set to 60000, fragmentation resolution to 30000, fragmentation intensity threshold to 5.0e3. Dynamic exclusion was enabled after 1 time and exclusion duration was 10s. Mass tolerance was set to 5ppm. Isolation window was set to 1.2 m/z . Normalized HCD collision energies were set to stepped mode with values at 30, 50, 150. Fragmentation scan range was set to auto, AGC target at standard and max IT at auto. Xcalibur AcquireX method modification was on. Mild trapping was enabled.

Metabolite identification was performed in the Compound Discoverer software (v 3.2, Thermo Fisher Scientific). Metabolites were annotated at the MS2 level using both an in-house mzVault spectral database curated from 1051 authentic compound

standards and the online spectral library mzCloud. The precursor mass tolerance was set to 5 ppm and fragment mass tolerance set to 10 ppm. Only metabolites with mzVault or mzCloud best match score above 50% and 75%, respectively, and RT tolerance within 0.5 min to that of a purified standard run with the same chromatographic method were exported to generate a list including compound names, molecular formula and RT. The curated list was then used for further processing in the Tracefinder software (v 5.0, Thermo Fisher Scientific), where extracted ion chromatographs for all compound were examined and manually integrated if necessary. False positive, noise or chromatographically unresolved compounds were removed. The peak area for each detected metabolite was then normalized against the total ion count (TIC) of that sample to correct any variations introduced from sample handling through instrument analysis. The normalized areas were used as variables for further statistical data analysis. Metabolomics analysis was performed in collaboration with Dr. Ming Yang and Dr. Cristian Frezza.

2.6.3 Complexomics

Mitochondria from hearts from wild type hearts at following timepoints: E18.5, P0, P2, P4, P5, P6, P7, P9, and P10 and COX7C^{MKO} mice at P10 were isolated by pooling multiple hearts to obtain 500ug of mitochondria. Mitochondria were then solubilized with 6 g digitonin/g protein and native complexes were separated by BN-PAGE. Lanes were cut into 60 even slices that were digested with trypsin. Peptides were separated by liquid chromatography and analyzed by online tandem mass spectrometry (LC-MS/MS) in a Q-Exactive mass spectrometer equipped with an Easy nLC1000 nano-flow ultra high-pressure liquid chromatography system (Thermo Fisher Scientific) at the front end. The mass spectrometer operated in positive ion mode switching automatically between MS and data-dependent MS/MS of the top 20 most abundant precursor ions. Full scan MS mode (400 to 1400 m/z) was set at a resolution of 70,000 m/μm with an automatic gain control target of 1×10^6 ions and a maximum injection time of 20 ms. Selected ions for MS/MS were analyzed using the following parameters: resolution 17,500 m/μm, automatic gain control target 1×10^5 ; maximum injection time 50 ms; precursor isolation window 4.0 Th. Mass spectrometry data were analyzed using MaxQuant (version 1.5.0.25). Spectra were matched against the human NCBI Reference Sequence Database with known contaminants added. The limit for the false-discovery rate determined by target-decoy database search was set to 0.01. Database searches were done with 20 ppm mass tolerance for precursor ions and fragmented ions, respectively. Two missed cleavages were allowed for tryptic digestion. Intensity based absolute quantification (iBAQ) values derived from the MaxQuant analysis were corrected for loading variation between different samples using the total iBAQ values of all detected proteins listed in MitoCarta 2.0. For each protein, gel migration profiles were created and normalized to the maximum abundance across all samples analyzed. Then the migration patterns of the identified proteins were hierarchically clustered by an average linkage algorithm with uncentered Pearson correlation distance measures. Heat maps of the resulting complexome

profiles consisting of a list of proteins grouped according to the similarity of their migration profiles in blue native gel electrophoresis were generated by representing the normalized abundance in each gel slice by a three color gradient (black/yellow/red). Microsoft Excel was used for visualization and analysis. Complexome profiling was performed in a collaboration with Dr. Ilka Wittig.

2.7 Image processing and data analyses

2.7.1 Image processing

Western blot films were digitalized with the V800 transparency scanner (Epson, Japan) at 800 dpi 16Bit colour depth. Fiji (Schindelin et al., 2012) was used for densitometric analyses. Graphpad Prism 9 was used for generating graphs and for statistical analyses (Student's t-tests). Adobe Illustrator was used for cropping scanned films and generating figures and figure arrangements. Instant Clue (Nolte et al., 2018) was used to generate hierarchical clusterings, heatmap illustrations, and volcano plots.

Histological sections were imaged by Brightfield images of H&E, and COX stained sections were taken with a slidescanner (Leica SCN400) at 20x magnification.

2.7.2 Statistical analysis

Data sets were analyzed for significance by two-tailed unpaired Student's t-test. All p-values < 0.05 were considered as statistically significant; all data represented as mean $\pm$ SD (t-test: *p<0.05, **<0.01, ***p<0.001, ****p<0.0001).

Statistical analysis data visualization and writing were performed using Microsoft Word, Excel and Graphpad Prism 9. Figures and Illustrations were made with Adobe Illustrator CS (Adobe). Western Blot quantification was performed with Fiji.

2.7.3 Enrichment analysis

Enrichment analysis for proteomics data was performed with DAVID enrichment tool. Enrichment analysis for metabolomics data was done by MetaboAnalyst tool. Figures and diagrams were created using Adobe Illustrator and BioRender tool.

2.8 Chemicals

[³⁵ S]-Methionine	Hartmann Analytic
2-Mercaptoethanol	Sigma Aldrich
2-Propanol	Applchem
Acetic Acid	AppliChem
Acetone	AppliChem
Adenosine-tri-phosphate	ATP Sigma Aldrich
ADP Adenosine 5'-diphosphate sodium Salt	Sigma Aldrich
Agarose NEE0	Ultra-Quality Roth
Alanine (L-)	Sigma Aldrich
Arginine mono-hydrochloride (L-)	Sigma Aldrich
Asparagine monohydrate (L-)	Sigma Aldrich
Aspartic Acid (L-)	Sigma Aldrich

Bradford	Sigma Aldrich
Bromophenol Blue	MERCK
BSA (Albumin from bovine serum) fatty acid free	Sigma Aldrich
Catalase, bovine liver	Sigma Aldrich
Coomassie Brilliant Blue R250	MERCK
Creatine Phosphokinase Type III, bovine	Sigma Aldrich
Cysteine hydrochloride monohydrate (L-)	Sigma Aldrich
Cytochrome c, equine heart	Sigma Aldrich
DAB	Vector
DAPI	AppliChem
DDM Dodecyl- β -maltoside	Roth
dNTPs	Promega
DreamTaq™ buffer (10x)	ThermoFischer Scientific
DreamTaq™ polymerase	ThermoFischer Scientific
EDTA Ethylenediaminetetraacetic acid	Sigma Aldrich
Entellan®	Merck Millipore
Eosin	Sigma Aldrich
Ethanol	AppliChem
Ethidium bromide	Sigma Aldrich
Glutamine (L-)	Sigma Aldrich
Glycerol	Sigma Aldrich
Glycine Molecular Biology grade	AppliChem
GoTaq® buffer (5x)	Promega
GoTaq® polymerase	Promega
GTP Guanosine 5'triphosphate sodium salt	Sigma Aldrich
HCl 37%	VWR
HEPES-Molecular biology grade	AppliChem
Histidine mono hydrochloride monohydrate (L-)	Sigma Aldrich
Isoleucine (L-)	Sigma Aldrich
Ketamin hydrochloride CIIN	Sigma Aldrich
Leucine (L-)	Sigma Aldrich
Lysine mono-hydrochloride (L-)	Sigma Aldrich
Mannitol	AppliChem
Mayer's haemotoxylin	Sigma Aldrich
Methionine (L-)	Sigma Aldrich
MgCl ₂ 25 mM	Promega
Milk powder	AppliChem
Nitrotetrazolium Blue	Sigma Aldrich
Phenylalanine (L-)	Sigma Aldrich
Ponceau S	Abcam
Potassium chloride	AppliChem
Potassium phosphate monobasic	Sigma Aldrich
Proline (L-)	Sigma Aldrich
Protease Inhibitor tablets EDTA free	Roche

Protein Standard	Sigma Aldrich
Proteinase K	AppliChem
SDS granules	Sigma Aldrich
Serine (L-)	Sigma Aldrich
Sodium azide	AppliChem
Sodium chloride for molecular biology	Sigma Aldrich
Sodium deoxycholate	AppliChem
Sodium hydroxide	Sigma Aldrich
Sodium phosphate dibasic	Sigma Aldrich
Sodium phosphate dibasic dihydrate	Sigma Aldrich
Sodium phosphate monobasic	Sigma Aldrich
Sodium succinate dibasic hexahydrate	Sigma Aldrich
Sucrose	Sigma Aldrich
TEMED	Sigma Aldrich
Threonine (L-)	Sigma Aldrich
Tri-sodium citrate dihydrate	AppliChem
Triton™ X 100	Sigma Aldrich
Trizma® Base (Tris)	Sigma Aldrich
Tryptophan (L-)	Sigma Aldrich
Tween® 20	VWR
Tyrosine (L-)	Sigma Aldrich
Valine (L-)	Sigma Aldrich
Xylazine Hydrochloride	Sigma Aldrich
Xylol	Roth

3 Results

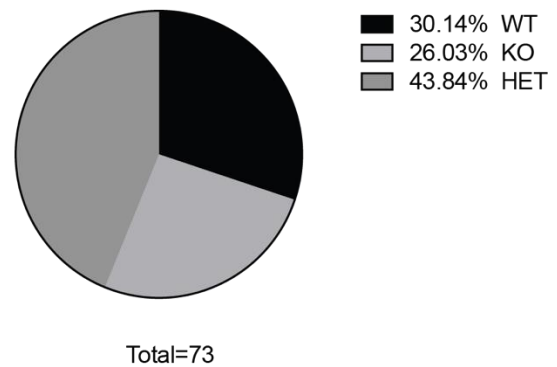
3.1 Full body depletion of COX7C

Studies on COX7C are scarce. No function has been attributed to COX7C, nor has it been investigated *in-vivo*. It has been proposed as a prognostic marker for cancer and diabetes-related sepsis (Dang et al., 2006, Wang et al., 2022, Wu et al., 2020), indicating its importance in human disease. Further on, gene variants in *Cox7c* have been connected with chronic myeloid leukemia (El-Masry, 2023). To elaborate on the role of COX7C in CIV function and stability, we developed a whole-body mouse KO model of *Cox7c*. We ubiquitously disrupted *Cox7c* gene in mice using β -Actin promotor for Cre expression. To obtain COX7C KO mice, we crossed *Cox7c^{loxp/loxp}* mice with *Cre^{+tg}* mice to produce offspring with deletion in *Cox7c* intron 6, a functional knockout of *Cox7c* gene (COX7C KO mice).

A.



B.



C.

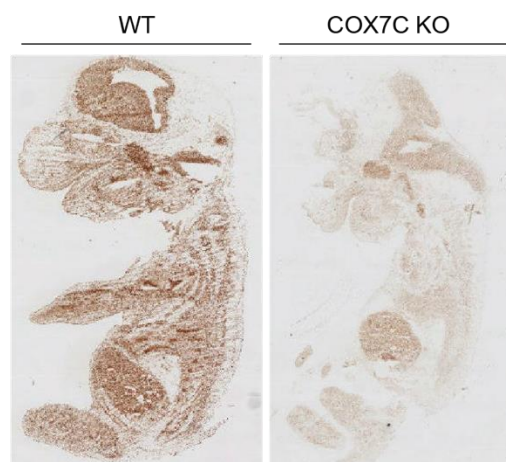


Figure 3.1. Whole body deletion of *Cox7c* leads to late embryonic lethality. **A.** Image of WT and COX7C KO embryo for comparison of size and developmental stage at E13.5-14.5. **B.**

Percentage of recovered genotypes of embryos at E13.5- E14.5. **C.** CIV deficiency shown by COX staining of fresh frozen whole embryo sections.

Viable COX7C KO mice could not be recovered, so we investigated embryos at earlier stages of development. At E13.5-E15.5 embryos are at the same stage of development as the wild-type littermates (WT), except for difference in size (Figure 3.1A). Percentage of recovered genotypes at E15.5 can be seen in Figure 3.1B. To determine the effect of *Cox7c* deletion to CIV activity, we performed COX enzymatic staining on whole embryo sections. COX staining is based on oxidation of cytochrome c by the CIV maintained within the fresh frozen tissue sections. Thus, dark brown staining, indicative of oxidized cytochrome c, represents intact CIV activity. In our COX7C KO embryos, low intensity stain indicated loss of CIV activity at E13.5 (Figure 1C).

Since we could not recover any COX7C KO mice, but could identify embryos at E15.5, we concluded that loss of *Cox7c* leads to embryonic death between E15.5 and E18.5. This indicates that COX7C is essential for late embryonic development. To investigate OXPHOS deficiency in COX7C KO embryos, we isolated mitochondria from whole embryos at E13.5 and solubilized mitochondrial OXPHOS complexes with a mild detergent dodecyl-maltoside (DDM). Other than existing as individual complexes, OXPHOS complexes form supramolecular structures such as the respirasome (RC), that are commonly named supercomplexes (SC). To investigate SC structures, mitochondria were solubilized with digitonin, a milder detergent that preserves respiratory supercomplexes (Schägger and von Jagow, 1991). After solubilization, samples were analyzed by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE). BN-PAGE analysis is based on separating solubilized native protein complexes on a gradient gel to investigate physiological protein-protein interactions, large protein complexes and their oligomeric status, including protein activity, import and assembly assays (Wittig et al., 2006). When followed with western blot analysis it also enables immunodetection of complexes (Wittig et al., 2006).

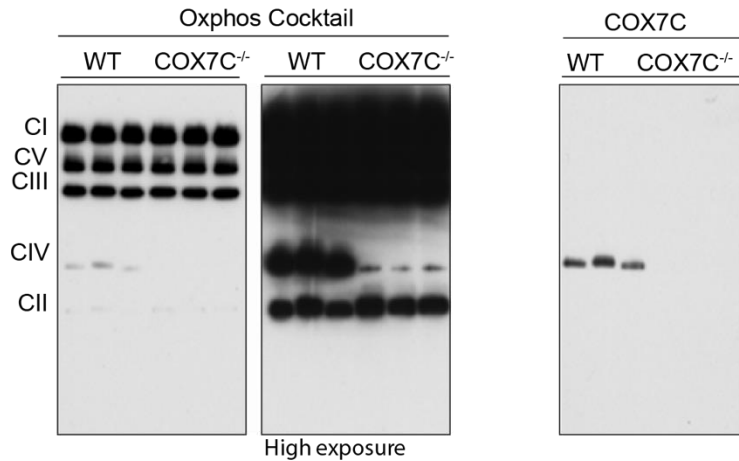
DDM BN-PAGE analysis of COX7C KO mitochondria, followed by western blot, shows a strong loss of CIV steady-state levels (Figure 3.2A). We could also confirm loss of steady-state levels of COX7C on western blot (Figure 3.2A, right panel). Furthermore, we could see no change in whole levels of complexes I - III (CI-CIII) (Figure 3.2A), concluding that loss of COX7C leads to an isolated CIV deficiency in embryos at E13.5.

Digitonin BN-PAGE shows again a strong loss of CIV, but also a loss of respirasome structure in COX7C KO (Figure 3.2B). Furthermore, CI and CIII show a change in migration pattern consistent with loss of respirasome structure, where neither complex could be detected at the size corresponding to the respirasome, but seem to redistribute to other structures (Figure 3.2B).

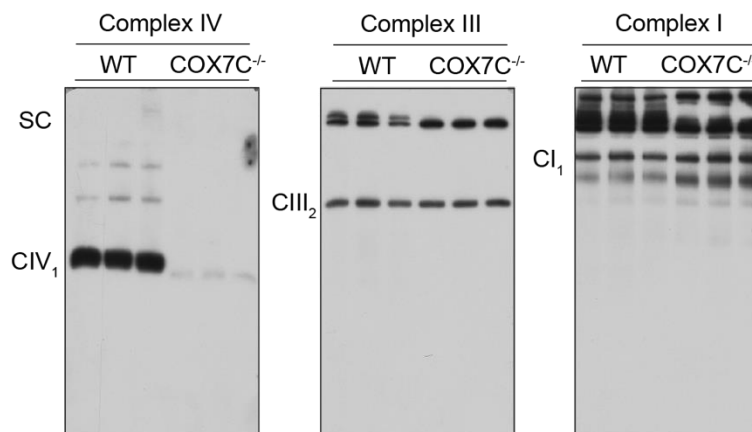
In our WT mice, we observed that CIV monomer (CIV₁) shows the highest abundance, while steady-state levels of SCs are very low (Figure 3.2, left panel), indicating that CIV involvement in SC structures in embryos is low. To further

investigate residual CIV activity shown on whole embryo sections (Figure 1A) we performed *in-gel* activity assay for CIV. While most of CIV activity was gone, we can still detect low activity for CIV monomer structure (CIV₁) (Figure 3.2A).

A.



B.



C.

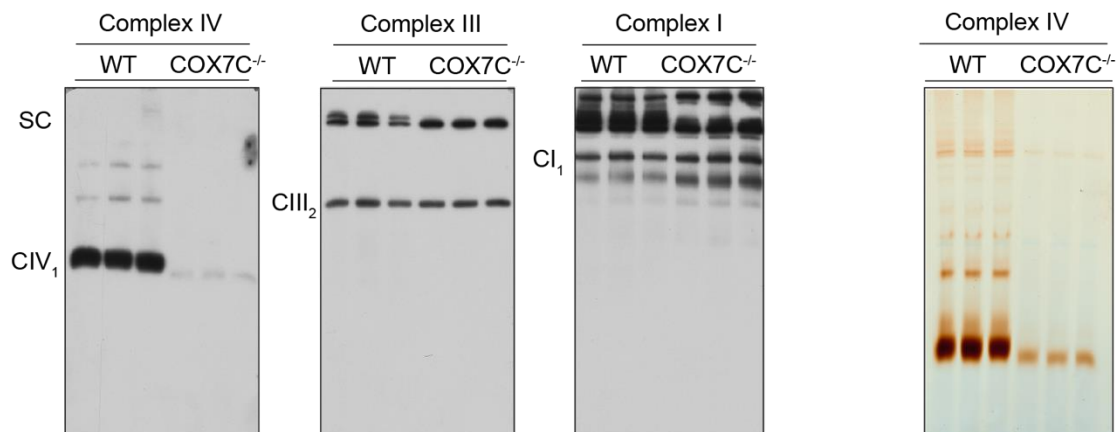


Figure 3.2. BN-PAGE analysis of mitochondria from whole embryos. **A.** Steady-state levels of complexes I-V in WT and COX7C KO embryo mitochondria (n=3). **B.** Steady-state levels of digitonin solubilized OXPHOS supercomplexes in WT and COX7C KO embryo mitochondria (n=3). **C.** CIV *in-gel* activity assay of digitonin solubilized mitochondria for WT and COX7C KO embryo. Antibodies used for A. OXPHOS cocktail; B. COX1, UQCRC1, NDUFA9. OXPHOS complexes I-V are labeled as CI-V; SC – supercomplex structures; Subscript number indicates oligomeric status of corresponding complex.

3.2 Heart specific COX7C deficiency

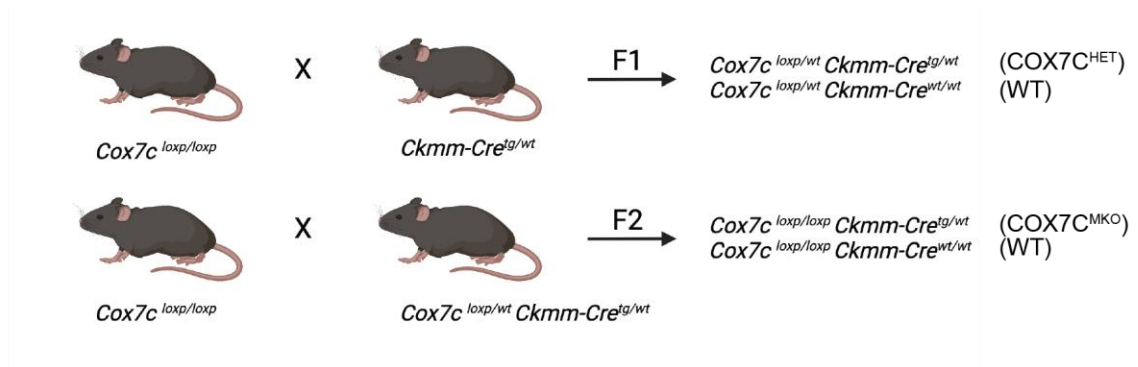
More recently, COX7C has been implicated in maintaining mitochondrial function during regenerative window of the neonatal heart, which is until postnatal day 8 (P8). Cardiac arrest in *Cox7c* heterozygous mice at P2 after birth lead to severe lipid accumulation and cardiac dysfunction (Li et al., 2018). Further on, *Cox7c* was reported to be under regulation of PITX2, a transcription factor essential for regenerative capacity of cardiomyocytes during development (Tao et al., 2016).

To investigate further how COX7C affects CIV in the heart and how it relates to heart physiology, we developed a heart-specific model of COX7C deficiency. We

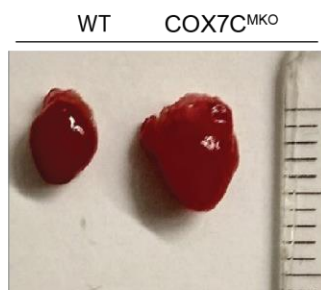
disrupted *Cox7c* in the heart by crossing *Cox7c^{loxp/loxp}* mice with mice expressing Cre recombinase under muscle creatine kinase (*Ckmm*) promoter active in the heart and muscle from embryonic day 15 (Lyons et al., 1991).

This breeding strategy provided *Cox7c^{-/-}Cre^{+tg}* mice (labeled as COX7C^{MKO}) in F2 generation (Figure 3.3A).

A.



B.



C.

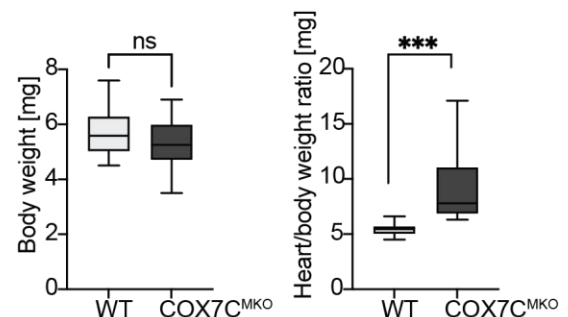


Figure 3.3. Heart-specific mouse model of COX7C develops early onset cardiomyopathy. **A.** Breeding strategy for obtaining heart tissue conditional disruption of *Cox7c* gene. **B.** Heart size comparison of WT and COX7C deficient hearts at P10. **C.** Changes in bodyweight and heart weight/body weight ratio of COX7C^{MKO} mice (p-val>0,05; n=30)

COX7C^{MKO} mice present an abrupt loss of general fitness and failure to thrive in the first two weeks after birth, so the humane endpoint was determined to P10. To investigate the cause of such a severe phenotype, mice were sacrificed and most of the subsequent analysis was performed at P10. We could determine that COX7C^{MKO} mice developed increased hearts by P10 (Figure 3.3B). Heart mass increase was evident by heart-weight-to-body-weight ratio, which was significantly higher in COX7C^{MKO} than in wild-type littermates (WT) (Figure 3.3C, right panel). No significant change in body weight could be observed at P10 (Figure 3.3, left panel). Our

observations indicate that heart-specific conditional disruption of *Cox7c* leads to a severe early onset cardiomyopathy, with life expectancy of less than two weeks.

Mitochondrial cardiomyopathy occurs in 20-40% of children with mitochondrial diseases, making it a common consequence of mitochondrial dysfunction (Holmgren et al., 2003). Other than causing metabolic alterations and changes to bioenergetics of the heart, also leads to activation of fetal gene expression program, responsible for remodeling of cardiac tissue (Ingles et al., 2019, Dirkx et al., 2013). To examine histological changes to the heart tissue, we performed hematoxylin-eosin (H&E) staining of mice heart sections (Figure 3.4A). We could not detect any significant perturbations in structure and general morphology of the heart tissue of $COX7C^{MKO}$.

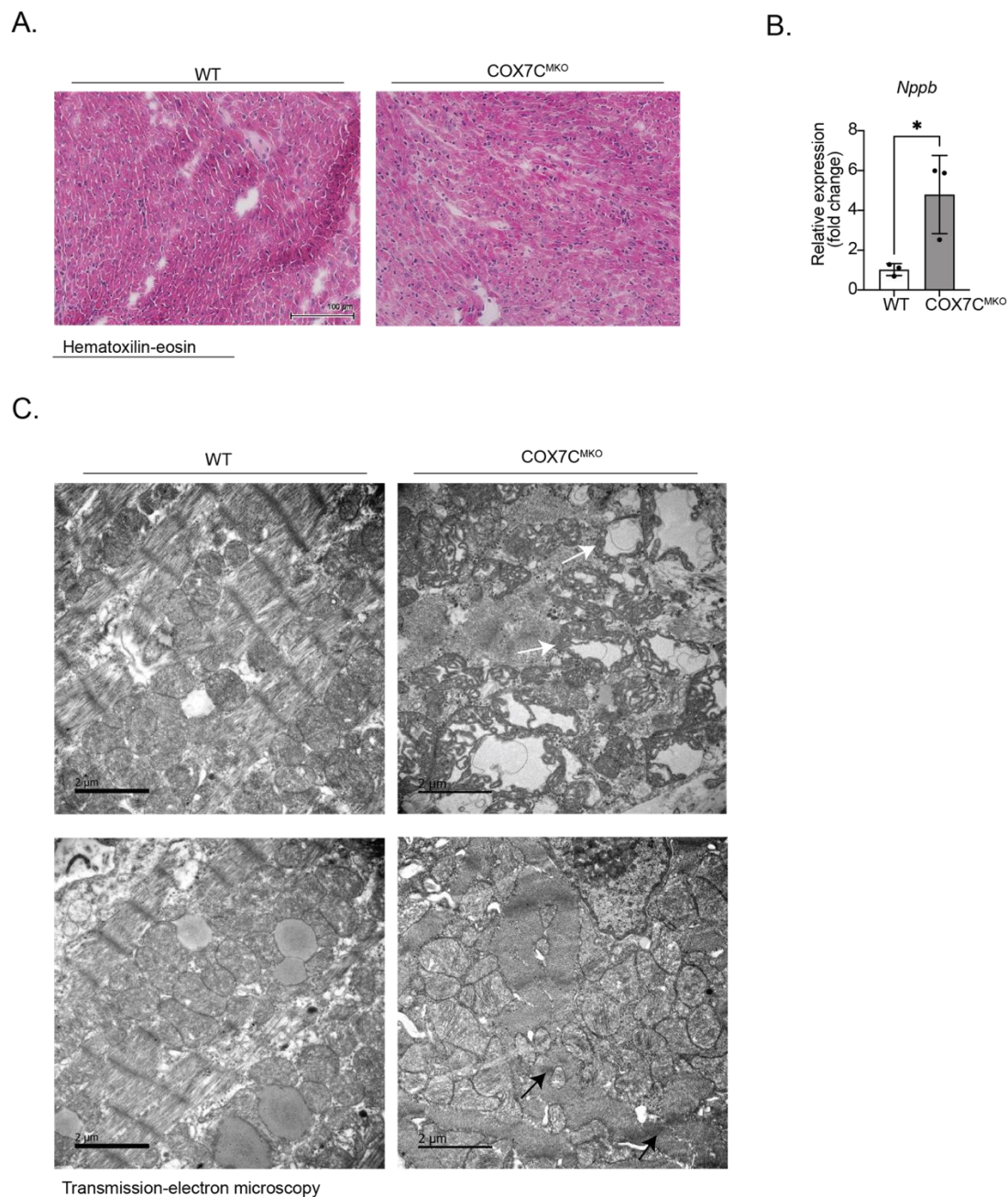


Figure 3.4. Changes to cardiac tissue structure upon loss of COX7C. **A.** Hematoxylin-eosin staining of fresh frozen heart sections of WT and $COX7C^{MKO}$ at P10. **B.** Steady-state transcript

levels for *Nppb* measured by quantitative PCR ($n=3$, $p\text{-value}>0,05$). **C.** Ultrastructure of COX7C^{MKO} and WT cardiomyocytes at P10 as imaged by TEM (black arrow – Z disks; white arrow – disrupted mitochondria)

To confirm cardiomyopathy in our COX7C^{MKO} we looked at steady state transcript levels for *Nppb*, known marker of hypertrophic cardiomyopathy. Indeed, we see a significant increase of transcript levels for *Nppb* upon loss of COX7C (Figure 3.4B).

We further looked at the ultrastructure of cardiac tissue of WT and COX7C^{MKO} animals at P10 by transmission electron microscopy imaging (TEM). TEM imaging reveals disruption of cardiac muscle structure in COX7C^{MKO} hearts. Irregular arrangement of muscle fibers, with disorganized and thickened Z-disks can be seen in COX7C^{MKO} animals (Figure 3.4C, black arrows). Further on, in COX7C^{MKO} animals we could identify swollen and disrupted mitochondria throughout the sample (Figure 3.4C, white arrows), concluding that loss of COX7C in the heart leads to early onset cardiomyopathy, causes abnormal mitochondrial morphology and affects cardiomyocyte contractile machinery.

3.2.1 Effect of COX7C depletion to OXPHOS

From the disrupted and swollen mitochondria seen on TEMs (Figure 3.4C) and a fast-progressing phenotype of COX7C^{MKO} mice, we proceeded to investigate mitochondrial dysfunction in COX7C^{MKO}. Elucidating further how loss of COX7C might affect complex IV and OXPHOS in heart mitochondria, we investigated steady-state levels and organization of all OXPHOS complexes on the inner mitochondrial membrane by BN-PAGE analysis.

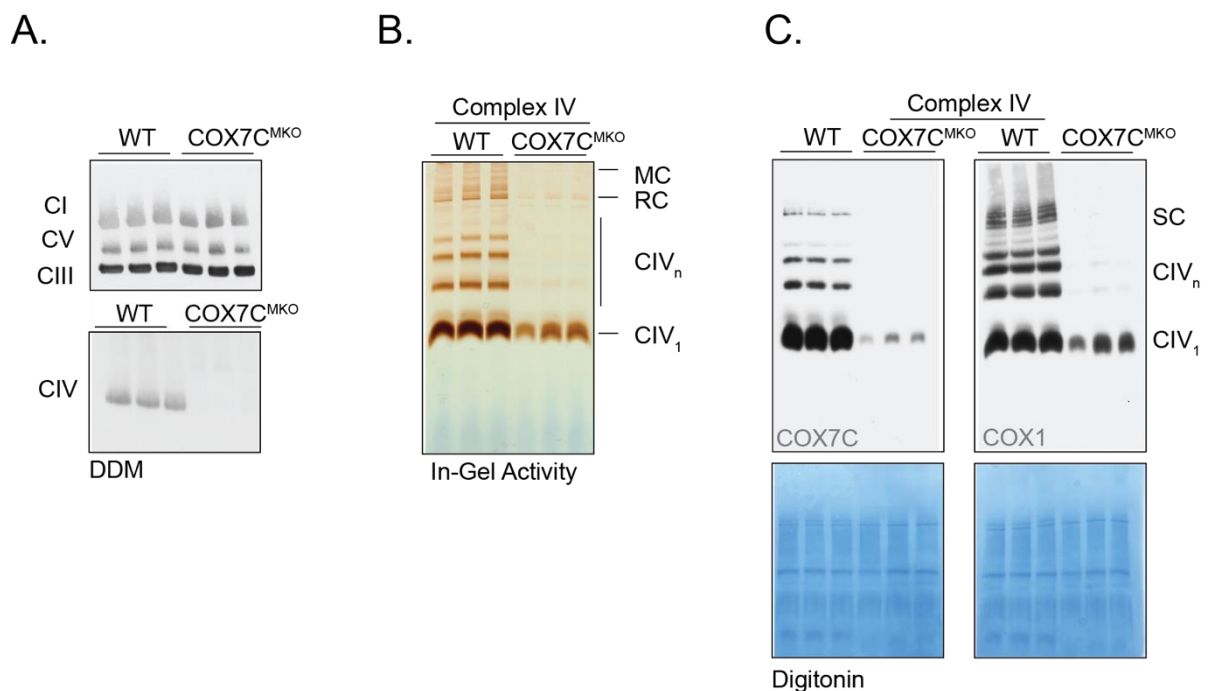


Figure 3.5. Loss of COX7C in the heart leads to an isolated complex IV deficiency. **A.** Steady-state levels of OXPHOS complexes I-IV in WT and COX7C^{MKO} heart mitochondria, separated on DDM BN-PAGE, followed by western blot and immunodetection. **B.** In-gel activity for

complex IV in WT and COX7C^{MKO} heart mitochondria separated on digitonin BN-PAGE. **C.** Steady-state levels of Complex IV-containing supercomplexes, separated on digitonin BN-PAGE followed by western blot and immunodetection for COX7C (left panel) and COX1 (right panel). Coomassie-stained membrane below as loading control.

DDM BN-PAGE analysis showed steady-state levels of OXPHOS complexes I-III did not change upon loss of COX7C (Figure 3.5A, upper panel).

Only a strong loss of complex IV can be observed (Figure 3.5A, lower panel), recapitulating the results obtained from whole body depletion of *Cox7c* (Figure 3.2). To investigate how COX7C might contribute to SC structures, we analyzed heart mitochondria of P10 WT and COX7C^{MKO} animals on a digitonin BN-PAGE (Figure 3.5B and C). BN-PAGE analysis was followed with *in-gel* activity assay for complex CIV, showing that complex CIV activity is also very low, albeit still maintained a mild signal of activity for monomer complex CIV (CIV₁) (Figure 3.5B). Western blot analysis following BN-PAGE showed a similar pattern to *in-gel* activity. Strong decrease in steady state levels of all structures containing complex IV can be observed, with a mild signal remaining for monomer of complex IV (Figure 3.5C). Further on, we also detected low amount of COX7C in P10 COX7C^{MKO} heart mitochondria (Figure 3.5C, left panel).

We conclude that COX7C^{MKO} mitochondria recapitulate the OXPHOS defect shown in COX7C KO embryos, showing that loss of COX7C leads to strong isolated CIV deficiency, with a loss of SC structures containing CIV.

Consistently, our BN-PAGE analysis of OXPHOS upon loss of COX7C shows an isolated CIV deficiency, with almost a complete loss of higher molecular structures of Complex IV. We further wanted to investigate in detail the dynamics of complex CIV depletion upon loss of COX7C, and whether supercomplex structures are more sensitive to loss of COX7C. Additionally, our mice have a very severe phenotype that coincides with postnatal heart development and heart regenerative window, so we decided to follow changes to OXPHOS complexes in a time resolved manner. For this reason, we performed complexome profiling analysis of heart mitochondria from COX7C^{MKO} mice. Complexome profiling is a method that couples mass spectrometry with BN-PAGE analysis to provide high resolution of mitochondrial native complexes. Firstly, mitochondrial complexes are separated on a BN-PAGE gel, then followed by slicing of the gel to 45-60 slices per sample, each gel cut is analyzed by mass spectrometer. This way, complexome profiling provides an in-depth composition for each protein complex structure within mitochondria (Wittig and Malacarne, 2021).

Complexome profiling was done in three timepoints for WT and COX7C^{MKO} heart mitochondria: P2, P5, P10. Mitochondrial complexes were solubilized with digitonin and separated on BN-PAGE (Figure 3.6A). Each sample then provided 48 gel slices which were further analyzed by mass spectrometry to obtain relative abundance for mitochondrial complexes in all timepoints.

Relative abundance heatmap shows a progressive loss of CIV in all structures from P2 to P10 (Figure 3.6B). At P2 we see a decrease of CIV structures by about 30% (Figure 3.6C). Until P5 about 50% of CIV is still maintained (Figure 3.6D). At P10 the

loss is around 80%, and loss of CIV seems to happen in all oligomeric states of CIV consistently, including supercomplexes (Figure 3.6E). This result confirms what we see on BN-PAGE as well - there is still about 20-25% of CIV at the terminal stage of COX7C^{MKO} in hearts. We can confirm that most CIV is within individual CIV, while just a small amount of CIV is contained in supercomplex structures. Since loss of CIV seems to happen equally in all oligomeric states of complex IV and in SC structures, supercomplex structures do not seem to provide additional stability to complex CIV upon loss of COX7C.

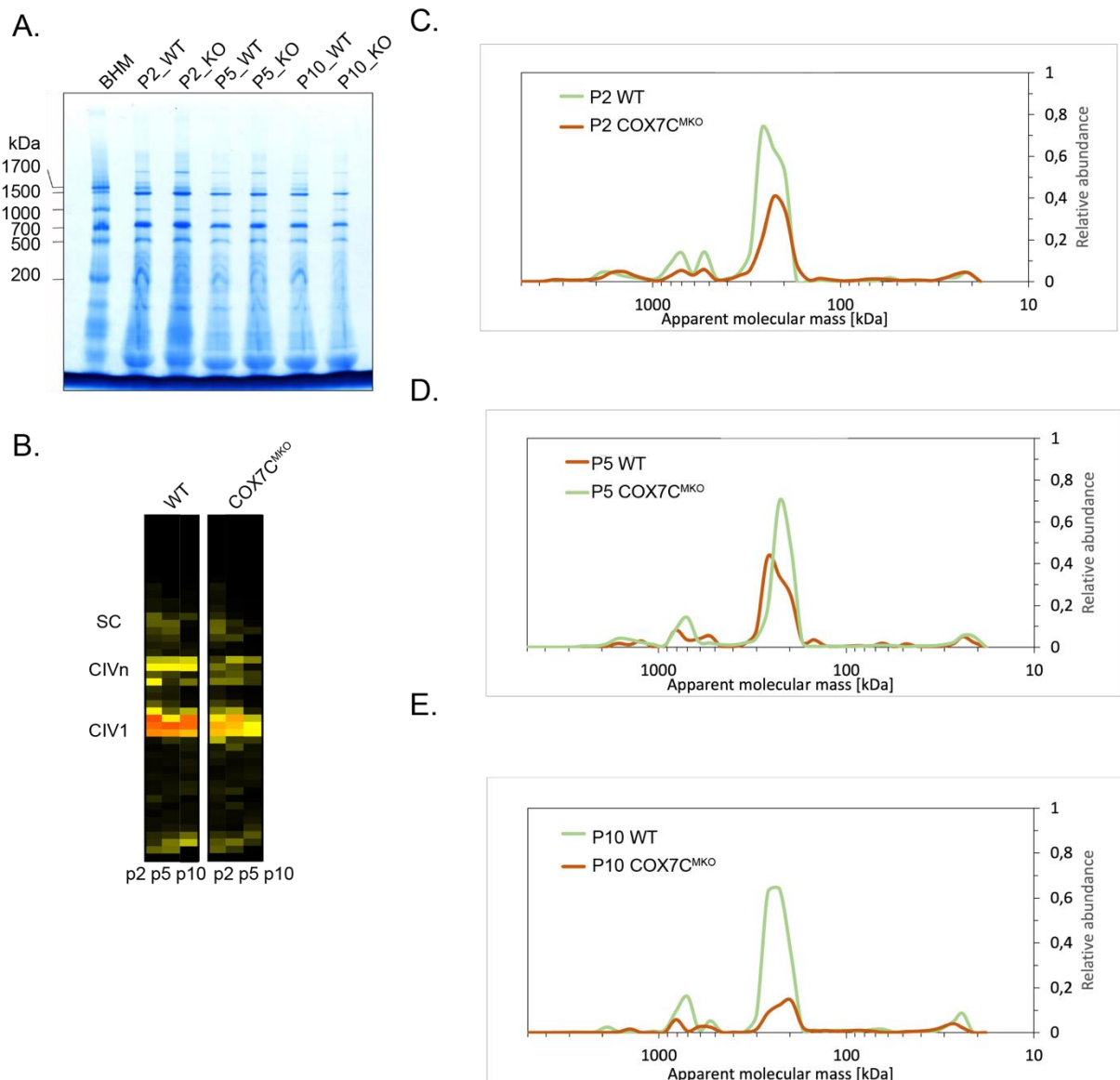


Figure 3.6. Time-resolved complexome profiling of COX7C^{MKO}. **A.** Coomassie-stained BN-PAGE gel of heart mitochondria from timepoints P2, P5, P10. Mitochondria have been pooled for each sample (n=7). **B.** Relative abundance (max normalization) of complex IV structures as they change during postnatal development. Line graph depicting relative abundance peaks for complex IV at **C.** P2 **D.** P5 **E.** P10.

As we could conclude, CIV is most abundant as a monomer, and upon COX7C loss we see a decrease primarily on SC structures containing CIV, while a low signal

remains within individual CIV (Figure 3.6). Moreover, we could detect very low levels of COX7C protein on western blot analysis (Figure 3.5C). Our complexomics data also shows that COX7C protein can be detected at P10 (Figure 3.7A) and shows the same pattern as relative abundance for CIV (Figure 3.6B), indicating that, as COX7C is depleted, levels of CIV go down as well.

Seeing how loss of CIV structures develops upon depletion of COX7C prompted us to investigate whether this phenotype is specific for COX7C, or if it's a common effect to lose SCs and CIV the same way upon destabilization of CIV. For this reason, we compared our COX7C^{MKO} model to mice deficient in COX10. COX10 is an early assembly factor for CIV, involved in synthesis of heme cofactor for COX1 subunit. Thus, we targeted very early stages of CIV biogenesis by deleting *Cox10*.

COX10 was disrupted in heart and skeletal muscle by crossing *Cox10*^{loxp/loxp} mice with mice expressing Cre recombinase under muscle creatine kinase (Ckmm) promoter, same strategy as for COX7C^{MKO} (Figure 3.3). In F2 generation we could obtain *Cox10*^{loxp/loxp} *Cre*^{wt/tg} mice (COX10^{MKO}). COX10^{MKO} mice are born and develop mitochondrial cardiomyopathy, with life expectancy of 4-5 weeks (Ahola et al., 2022).

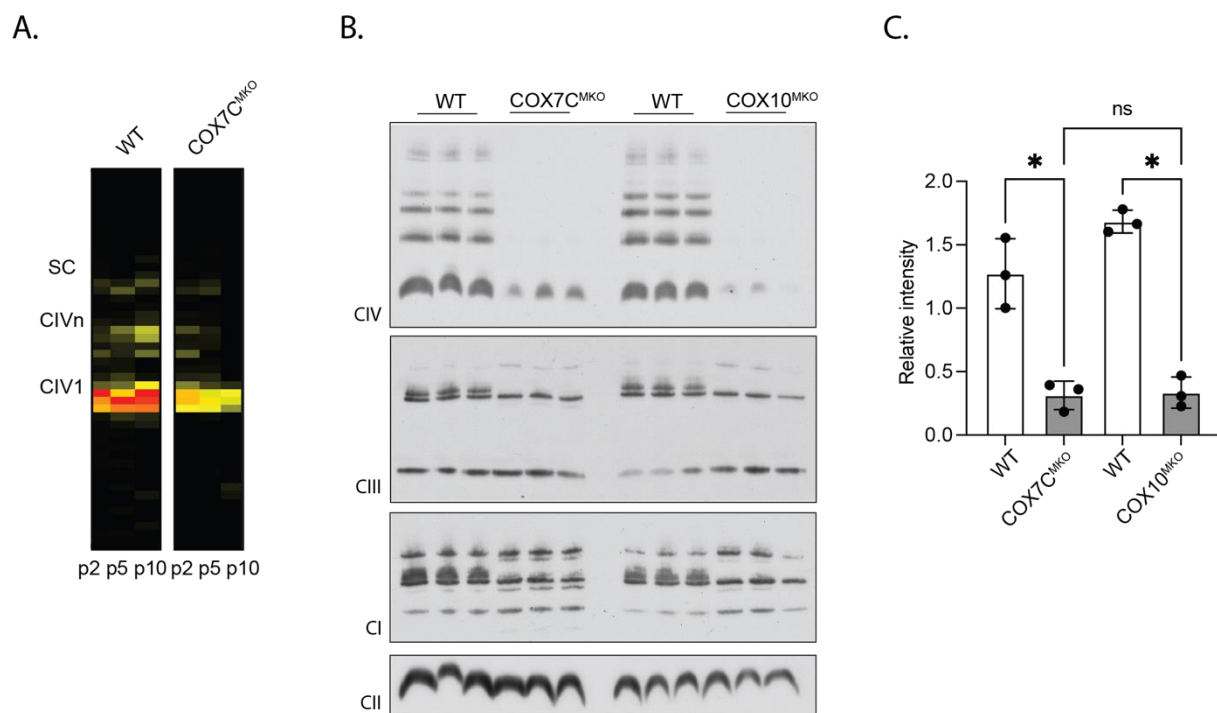


Figure 3.7. Dynamics of CIV loss are similar between COX7C^{MKO} and COX10^{MKO}. **A.** Relative abundance of COX7C protein obtained through complexome profiling; **B.** Steady-state levels of SC structures in heart mitochondria of COX7C^{MKO} and COX10^{MKO}, analyzed by digitonin BN-PAGE followed by western blot for CIV (COX1), CIII (UQCRC1), CI (NDUFA9), CII (SDHA); **C.** Relative protein abundance of CIV in COX7C^{MKO} and COX10^{MKO}, quantification of B. (p-value>0.05)

To investigate whether targeting late (COX7C) or early (COX10) assembly step of CIV affects CIV and OXPHOS the same way (Figure 3.8), we performed BN-PAGE analysis on heart mitochondria from the two models. We isolated both COX7C^{MKO} and

COX10^{MKO} heart mitochondria at p10 and 3 weeks, respectively, and compared them on a digitonin BN-PAGE (Figure 3.7B). We could confirm that loss of CIV and distribution of CIV were comparable between two models (Figure 3.7B). Residual CIV signal is present in both mouse models (Figure 3.7C). Additionally, SC structures show the same pattern, including SC structures containing CI and CIII (Figure 3.7B, lower panels).

Loss of CIV was comparable between COX7C^{MKO} and COX10^{MKO} mice indicating there is a similar loss and distribution of remaining CIV regardless of which step of assembly is disrupted.

OXPHOS complexes CI and CIII also show the same pattern of reorganization, making it a conserved phenotype for heart OXPHOS maintenance and organization upon loss of CIV. While COX7C seems to be essential for stability of CIV, and COX10 for early assembly steps of CIV, they should affect CIV differently. However, we were focused on following steady-state levels for OXPHOS structures between two models, and to pinpoint the difference in effect on CIV stability or assembly, further studies are needed.

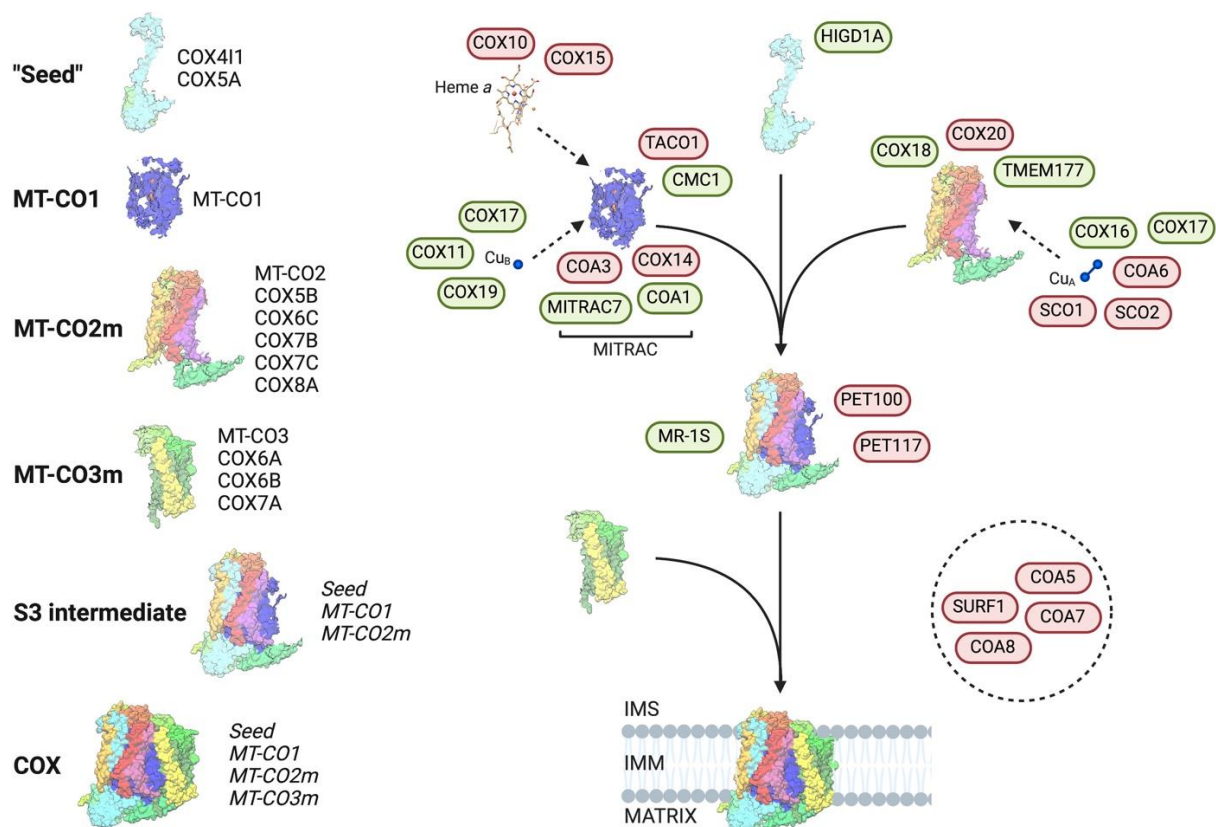


Figure 3.8. Assembly of CIV. (Brischigliaro and Zeviani, 2021)

Since COX7C belongs to later stages of CIV assembly (Figure 3.8), we wanted to determine if gradual loss of COX7C during postnatal development would condition formation of functional subassemblies of CIV. To look at our complexomics data in higher detail and find hints of whether loss of CIV in COX7C^{MKO} could be attributed to

stability or to assembly defects, we investigated intensities for every detected subunit of CIV throughout postnatal development (Figure 3.9).

At P10 we could not detect any stable subassemblies of CIV that correspond to the reported structures of CIV subassembly (Vercellino and Sazanov, 2022). Core subunits mtCO2 and mtCO3, which are main components of modules of assembly for CIV, do not show a signal below fully assembled CIV in our KO mice in any timepoint (Figure 3.9A). However, at P2 COX shows an additional peak at 120kDa and at 70kDa, also present at P5 (Figure 3.9B, lower panel). 70kDa structure is present also in the WT mice at the same timepoints but to lesser extent (Figure 3.9B, upper panel).

Other subunits accumulated below fully assembled CIV monomer include COX4i1 and COX5A, components of CIV assembly seed (Figure 3.9A). However, they do not seem to be interacting with mtCO1 since their peaks do not overlap. These subunits are not present at 120kDa or 70kDa peaks observed for mtCO1 (Figure 3.9B, lower panel). Further on, we observed that COX6B, an end stage assembly subunit, is absent at P10 in COX7C^{MKO} mice, while it shows a clear signal on the bottom of the gel in the WT mice (Figure 3.9A).

To investigate whether these are indications of stalled assembly of CIV, we performed *in-organello* translation experiment. In-organello experiment allows us to follow protein synthesis within mitochondria, by providing radioactively labeled methionine to isolated mitochondria, which gets incorporated into newly formed proteins. Since genes for core subunits of CIV are encoded within mitochondrial DNA, we wanted to confirm if synthesis of these subunits is affected upon loss of COX7C and subsequent CIV deficiency. At P10, we could detect all newly synthesized subunits encoded within mitochondria, and there was no difference upon loss of COX7C (Figure 3.9C). All three core subunits of CIV could be synthesized in COX7C^{MKO} hearts.

In the WT hearts at P2 we see subassembly intermediate containing COX1, indicating increased assembly of CIV, consistent with mitochondrial remodeling at early postnatal heart development, starting with mitochondrial biogenesis (Lehman et al., 2000). This intermediate is more abundant in COX7C^{MKO} at P2 and P5, indicating that assembly of CIV might have been initiated in these mice as well, as a part of mitochondrial remodeling in heart development. However, it might be that in absence of COX7C, assembly of CIV cannot be completed, since COX7C is part of the next module in the assembly line of CIV, mtCO2 module (Figure 3.8). Thus, this 70kDa assembly intermediate accumulates at P2 and P5 in COX7C^{MKO}. This is further corroborated by accumulation of COX4i1 and COX5A, which form the seed of early CIV assembly (Figure 3.8).

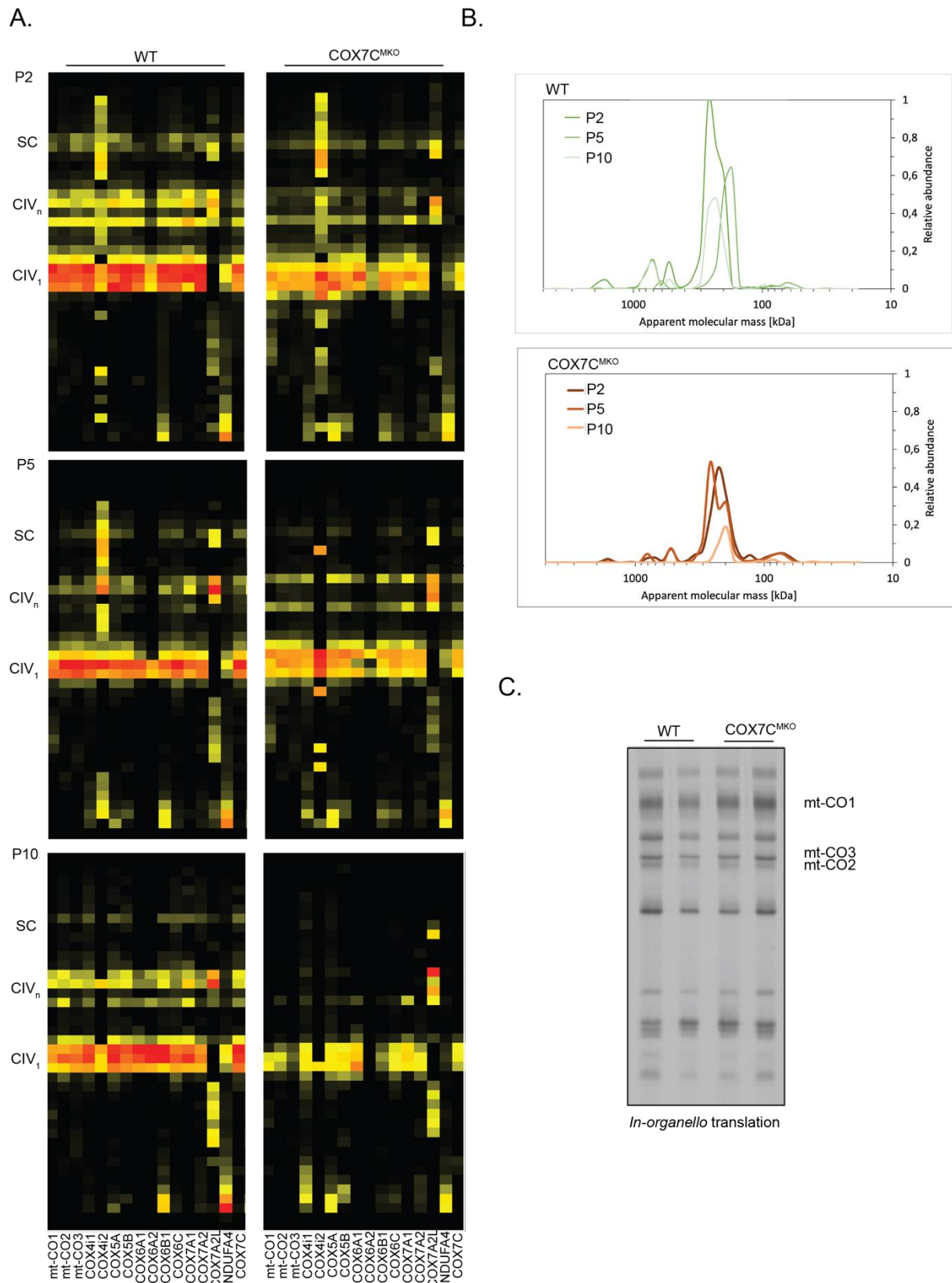


Figure 3.9. Complexome profiling for CIV during postnatal development of COX7C^{MKO} mice. **A.** Relative protein abundance for all detected CIV subunits during postnatal development of WT (left panel) and COX7C^{MKO} mice (right panel), visualized as a heatmap. **B.** Line graphs for relative protein abundance for mtCO1 of CIV during postnatal development. **C.** *In-organello* translation of mitochondrial encoded proteins of heart mitochondria from WT and COX7C^{MKO} mice.

One of the observations we saw consistently upon loss of CIV is the loss of respirasome (RC) and reorganization of SC structures of CI and CIII (Figure 3.2, Figure 3.5, Figure 3.7). As mentioned, respirasome is an SC structure containing CI, CIII₂, and CIV. It is considered a respiratory unit since it contains a complete electron transport chain (Acin-Perez et al., 2008). Since one of the proposed roles of SCs is to provide stability to individual OXPHOS complexes, we investigated how CI and CIII rearrange upon loss of CIV.

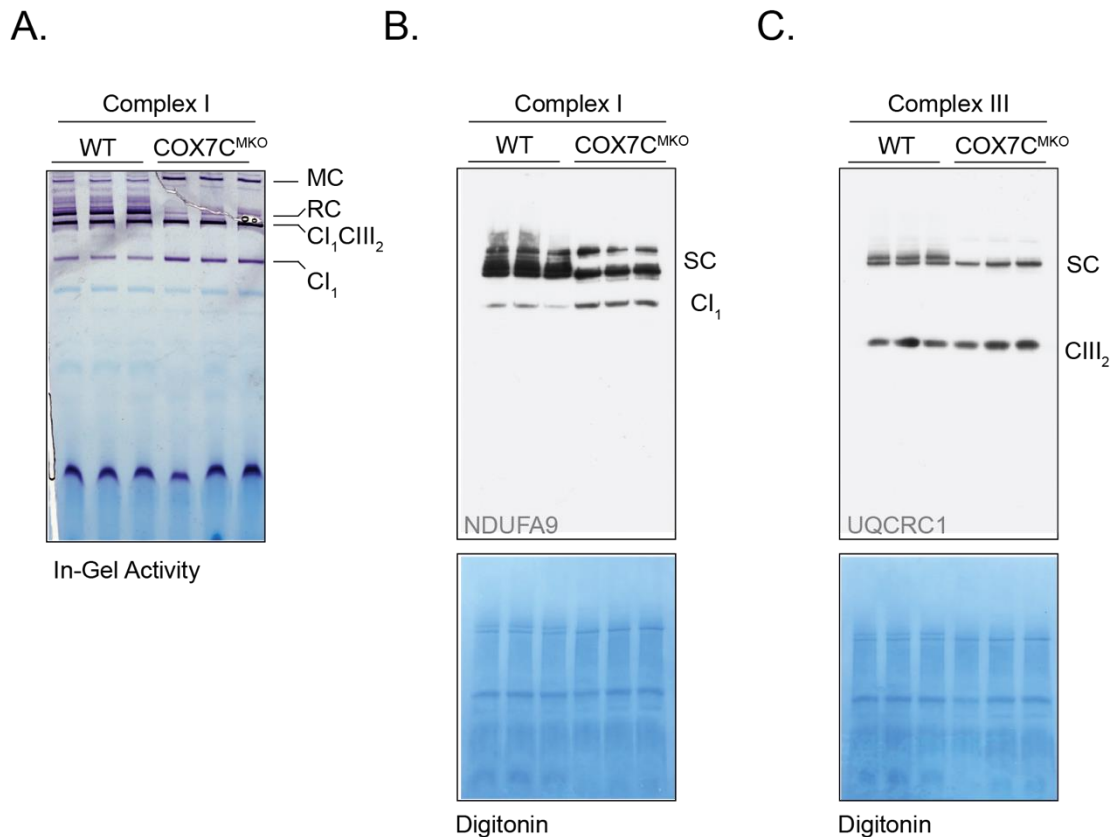


Figure 3.10. Complex I and Complex III show a change in distribution upon loss of CIV. **A.** Digitonin BN-PAGE followed by in-gel activity assay for CI; **B.** Steady state levels of Complex I structures: Supercomplexes (SC), Complex I monomer (CI₁); analyzed by digitonin BN-PAGE followed by western blot. Immunodetection on NDUFA9 subunit of CI. **C.** Steady state levels of Complex III structures: Supercomplexes (SC), Complex III dimer (CIII₂); analyzed by digitonin BN-PAGE followed by western blot. Immunodetection on UQCRC1 subunit of CIII

Firstly, we observed no change to whole levels of CI or CIII in COX7C^{MKO} heart mitochondria on DDM BN-PAGE (Figure 3.5). Complex I in-gel activity following digitonin BN-PAGE showed a redistribution of SC structures containing CI (Figure 3.10A). While respirasome structure seems to be completely disrupted, coinciding with loss of CIV, remaining CI distributes to CI-CIII₂, CI₁ and to megacomplex structures (Figure 3.10A). Western blot analysis for Complex I showed the same pattern (Figure 3.10B). Complex III also shows a change in distribution within SC structures upon loss of CIV, where CIII₂ rearrangement seems less obvious. Since BN-PAGE could not reveal the extent of changes to distribution and composition of CI and CIII upon loss of CIV, we investigated how these complexes change on our complexome profiling data.

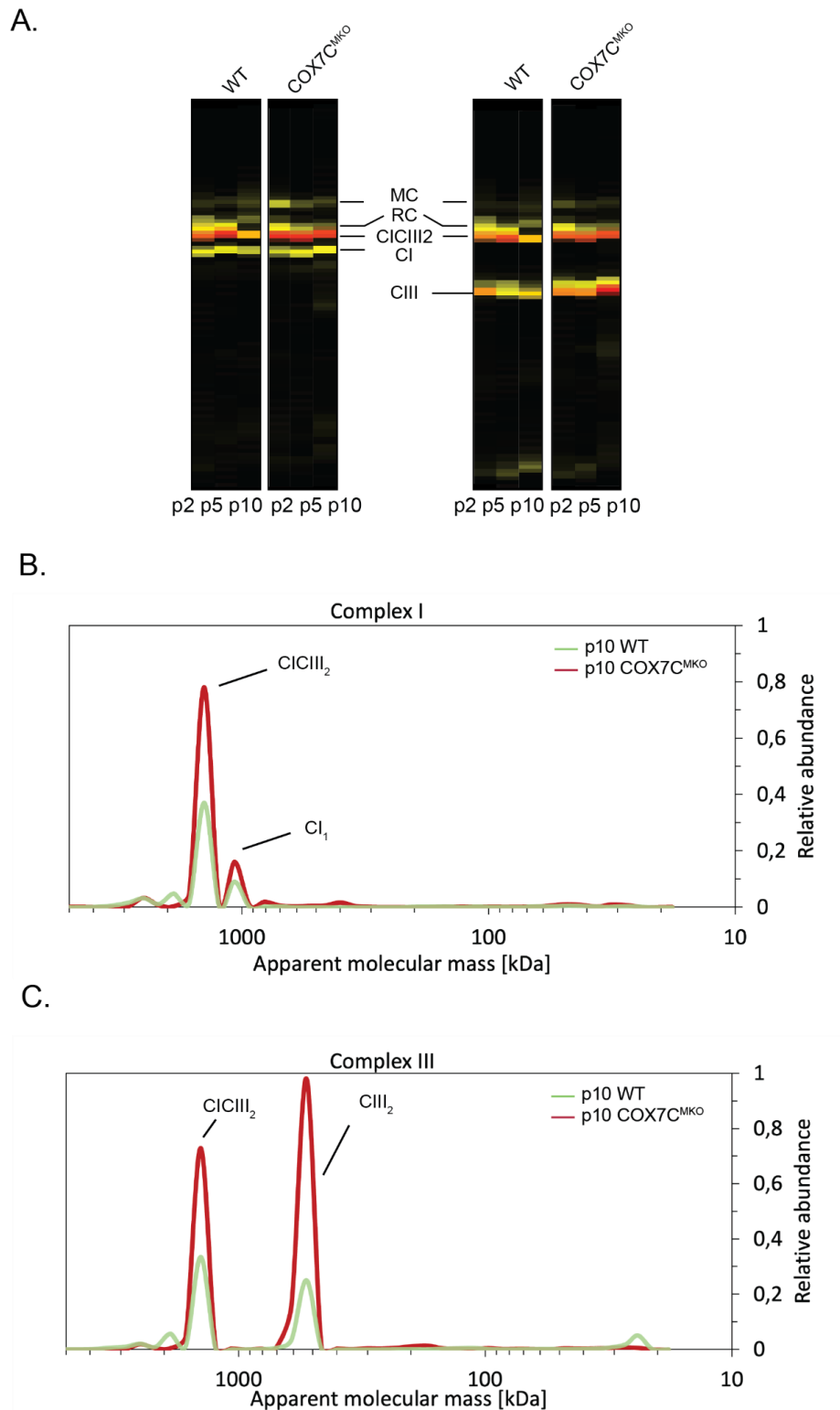


Figure 3.11. Complexome profiling confirms redistribution of CI and CIII. **A.** Heatmap of relative abundances for CI (left) and CIII (right) in WT and COX7C^{MKO} heart mitochondria at P10. **B.** Relative abundance for CI and **C.** CIII in heart mitochondria of WT and COX7C^{MKO} mice at P10.

We could observe a clear increase for CICIII₂ and CIII₂ in COX7C^{MKO} mice compared to WT (Figure 3.11A). Further on, reorganization of CI and CIII follows the

loss of respirasome and develops progressively as CIV disappears (Figure 3.11A, right panel).

The strongest difference is visible at P10. CI shows a 30-40% increase in CII₂ and CI₁ structures, and a complete absence of the respirasome (Figure 3.11B). Similar can be observed for CIII where CII₂ structure shows a strong increase in relative abundance in comparison to the WT mice (Figure 3.11C). While CI seems to preferentially distribute to CII₂ structure, CIII shows a strong increase in CII₂ upon loss of CIV. Reorganization of CI and CIII structures on the inner mitochondrial membrane upon loss of CIV seems to be a compensating mechanism due to disruption of respirasome structure. Whether this reorganization has any physiological significance was beyond our interest, but it is very plausible that the redistribution of CI and CIII is coincidental, due to the dissociation of CIV, and not a regulated process.

3.3 Stress responses of the heart upon loss of COX7C in the heart

Regardless of which complex is affected, when stress within mitochondria is sensed, it leads to activation of the integrated stress response (ISR). Other than ISR activation, different tissues have different coping strategies for mitochondrial stress. Mitochondrial dysfunction in the heart usually leads to decrease of ATP levels, ROS production and metabolic changes, compromising contractility of the cardiac muscle. Compromised contractile activity of the heart leads to activation of compensatory heart remodeling, which can lead to pathological consequences and development of hypertrophic or dilated cardiomyopathy. To further elucidate physiological consequences of CIV loss in COX7C^{MKO} and COX10^{MKO} hearts, we investigated these common stress pathways, and described in more detail the molecular changes to cardiac tissue between the two models.

COX7C^{MKO} mice present with fast progressing phenotype and early onset cardiomyopathy and abrupt death in less than two weeks after birth (Figure 3.3). Thus, describing and finding the cause for such a severe early development of phenotype was our next goal. To investigate when changes to cardiomyocyte function happen and how they develop towards early onset cardiomyopathy, we investigated three timepoints in COX7C^{MKO} mice: P2, P5, P10 (Figure 3.12). The first sign of cardiomyopathy is visible at P5, evidenced by a significant increase in transcript levels of *Nppb*, a cardiomyopathy marker, in COX7C^{MKO} mice hearts (Figure 3.12B).

As mentioned before, increase in mitochondrial biogenesis happens during postnatal heart development (Lehman et al., 2000) and is also a strategy to compensate for mitochondrial dysfunction in heart failure (Rimbaud et al., 2009). To test whether CIV deficiency leads to changes in mitochondrial amount, we measured TOMM20 protein levels in all timepoints and found no change between WT and COX7C^{MKO} (Figure 3.12A).

Phosphorylation of elongation initiation factor 2-alpha (eIF2α) through one of four known kinases is considered the main event in activation of ISR (Donnelly et al., 2013). For this reason, we investigated the phosphorylation status of eIF2α. The first signs of ISR activation are noticeable at P2 already, with a slight increase in phosphorylation

of eIF2a (Figure 3.12A). Additionally, the signal increases steadily in a reverse correlation to levels of COX1 (Figure 3.12). While the phosphorylation of eIF2a is observed at P2 and P5, we could not detect any significant changes in transcript levels for *Atf4*, the downstream effector of ISR (Figure 3.12C).

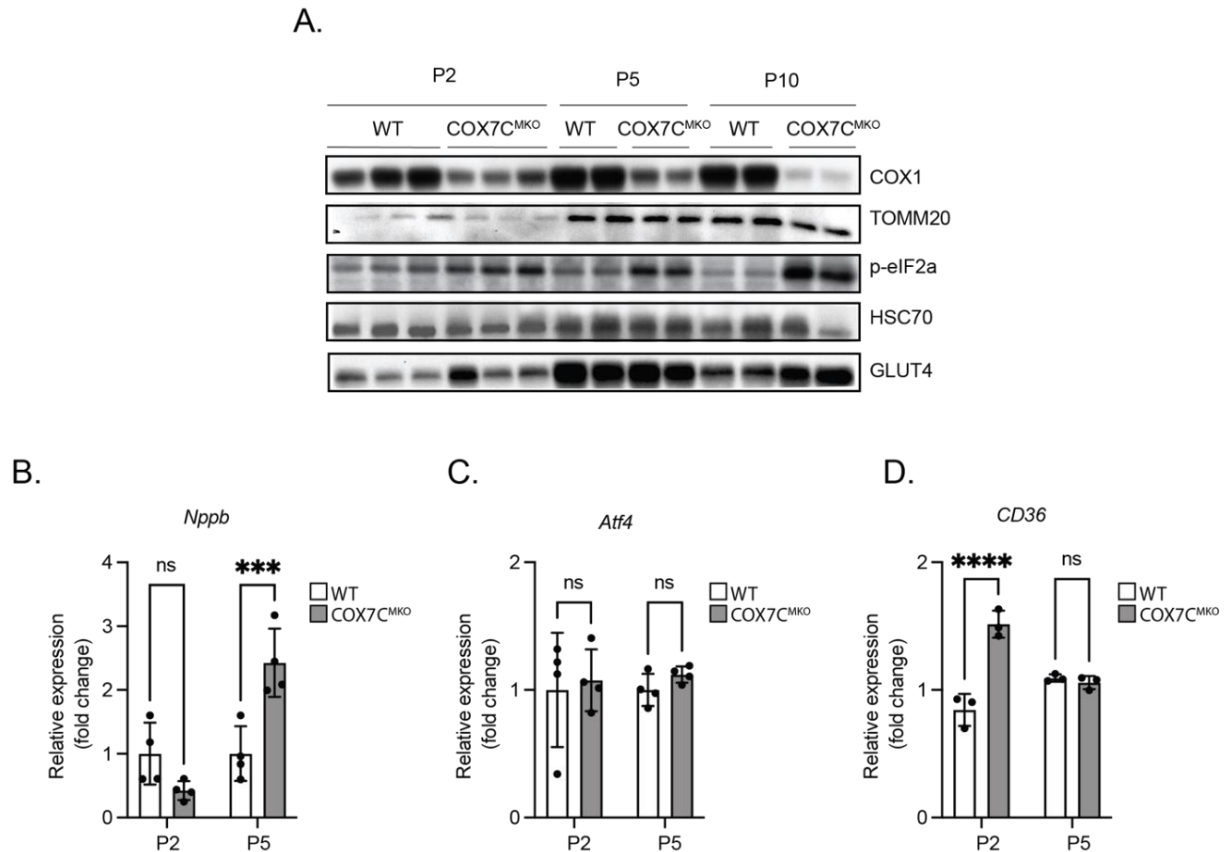


Figure 3.12. Development of heart mitochondrial phenotype in COX7C^{MKO} mice. **A.** Steady-state protein levels for COX1, phosphorylated eIF2a, TOMM20, and GLUT4 from P2, P5, P10 whole heart lysate of WT and COX7C^{MKO} animals; Steady-state transcript levels of P2 and P5 hearts for **B.** *Nppb* (n=4); **C.** *Atf4* (n=4); **D.** *Cd36* (n=3, Student t-test, p-val<0,05)

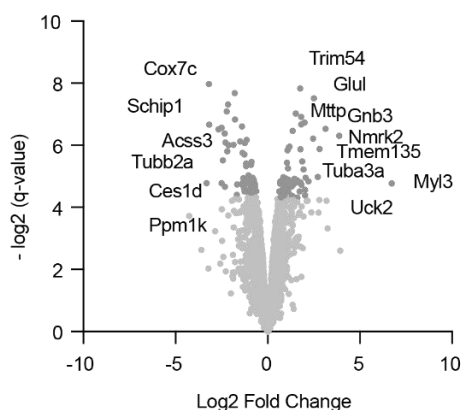
We additionally followed markers to investigate metabolic status of COX7C^{MKO} cardiomyocytes, such as GLUT4 protein levels and *Cd36* transcript level, both responsive upon cardiomyopathy (Stanley et al., 2005). GLUT4 increase could be observed at P10, indicating changes to glucose handling and upregulation of glycolysis (Figure 3.12A). However, *Cd36* transcript levels, indicative of changes to fatty acid oxidation, show an increase as early as P2 in COX7C^{MKO} (Figure 3.12C). We could conclude that markers for cardiomyopathy and ISR seem to be present early after birth.

3.3.1 Proteomics of COX7C^{MKO} hearts

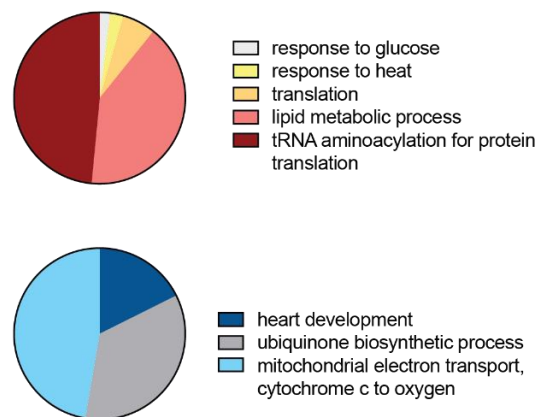
Since we could observe some changes in COX7C^{MKO} mice early on, we set out to describe in detail the changes to the heart at the late stage of cardiomyopathy. To further describe the consequences of COX7C depletion to the heart, we performed label-free proteomics analysis of whole heart tissue lysate from COX7C^{MKO} animals

(Figure 3.13). Whole heart proteomics yielded 5902 identified proteins, from which there were 154 significantly changed proteins. Out of 154 significantly changed proteins, 77 of them upregulated and 77 are downregulated (Figure 3.13A).

A.



B.



C.

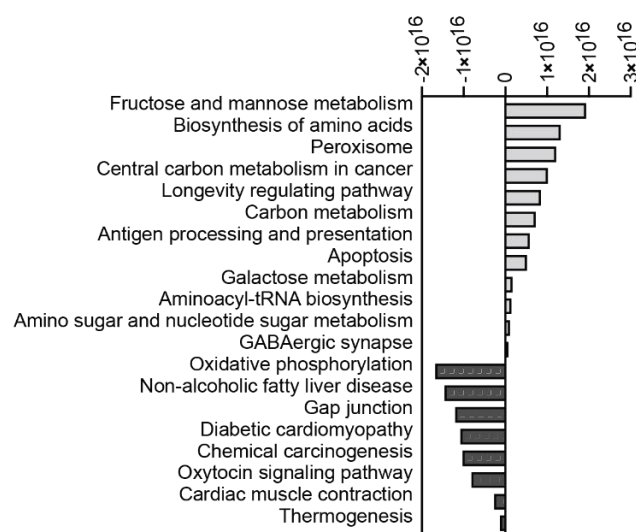


Figure 3.13. Proteomics analysis of COX7C^{MKO} mice. **A.** Volcano plot of protein fold change for whole heart proteome of COX7C^{MKO}. Significantly changed proteins shown in dark grey (Student t-test, $q\text{-val} < 0.05$) **B.** Go-Term enrichment analysis for upregulated (red pie chart) and downregulated proteins (blue pie chart) in COX7C^{MKO} hearts; **C.** KEGG pathway enrichment analysis of upregulated and downregulated proteins in COX7C^{MKO} hearts.

To annotate these changes to corresponding molecular pathways and processes, we performed GO term enrichment analysis and KEGG pathway enrichment analysis. GO term analysis shows significant enrichment in tRNA aminoacylation pathway and lipid metabolic processes among the upregulated proteins. Enrichment of downregulated proteins shows significant enrichment in only three processes,

mitochondrial electron transport chain, ubiquinone biosynthesis and heart development (Figure 3.13B). Additionally, KEGG pathway analysis identified central carbon metabolism and amino acid metabolism to be the most enriched pathways (Figure 3.13C).

By the differentially changed proteins we can identify changes in lipid metabolism: upregulation of microsomal triglyceride transfer protein (MTTP) which catalyzes the transport of triglycerides, acyl-CoA synthetase short chain family member 3 (ACSS3) - involved in ketone body synthesis and carboxylesterase 1D (CES1D) which hydrolyzes triacylglycerols and monoacylglycerols. Changes in cardiac muscle signaling and structure are also evidenced by differential regulation of myosin light chain isoform 3 (MYL3), tubulin alpha and beta isoforms (TUBB2A, TUBA3A).

Although our enrichment analysis fails to emphasize, an interesting observation is the downregulation of protein phosphatase 1K, a key regulator of branched chain amino acid metabolism (Figure 3.13A). PPM1K leads to an activation of branched-chain α -ketoacid dehydrogenase complex (BCKDH), a rate limiting enzyme in BCAA metabolism, and its downregulation is associated with defects in the catabolism of BCAAs (Yang et al., 2022). So far, proteome remodeling indicates mild changes related to ISR activation, changes in peroxisomal fatty acid oxidation and BCAA metabolism.

3.3.2 ISR in hearts upon loss of COX7C

Since we could observe phosphorylation of eIF2 α in our P10 COX7C^{MKO} hearts, we proceeded to describe the contribution of ISR activation in CIV deficiency upon loss of COX7C. In our proteomics data of COX7C^{MKO} hearts we identified some of ISR targets among the upregulated proteins (Figure 3.14A). The targets we identified are phosphoglycerate dehydrogenase (PHGDH), involved in serine metabolism, amino acid transporters such as solute carrier family 7 member 5 (SLC7A5), tRNA acetyltransferases, and CCAAT enhancer binding protein (CEBPB). At P10, phosphorylation of eIF2 α is evident (Figure 3.14B). However, we see only a slight increase of *Atf4* steady-state transcript level (Figure 3.14C). To identify which transcription factor is regulating the ISR in our model, we analyzed our proteomics data set with iRegulon. We identified CEBPB, one of the dimerizing factors of ATF4, which shows a complete overlap with ATF4 targets identified in our dataset (Figure 3.14E), indicating its involvement in ISR activation in COX7C^{MKO}.

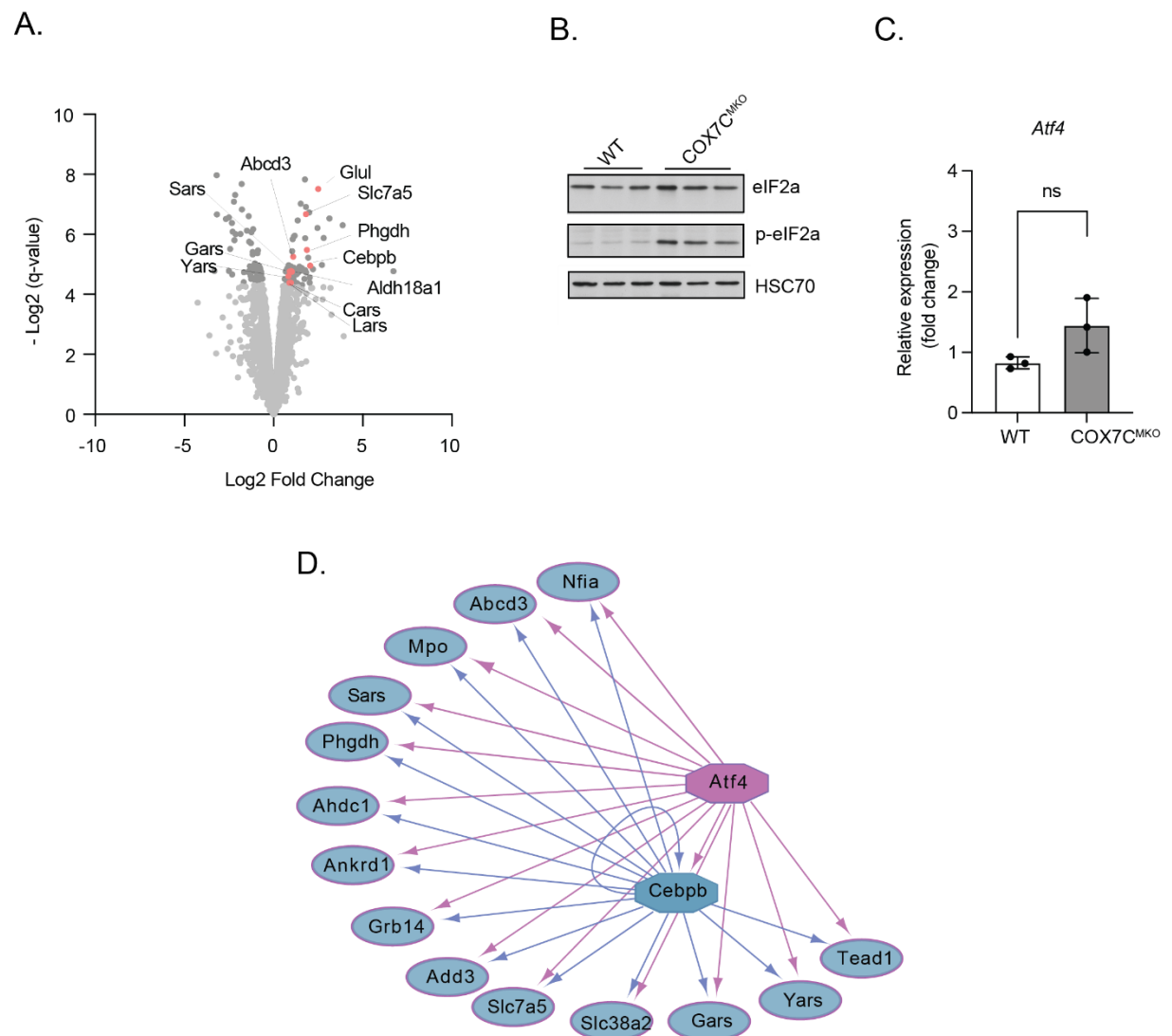


Figure 3.14. ISR and OXPHOS changes in COX7C^{MKO} mice. **A.** Volcano plot of protein fold changes of known ISR targets in whole heart proteomics data from COX7C^{MKO} mice; ISR targets highlighted in red. **B.** Steady-state protein levels of phosphorylated eIF2a; **C.** Steady-state transcript levels for *Atf4* (p-val>0.05); **D.** Diagram of iRegulon transcriptional regulation analysis.

3.3.3 Proteomics of COX10^{MKO} hearts

To see if CIV deficiency in the heart generally involves CEBPB upon ISR activation, we compared COX7C^{MKO} to COX10^{MKO} model. Firstly, we performed whole heart proteome analysis of COX10^{MKO} (Figure 3.15). We identified 880 changed proteins, out of which 685 are upregulated and 195 are downregulated. KEGG enrichment analysis reveals that most upregulated proteins belong to amino acid metabolism, fructose and glucose metabolism, pentose-phosphate pathway, followed by serine and glycine metabolism, nucleotide metabolism and carbon metabolism (Figure 3.15B). Western blot analysis of ISR targets shows a clear and strong upregulation of proline, serine and 1C metabolism (Figure 3.15C), further confirming what we see on proteomics data. Additionally, qPCR analysis shows upregulation of transcript levels of *Atf4*, *C-Myc* and their downstream targets (Figure 3.15D). Unlike COX7C^{MKO},

COX10^{MKO} hearts show a more canonical ISR activation, with upregulation of *Atf4* and increase of protein levels for many ISR targets. Analysis on COX10^{MKO} hearts was performed by Lea Isermann.

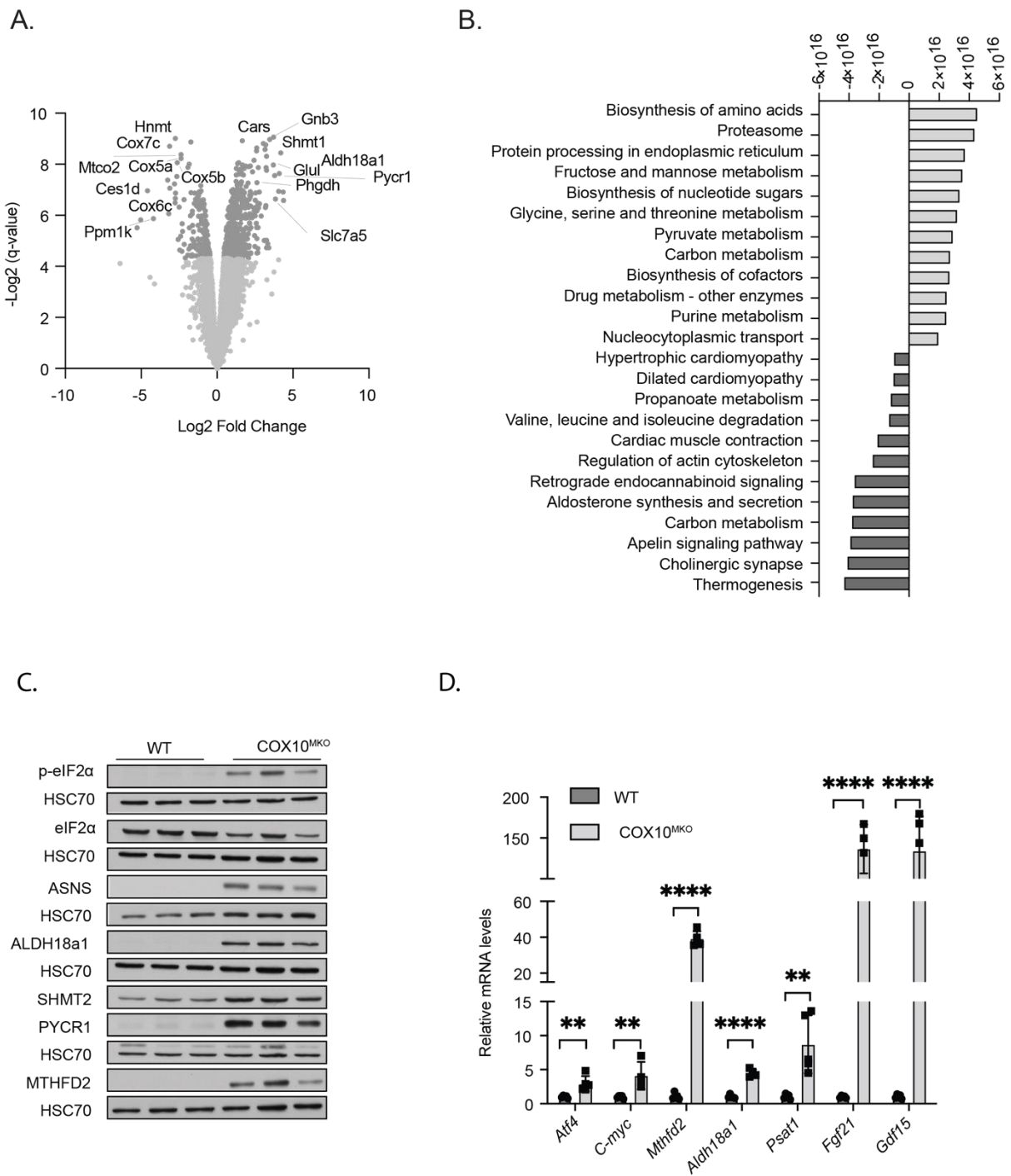


Figure 3.15. COX10^{MKO} proteomics and ISR activation. **A.** Vulcano plot of protein fold changes in COX10^{MKO} heart proteomics at 3 weeks. Significantly changed proteins shown in dark grey (Student t-test, q-val<0,05). **B.** KEGG pathway enrichment for COX10^{MKO} heart proteome; **C.** Steady-state protein levels of ISR targets in 3-week-old COX10^{MKO} hearts; **D.** Steady-state transcript levels of ISR targets in 3-week-old COX10^{MKO} hearts (Student’s t-test, p-val < 0,05).

We observed that COX10^{MKO} mice show more changes to the proteome than COX7C^{MKO}. While proteomics analysis of COX7C^{MKO} showed 154 proteins to be

differentially regulated (Figure 3.13), COX10^{MKO} had 880 proteins significantly changed (Figure 3.15). We proceeded to investigate where some of the changes in proteome response come from.

Firstly, we observed that COX10^{MKO} show significant upregulation of ribosomal proteins (Figure 3.16A). Moreover, mitochondrial ribosomal proteins are also upregulated in COX10^{MKO} (Figure 16B). Since changes in ribosomal and mito-ribosomal subunits indicates changes to protein translation, we next looked at proteins associated with translation and observed a general upregulation as well (Figure 16C)

Other than seemingly having a stronger ISR response, COX10^{MKO} mice show an extensive change in translational machinery, ribosomal and mito-ribosomal proteins, not present in COX7C^{MKO}.

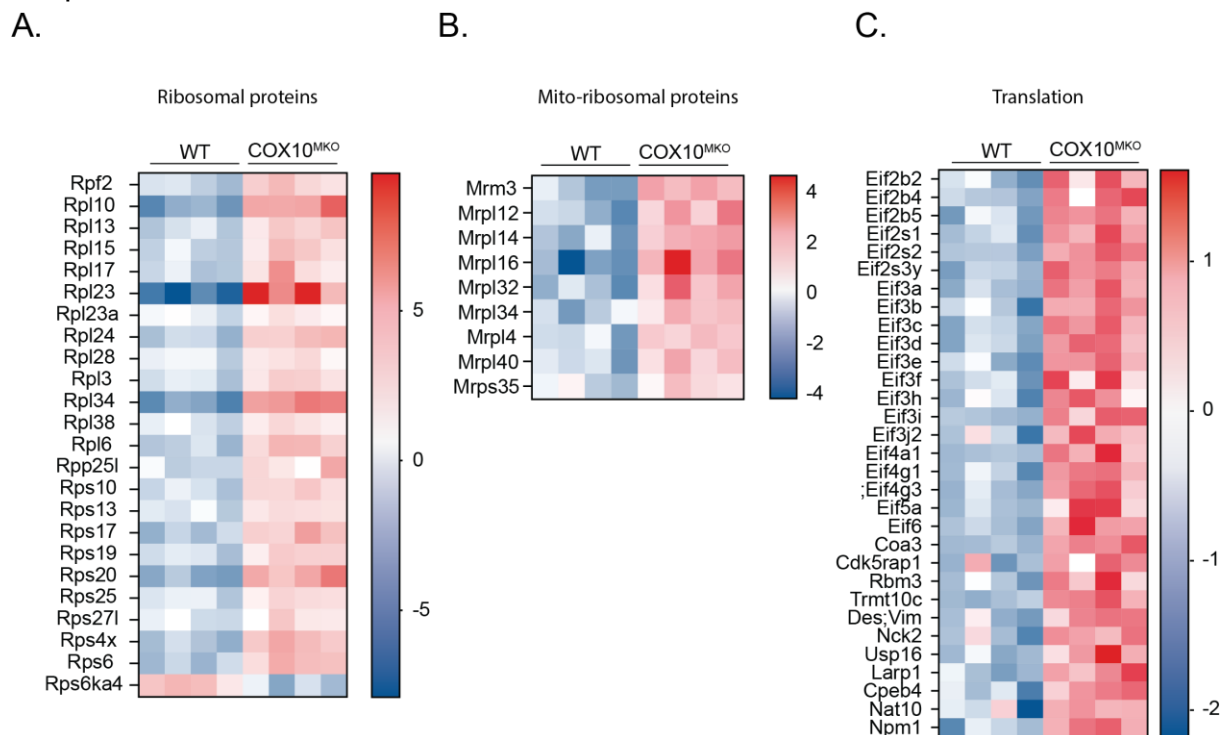


Figure 3.16. Proteome changes seen only in COX10 mice. Fold changes for proteins belonging to **A.** Ribosomal proteins; **B.** Mitochondrial ribosomal proteins; **C.** Translation machinery, identified in COX10^{MKO} mouse hearts at 3 weeks (n=4, q-val > 0,05).

3.3.4 ISR activation differences between COX7C^{MKO} and COX10^{MKO}

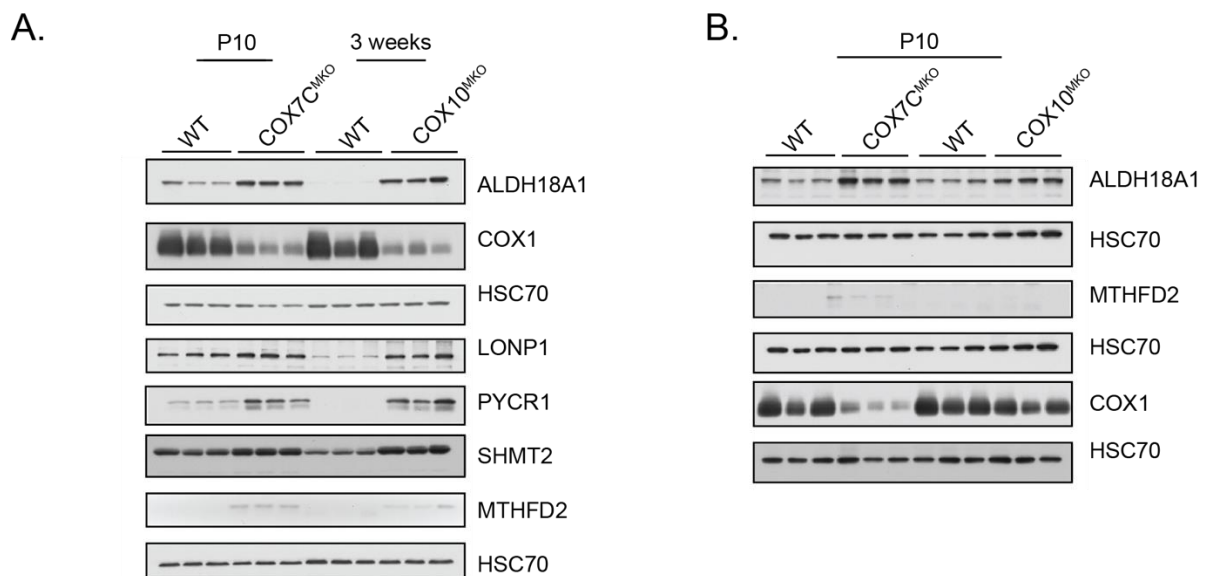
Since COX7C^{MKO} and COX10^{MKO} show activation of ISR to a differing extent, we decided to directly compare the ISR response in the two models. Firstly, we compared steady state protein levels for ISR markers by western blot analysis at terminal point of cardiomyopathy for both models, which is P10 for COX7C^{MKO} and 3 weeks for COX10^{MKO}. At P10 and 3 weeks, respectively, COX7C^{MKO} and COX10^{MKO} hearts show comparable signals for all checked markers of ISR (Figure 3.17A).

Since COX10^{MKO} mice live much longer and are older than COX7C^{MKO} mice, we compared both models in the same timepoint – P10 (Figure 3.17B.). We could observe that at P10 COX10^{MKO} mice still maintain high levels of COX1 protein, indicative of CIV levels (Figure 3.17B). Further on, at P10 COX10^{MKO} mice do not show upregulation of

any ISR markers (Figure 3.17B). This indicates that loss of CIV in COX10^{MKO} mice hearts happens later than in COX7C^{MKO} mice, outside the window of postnatal development. Since at P10 they do not show downregulation of COX1 nor activation of ISR, we conclude that COX10^{MKO} mice do not have any significant dysfunction during postnatal development.

Further on, we wanted to identify which transcription factors can be identified as regulators of ISR in 3 weeks old COX10^{MKO} mice. We analyzed COX10^{MKO} heart proteome with iRegulon and could identify ATF4 as the sole regulator of ISR response (Figure 3.17C). Seeing that iRegulon analysis for COX7C^{MKO} (Figure 3.14D) and COX10^{MKO} mice (Figure 3.17C) gave us quite different result, we decided to compare known ATF4 targets confirmed by ChipSeq analysis (Han et al., 2013) and their fold changes identified on proteomics data for both models (Figure 3.17D). We observe four times more targets significantly changed in COX10^{MKO} than in COX7C^{MKO} (Figure 3.17D).

We can conclude that ISR activation happens in different timepoints in COX10^{MKO} and COX7C^{MKO}. COX7C^{MKO} mice develop CIV deficiency and activate ISR during differentiation of cardiomyocytes, in postnatal heart development, while COX10^{MKO} mice develop the same CIV deficiency phenotype in adult and differentiated hearts. Further on, while COX7C^{MKO} show involvement of CEBPB in ISR response, COX10^{MKO} show canonical ATF4 dependent ISR. Analysis on COX10^{MKO} hearts was performed by Lea Isermann.



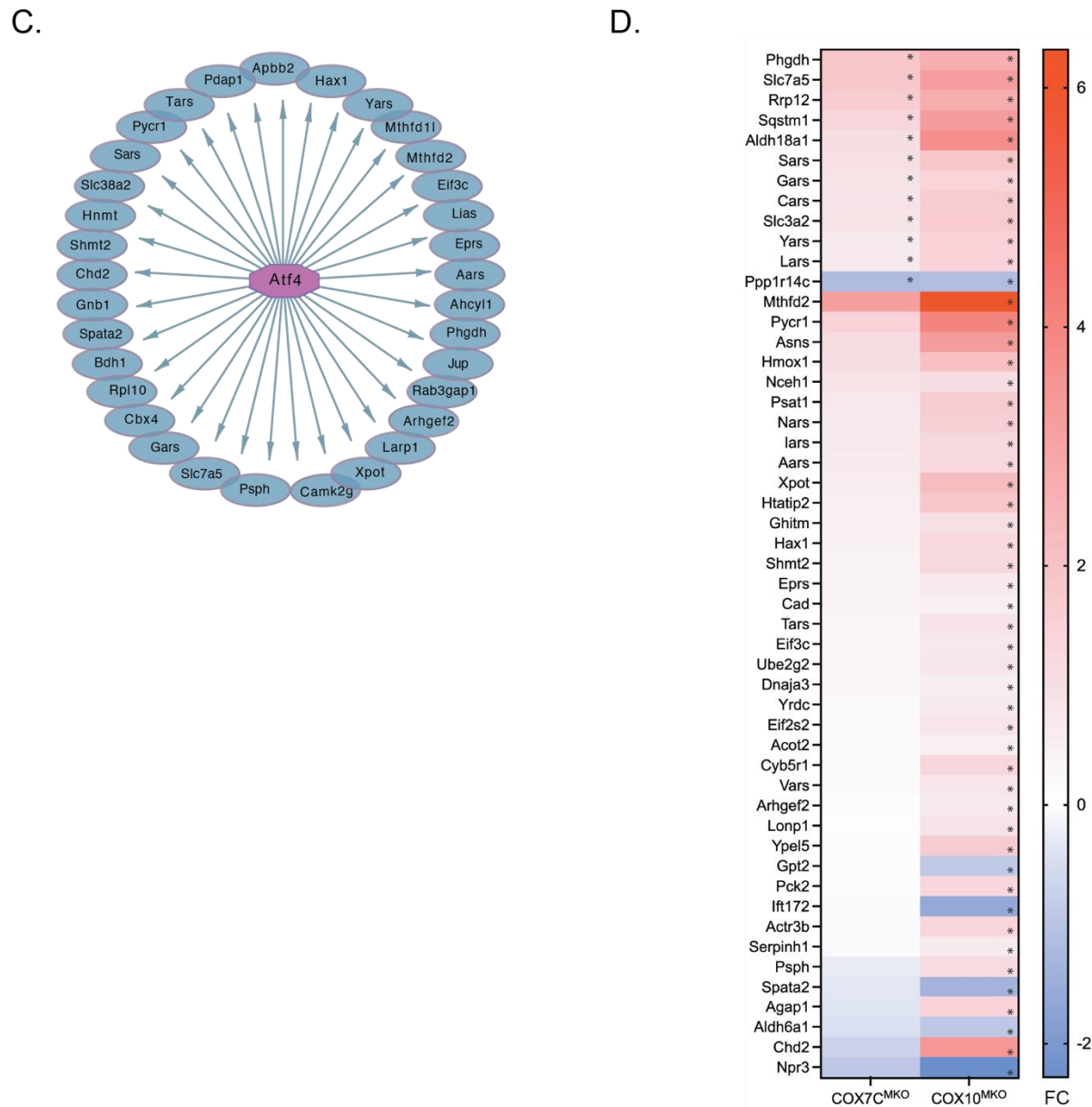


Figure 3.17. ISR activation differs between COX7C^{MKO} and COX10^{MKO}. **A.** Steady state protein levels of ISR targets in COX7C^{MKO} and COX10^{MKO} at p10 and 3 weeks of age, respectively; **B.** Steady state protein levels of ISR targets in COX7C and COX10 mice at p10; **C.** iRegulon analysis of COX10 proteomics at 3 weeks; **D.** Fold change (FC) ATF4 targets in COX7C^{MKO} and COX10^{MKO} at p10 and 3 weeks. Significantly changed proteins labeled with * (Student's t-test, q-val < 0,05)

3.4 Heart metabolome changes upon loss of COX7C

Since both mouse models develop essentially the same CIV deficiency but in different developmental stages of the heart, we proceeded to investigate metabolic changes between the two models. To try to narrow down to differences that might be driving the change in life expectancy and phenotype differences, we perform an in-depth analysis of the metabolic profile of the two models.

To describe metabolic changes in COX7C^{MKO}, we looked at known master regulators of metabolic rewiring upon cardiomyopathy. Western blot analysis shows a significant increase of AMPK phosphorylation (Figure 3.18A). Since AMPK responds to a change in energy status of the cell, we looked at changes to fatty acid oxidation regulators on transcript level, and we could identify a general decrease of *Ppar-a*, *Ppar-g* and *Cd36* mRNA (Figure 3.18B). In our proteomics data, we could identify an increase of peroxisomal fatty acid oxidation. Additionally, we identified changes in known regulators of heart glucose metabolism, and among upregulated proteins phosphofructokinase 2 (PFK2), a target of AMPK (Figure 3.18C)

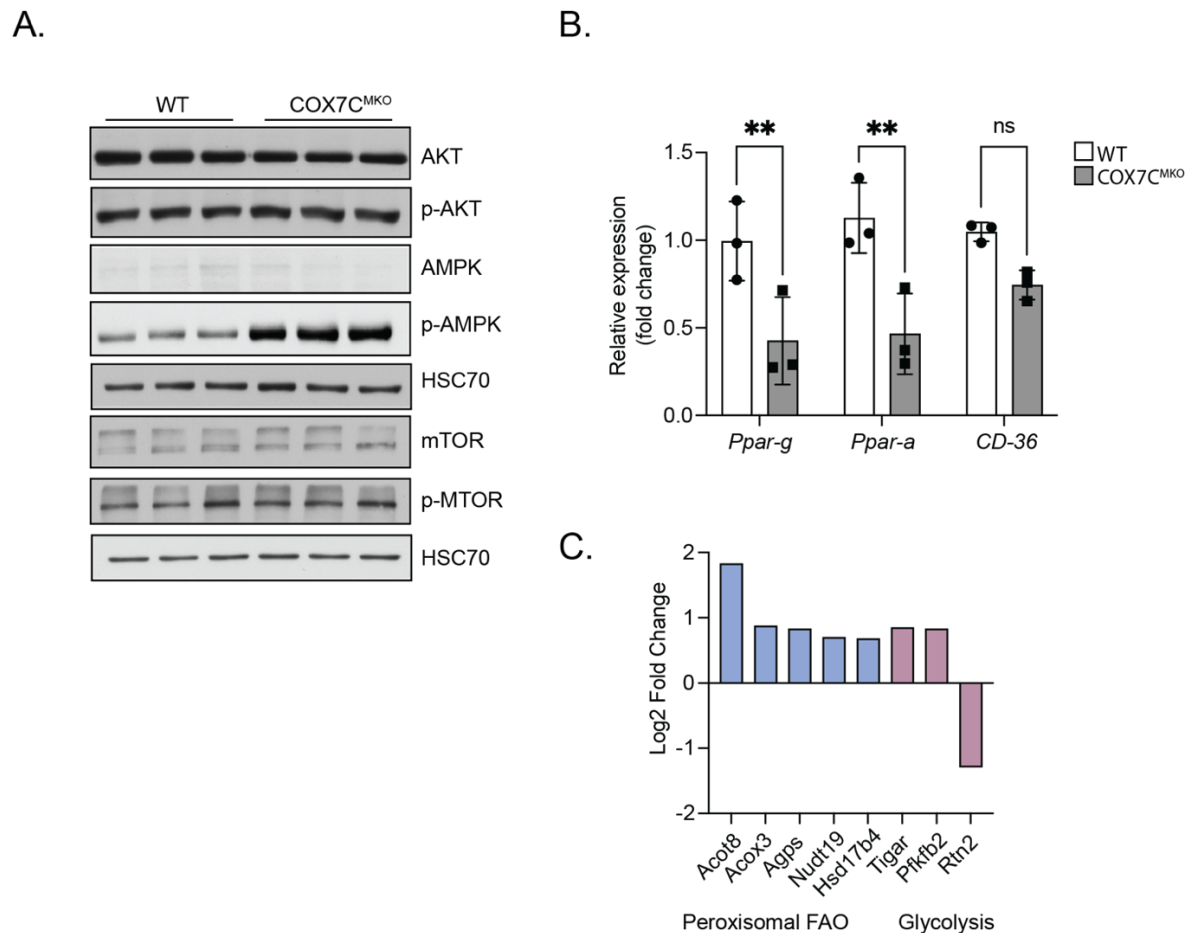


Figure 3.18. Changes in energetic status of COX7C mice is regulated by AMPK. **A.** Western Blot for phosphorylation status of AKT, AMPK and mTOR; **B.** qPCR for PPAR-g, PPAR-a and CD36 (n=3, (Student's t-test, p-val < 0,05); **C.** Proteomics data on significantly changed proteins of peroxisomal fatty acid oxidation (FAO) and glycolysis

To confirm changes on protein levels for metabolic pathways and regulators (Figure 3.13, Figure 3.18) we performed metabolomics analysis of COX7C hearts to further describe these changes. Out of 76 significantly changed metabolites, we found increase in 42 metabolites, while 34 are depleted (Figure 3.19A).

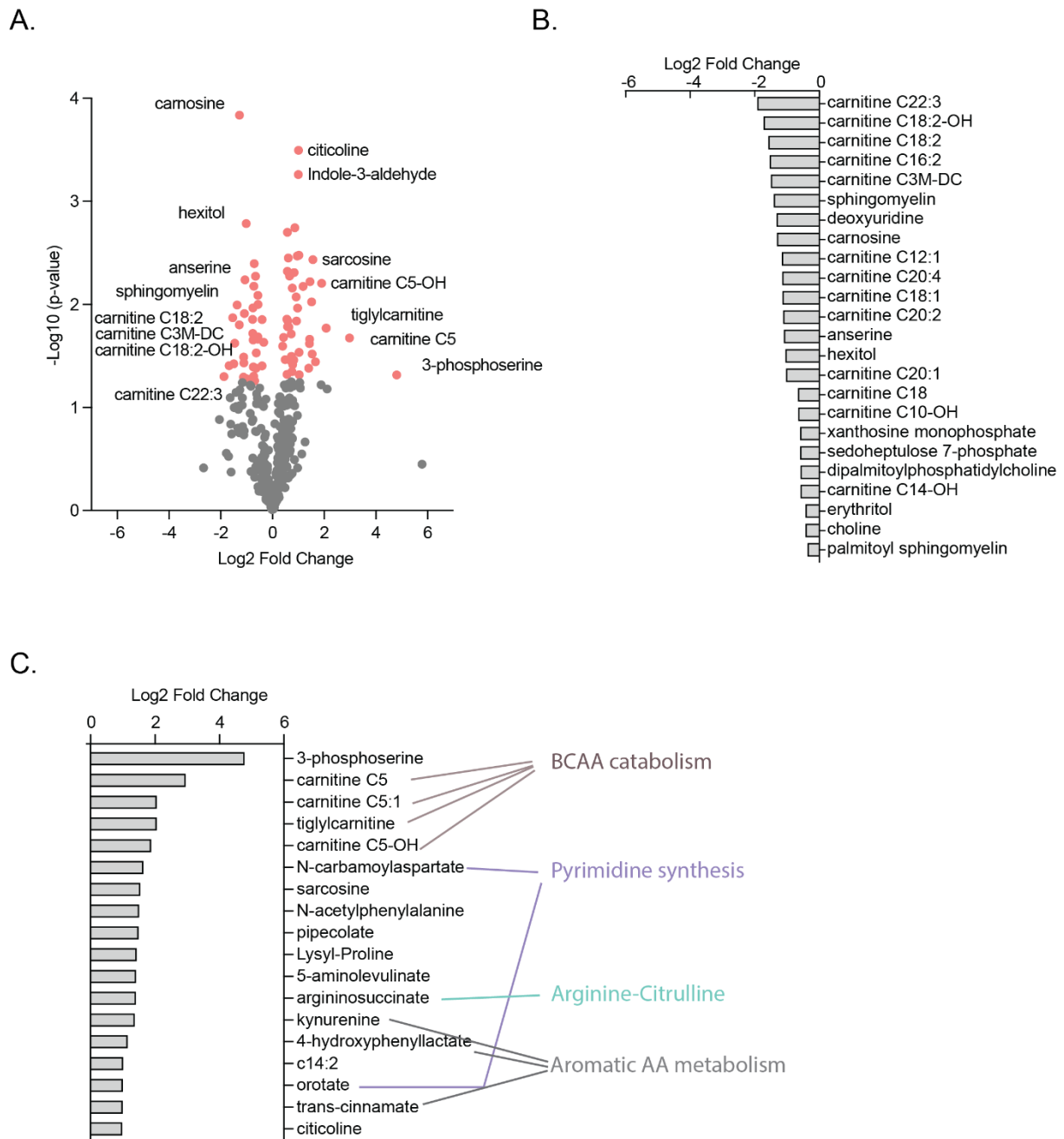


Figure 3.19. Metabolomics of COX7C^{MKO} hearts. **A.** Volcano plot showing significantly changed metabolites (in red); **B.** Fold change for most depleted metabolites in COX7C^{MKO} hearts; **C.** Fold change for most accumulated metabolites in COX7C^{MKO} hearts and the metabolic pathways they belong to.

Among depleted metabolites we found long and middle-chain acyl-carnitines, carnosine, and components of sphingolipid metabolism (Figure 3.19B). We identify accumulation of 3-P-Serine, metabolites of BCAA catabolism together with metabolites of pyrimidine synthesis, amino acid metabolism and urea cycle (Figure 3.19C).

3.4.1 Changes in BCAA metabolism in COX7C^{MKO} mice

One of the most prominent changes on our metabolomics data is the accumulation of C5 carnitines (Figure 3.19). C5 carnitines are a side product of incomplete BCAA metabolism, caused by inhibition of key metabolic enzymes (Xiao and Guo, 2022, Li et al., 2017). Other than a strong accumulation of BCAA metabolism side products, we see changes on protein level for key regulators of BCAA metabolism; PPM1K is among most downregulated proteins in our dataset (Figure 3.20A). Amino acid transporters for BCAA are also upregulated in our dataset (Figure 3.20, Figure 13A). Changes in BCAA metabolism are summarized in Figure 3.20B; PPM1K is downregulated, indicating that BCKDH is phosphorylated and inhibited. So far, inhibition of BCKDH has been implicated in metabolic remodeling to provide glutamate and aspartate for nucleotide synthesis (Yang et al., 2022, Sciacovelli et al., 2022).

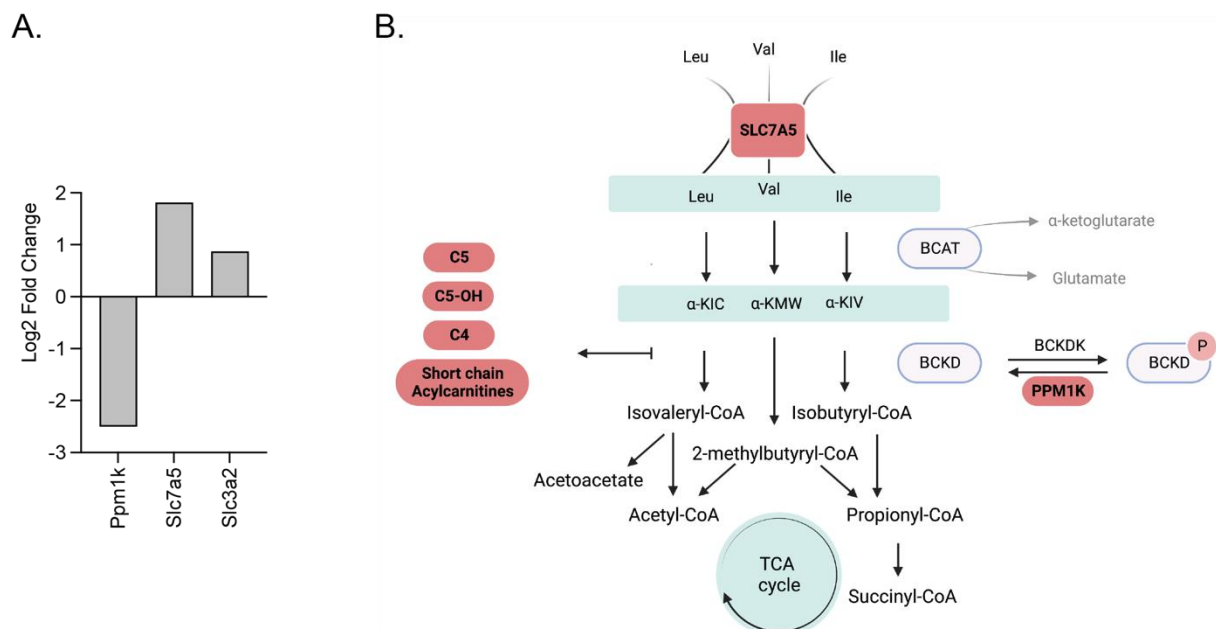


Figure 3.20. BCAA metabolism in COX7C mice. **A.** Proteins identified on proteomics analysis as significantly changed and are components of BCAA metabolism; **B.** Diagram of BCAA metabolism, with significantly changed proteins and metabolites labeled in red.

3.4.2 Pyrimidine synthesis in COX7C mice

Since both remodeling of 1C metabolism (Ben-Sahra et al., 2016) and changes to BCAA metabolism (Sciacovelli et al., 2022) have been implicated in nucleotide synthesis pathways, we further investigated changes to nucleotide metabolism in COX7C^{MKO} hearts at P10. We saw accumulation of metabolites for nucleotide synthesis, specifically pyrimidine metabolism (Figure 3.21A). Orotate, which is a component of de-novo pyrimidine synthesis, seems to be accumulated in COX7C^{MKO} (Figure 3.21B).

However, we also see accumulation of metabolites involved in salvage pathway of nucleotide synthesis (Figure 3.21B). Additionally, enzymes involved in salvage

pathway for pyrimidine synthesis show upregulation on proteomics data (Figure 3.21B, Figure 3.13). Even though we see orotate accumulation, at the same time we see an increase of salvage pathway for pyrimidine synthesis, indicating that orotate transport into cytosol might be affected. However, changes to nucleotide metabolism are evident in COX7C^{MKO}, although further flux analysis would be necessary to conclude on contribution from 1C and BCAA metabolism.

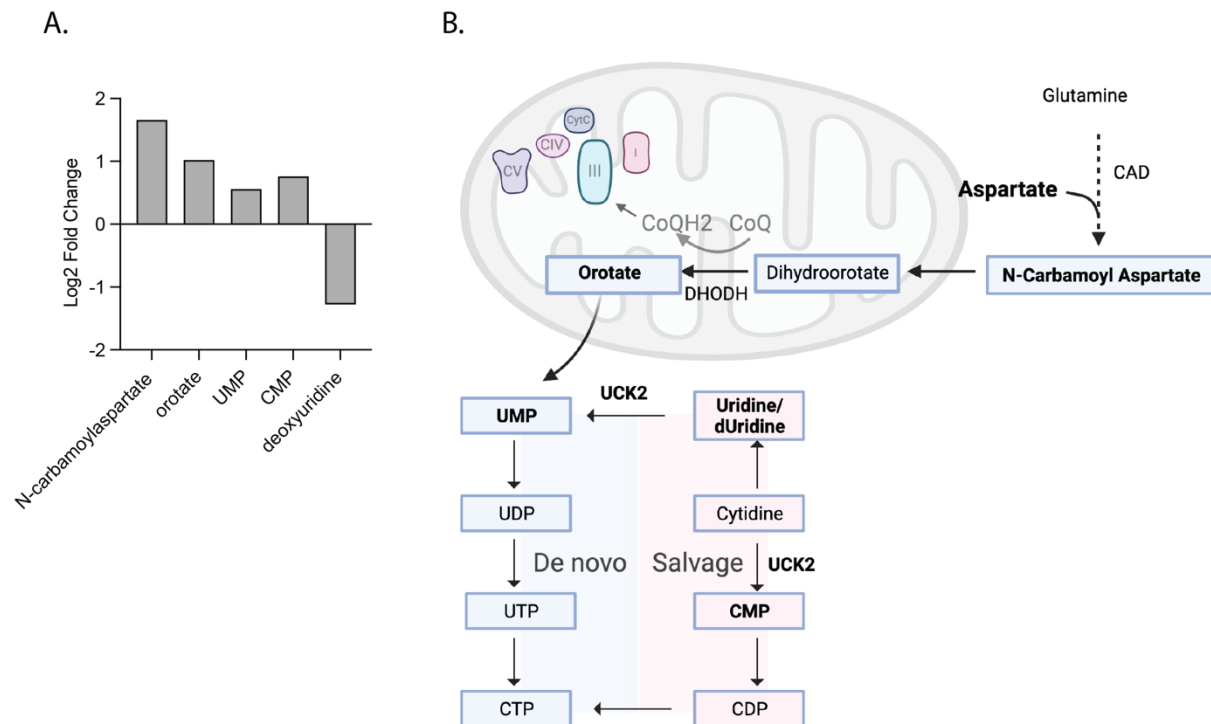


Figure 3.21. Pyrimidine synthesis in COX7C hearts. **A.** Fold changes for metabolites of pyrimidine metabolism identified; **B.** Diagram of pyrimidine metabolism, accumulated metabolites and upregulated proteins shown in bold.

3.4.3 Changes in COX10^{MKO} heart metabolism

Consistent with changes to the proteome, COX10^{MKO} metabolome remodeling shows more changes than COX7C^{MKO}, with 206 metabolites changed (Figure 3.22A).

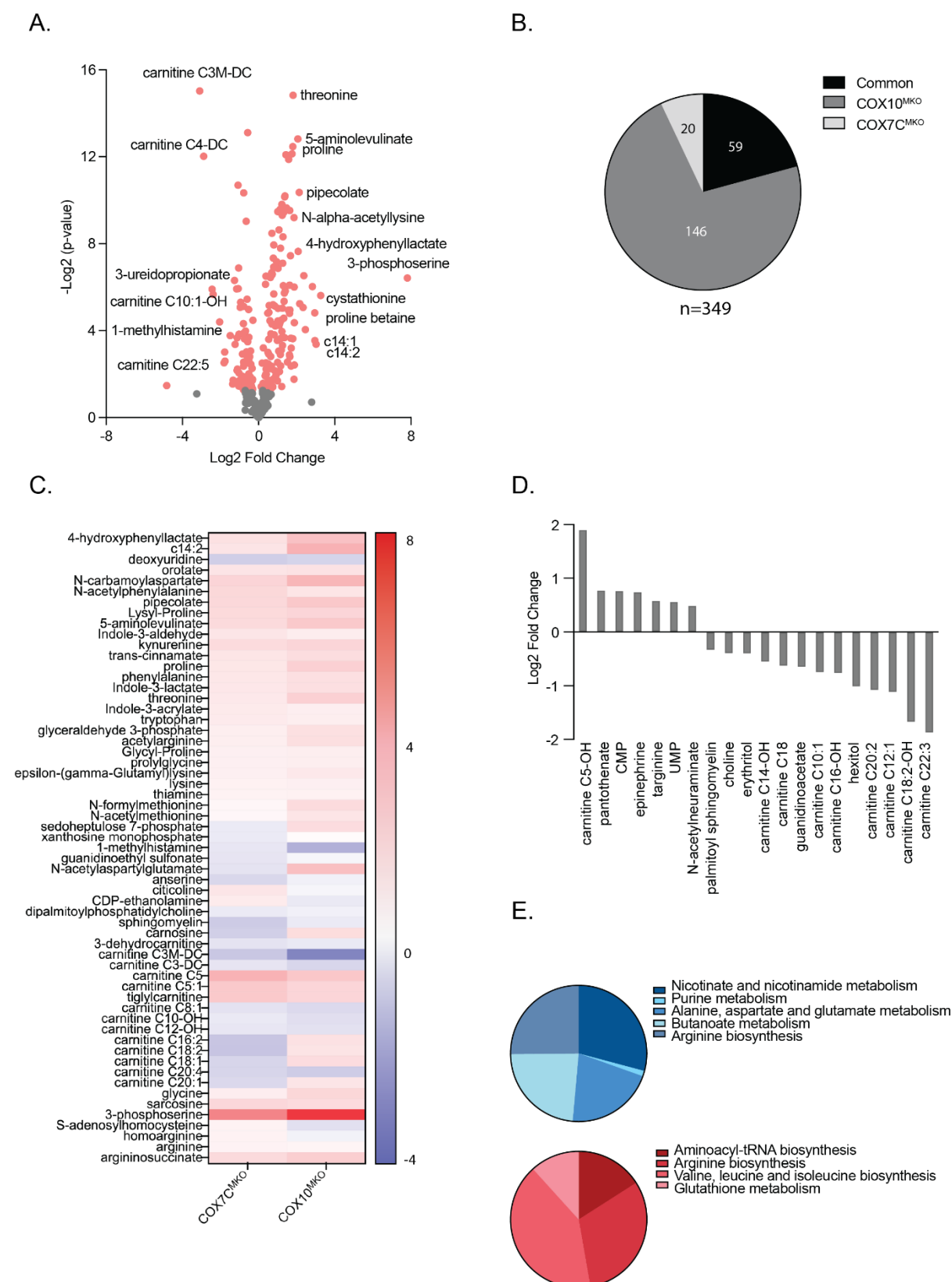


Figure 3.22. Comparison of metabolic profiles of COX7C and COX10 mice. **A.** Volcano plot of metabolites identified for COX10 mice hearts. Significantly changed metabolites labeled in red; **B.** Pie chart showing common metabolites between COX7C and COX10 mice; **C.** Log2 Fold changes for metabolites changed in both COX7C and COX10 mice; **D.** Log2 Fold change for metabolites changed only in COX7C^{MKO} **E.** MetaboAnalyst enrichment analysis of COX10 specific metabolites as a pie chart.

We could identify 59 common metabolites between the two models (Figure 3.22B). While 20 metabolites were specifically changed in COX7C^{MKO}, in COX10^{MKO} we saw 146 metabolites specifically changed (Figure 3.22B). Commonly changed metabolites are components of pyrimidine metabolism, BCAA catabolism intermediates, tryptophane, serine, proline, and urea cycle metabolites (Figure 3.22C).

In both models, 3-P-serine shows the strongest accumulation (Figure 3.22C). Most of the metabolites show the same direction of change, except for mid-chain acyl carnitines, which are depleted in COX7C^{MKO}, while accumulated in COX10^{MKO}, and components of Kennedy pathway (CDP- ethanolamine, citicoline: phospholipid synthesis). Additionally, S-adenosylhomocysteine (metabolite of methionine cycle) seems to be accumulating in COX7C^{MKO} but is depleted in COX10^{MKO} (Figure 3.22C).

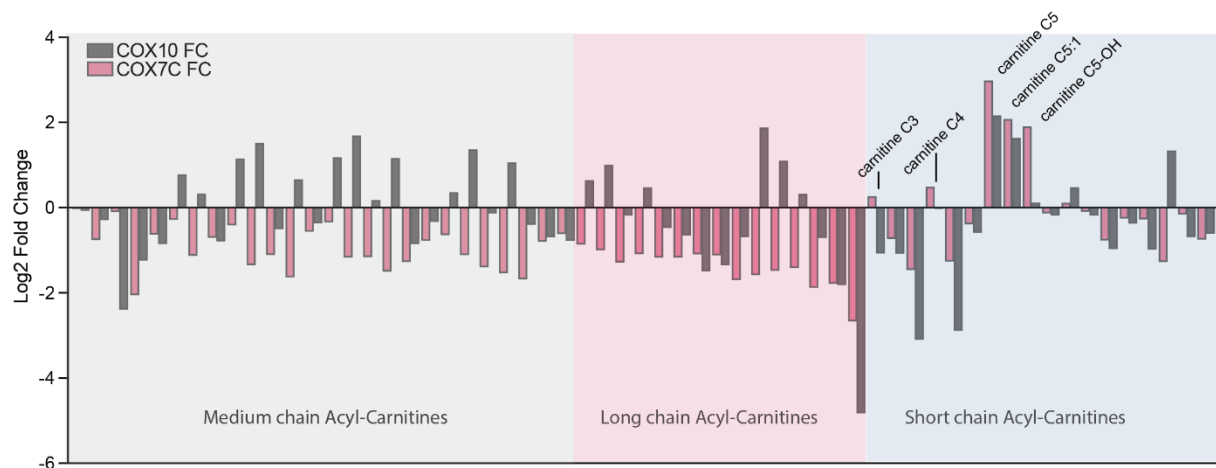
Metabolites changed only in COX7C^{MKO} include pantothenate, a committed step in CoA biosynthesis, carnitine C5-OH, pyrimidine nucleotides CMP and UMP, methylarginine which is a NOS inhibitor that feeds into arginine-citrulline cycle. Depleted metabolites are from the Kennedy pathway and mid- to long-chain acyl carnitines (Figure 3.22D).

For 146 metabolites specifically changed only in COX10^{MKO} we performed an enrichment analysis using MetaboAnalyst. Accumulated metabolites belong to glutathione metabolism, aminoacyl - tRNA biosynthesis and BCAA biosynthesis (Figure 3.22E, lower panel), while depleted metabolites belong to NAD metabolism, purine metabolism and amino acid metabolism, specifically alanine, aspartate, and glutamate (Figure 3.22E, upper panel), all playing key roles in anaplerotic pathways.

Taking a closer look into lipid metabolism, we investigated acyl-carnitine composition. Comparison between two models shows that only commonly accumulated acyl carnitines are C5 carnitines, products of incomplete BCAA catabolism (Figure 3.23A). Only exception is C5-OH, present in high amount only in COX7C^{MKO} (Figure 3.23A). While these are the only acyl-carnitines accumulated in COX7C^{MKO} and the rest are depleted, COX10 mice show accumulation of some mid-chain and long-chain acyl carnitines (Figure 3.23A).

Enzymes involved in fatty acid synthesis show upregulation in our proteomics data for COX10^{MKO}, while fatty acid oxidation enzymes are downregulated (Figure 3.23B). COX7C^{MKO} mice do not show change in any enzymes involved in mitochondrial FAS or FAO but do show changes in peroxisomal FAO (Figure 3.23B), indicating a significant difference in metabolic adaptations between two models.

A.



B.

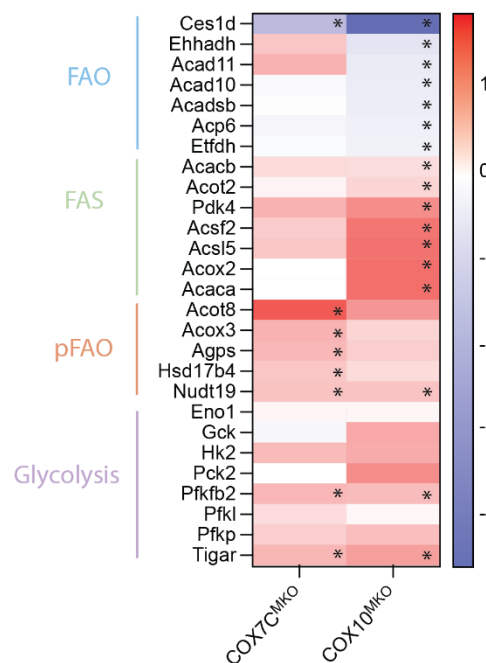
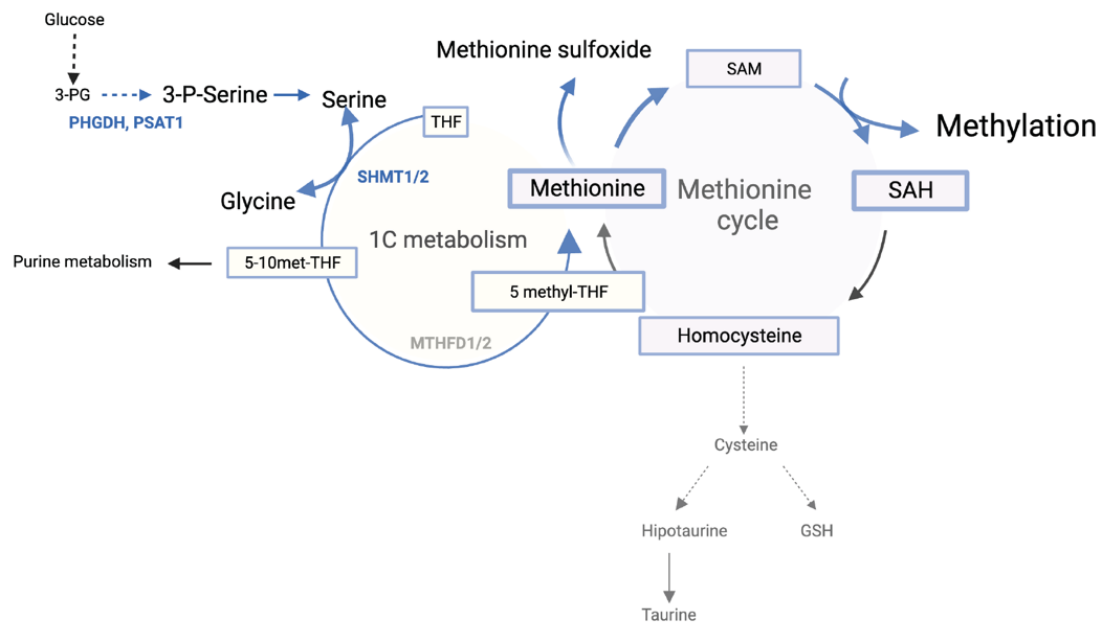


Figure 3.23. Fatty acid metabolism differs between COX7C^{MKO} and COX10^{MKO} mice. **A.** Acyl-carnitine profile for COX7C^{MKO} and COX10 mice, shown as log2 fold changes identified in metabolomics analysis; **B.** Proteins involved in fatty acid oxidation (FAO), fatty acid synthesis (FAS), peroxisomal fatty acid metabolism (pFAO), and glycolysis and their fold changes in COX7C^{MKO} and COX10^{MKO} mice, as identified in proteomics analysis.

Common and most striking change between COX7C^{MKO} and COX10^{MKO} mice is the accumulation of 3-P-Serine. 3-P-Serine is a product of rewiring of glycolysis for serine synthesis. We investigated how serine synthesis might be utilized for downstream metabolic processes in the two mouse lines.

A.



B.

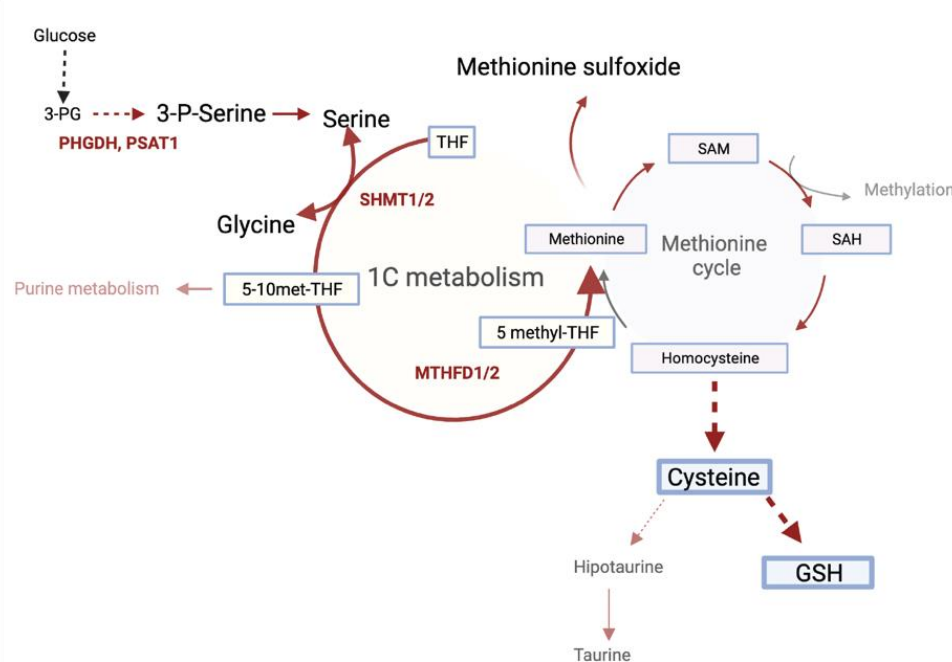


Figure 3.24. Schematic representation of ISR metabolic remodeling. **A.** $COX7C^{MKO}$ and **B.** $COX10^{MKO}$

While both mouse lines show activation of 1C metabolism and methionine cycle because of ISR activation, and both show accumulation of amino acids serine and glycine and key enzymes of their biosynthesis from glucose, in $COX7C^{MKO}$ mice we see an increase in SAH, while in $COX10^{MKO}$ mice we do not. Conversely, in $COX10^{MKO}$ mice we see downstream cysteine and GSH to be accumulated indicating an activation of antioxidant response, not present in $COX7C^{MKO}$ mice.

3.5 OXPHOS in postnatal heart development

To properly elucidate role of OXPHOS in postnatal heart development we performed in depth multi-omics analysis of postnatal heart development. Primarily, we focused on describing structural changes in all OXPHOS complexes during postnatal heart development. Heart postnatal development in mice lasts until cardiomyocytes are fully differentiated and exit mitotic cell cycle, which is until postnatal day 7. For this reason, we chose to investigate multiple timepoints in WT mice, from embryonic day 18.5 to p10. To describe changes in OXPHOS complexes, we performed complexome profiling analysis in a time-dependent manner (Figure 3.25.). We could identify all structures reported already for digitonin separated OXPHOS complexes (Vercellino and Sazanov, 2022).

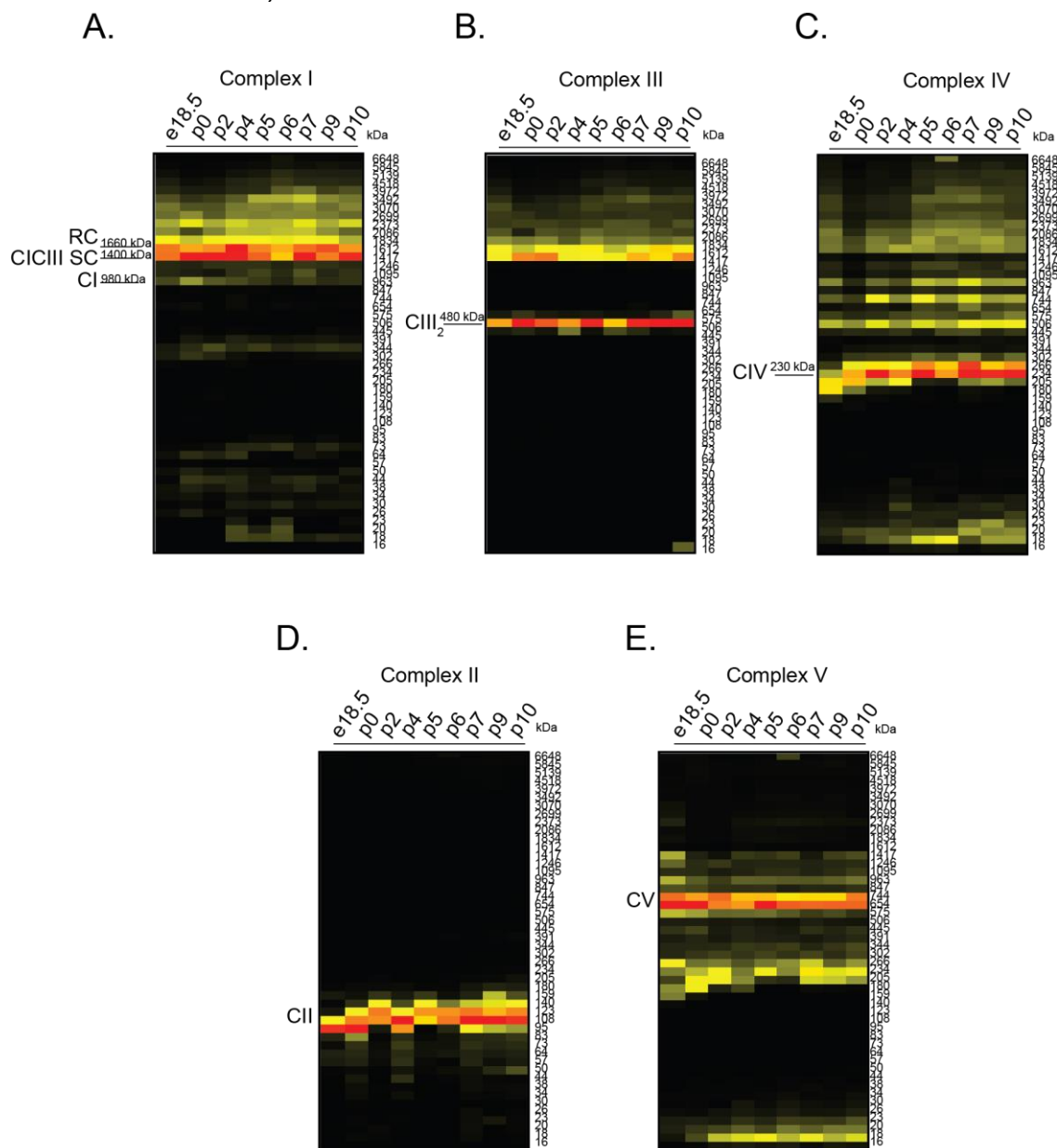
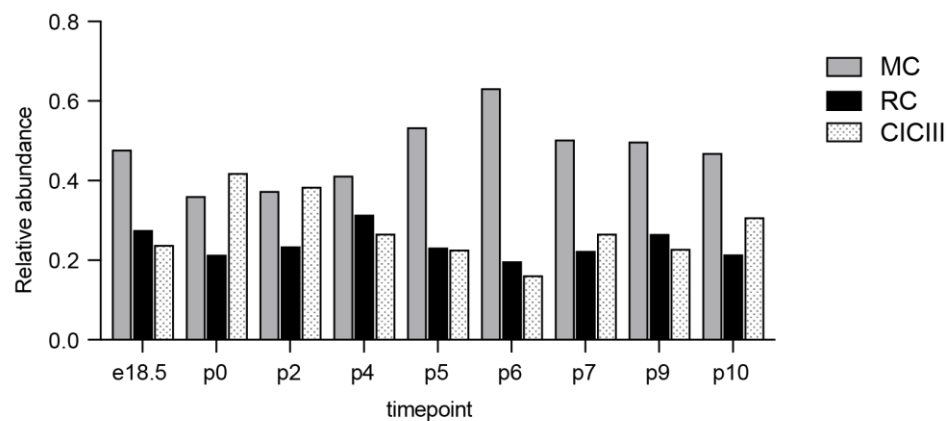


Figure 3.25. Time resolved complexome profiling of the heart during postnatal development. **A.** Heatmap of mean relative abundance for each complex at indicated timepoints during

postnatal development: E- embryonic day; P- postnatal day; **B.** Relative abundance peaks for each complex at indicated timepoints.

Complex I, identified as a monomer and as a component of supercomplexes, shows highest abundance within CICIII2 SC structure in all timepoints (Figure 3.25A). CIII, another SC- forming complex, shows highest abundance as a dimer, followed closely with its contribution to CICIII2 SC (Figure 3.25B). Most reports on ETC complexes during heart development have been on CIV, since CIV has tissue specific and developmentally regulated isoforms for some of its nuclear encoded subunits. We could detect at least six different structures containing CIV, from which monomeric structure is most abundant in all timepoints (Figure 3.25C). CIV monomeric structure shows a shift in size in the earliest timepoints, from E18.5 to p4. CII is only ever present as a monomer in all timepoints and shows a shift in size from E18.5 to P2 (Figure 3.25D). CV shows two peaks, one corresponding to fully assembled CIV at around 600-700kDa, and another one at 230kDa (Figure 3.25E). The structure of 230kDa seems to change dynamically as well in the earliest timepoints, E18.5 to P2.

A.



B.

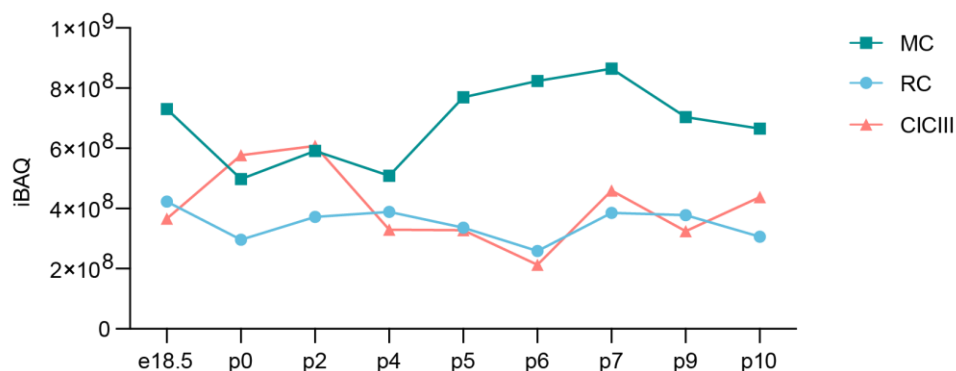


Figure 3.26. Dynamics of supercomplex structures during postnatal heart development. **A.** Bar graph of relative abundance for each SC structure: MC – megacomplexes containing CI,

CIII, CIV at molecular weight range from 1.8MDa to 3.4 MDa; RC – respirasome, containing CII₂CIV at 1.7MDa; CII₂ supercomplex at 1.4 MDa; **B.** iBAQ values for SC structures as defined for A., shown as a timeline graph.

We also analyzed how supercomplex structures change during development. We define three supramolecular structures that we follow: RC - respirasome, containing CII₂CIV, MC - mega complexes also containing CI, CIII and CIV but in higher stoichiometry, and CII₂ structure (Figure 3.26). While the respirasome does not fluctuate much in abundance, MC and CII₂ do change in inverse correlation (Figure 3.26A). Mega complexes seem to be highest in abundance from P5, while around P0 and P2 their abundance is the lowest. At those timepoints, CII₂ shows its highest peak, while it decreases post P5 (Figure 3.26A). Most dynamic period is right after birth until P5, after which abundance of each structure seems to stabilize.

3.5.1 Changes in heart OXPHOS complexes at birth

To further investigate dynamic changes in OXPHOS, we analyzed structures of individual complexes at early timepoints. CII shows a shift in size and abundance from P0 to P2 (Figure 3.27A). Further on, we could detect a shift in size for free CIV from E18.5 to P0, and another shift and increase in abundance at P2 (Figure 3.27B). For CV, we do not see change in fully assembled ATP synthase, but a shift in size for structure of 230kDa from E18.5 to P2 (Figure 3.27A). While CII, CIV and CV show dynamic changes size of detected structures during early timepoints in postnatal heart development (Figure 3.27A), for CI and CIII we could not detect any changes in size or any stable subcomplexes or assembly intermediates in the early timepoints (Figure 3.25B). This indicates that CII, CIV and CV composition, structure or assembly are regulated at birth.

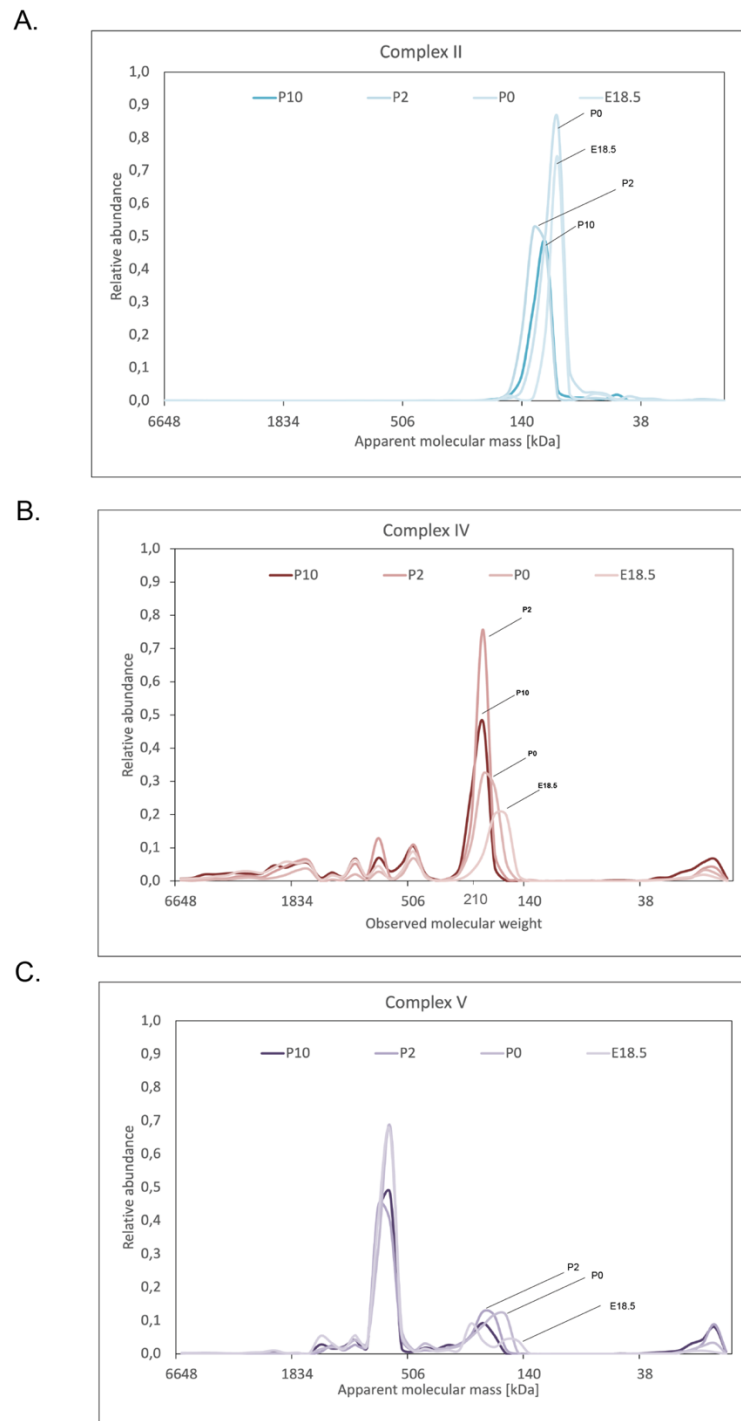


Figure 3.27. OXPHOS changes in the heart at birth. Shift in intensity peaks corresponding to a change in size and migration for A. CII, B. CIV and C. CV at E18.5, P0, P2 and at P10;

As mentioned, we could detect the following structures for CIV in our complexomics data: CIV monomer, migrating at 230kDa, CIV₂ at size of 500kDa, additional multimer structures of CIV at 750kDa and 1000kDa, RC at 1.6MDa and higher molecular weight megacomplexes (Figure 3.28A). For CIV monomer we detected a shift in size from E18.5, where we see a signal at 180-205kDa, to p2 where CIV migrates to its fully assembled size of 230kDa (Figure 3.28B). Highest abundance of CIV monomer is also detected at p2. To simplify the dynamics of CIV migration pattern through time, we define three structures that we follow at all timepoints: CIV – monomeric structure of

CIV, SC – supercomplex structures containing CIV, Sub CIV – lower molecular weight structures containing CIV subunits, i.e., not fully assembled CIV. At E.18.5 we observe a similar abundance for all three defined structures (Figure 3.28C)

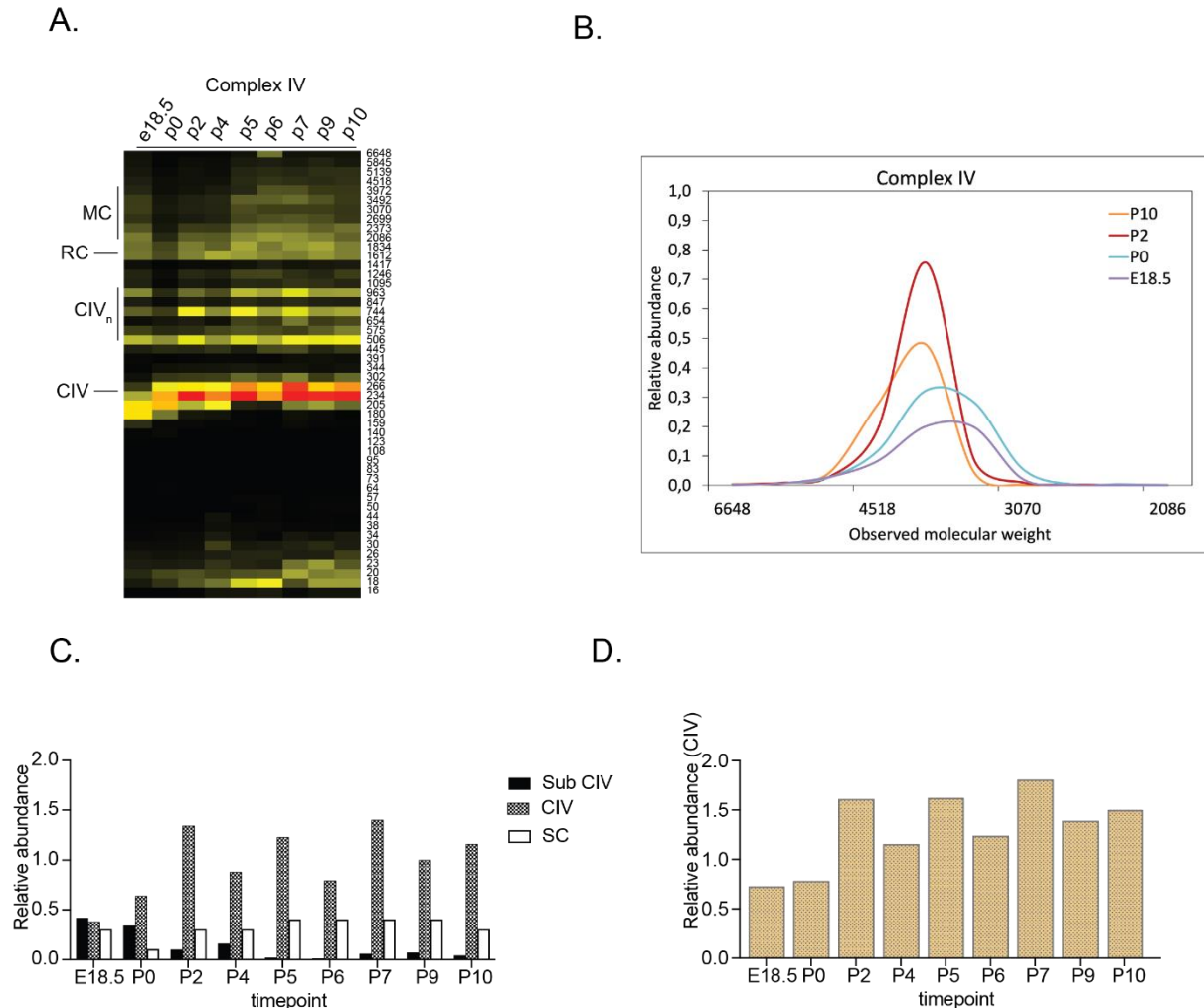


Figure 3.28. Dynamic changes in CIV - containing structures of the heart at birth. **A.** Time resolved heatmap of CIV mean relative abundance with indicated CIV structures; **B.** Line graphs for relative abundance peak for monomer CIV structure at indicated timepoints; **C.** Relative abundance of each CIV containing structure; SC – 1.6MDa, CIV – 230kDa, Sub CIV – 140 to 230 kDa; **D.** Relative abundance for sum of all CIV structures within a timepoint.

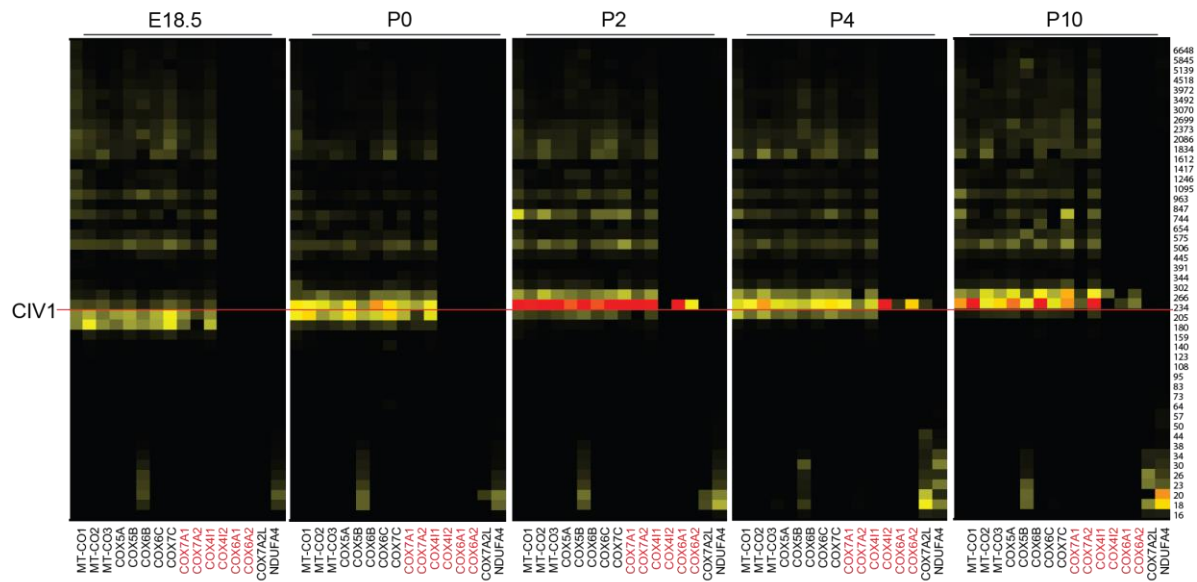
At P0 we see an increase in fully assembled CIV with concurrent decrease of SC structures. At P2 we see a peak in fully assembled CIV monomer, that maintains the highest abundance out of all three structures up until P10 (Figure 3.28C). Whole levels of CIV also strongly increase at P2 (Figure 3.28D). Since we see a change in size for free CIV, we can conclude that the structure and composition of free CIV seems to be regulated at birth.

3.5.2 Developmentally regulated isoforms responsible for CIV shift

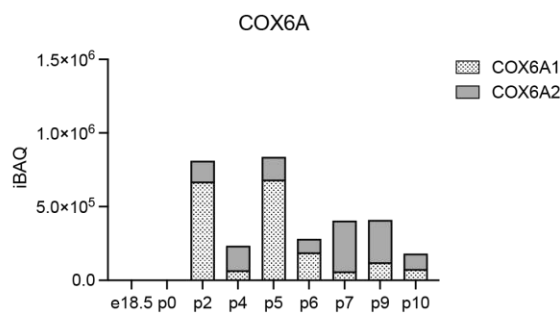
Developmentally regulated isoforms for CIV have been implicated in modulating activity of CIV. Gradual switch in ubiquitously expressed subunits of COX6A and COX7A subunits has been investigated in muscle and heart before, but never in detail

in the context of postnatal heart development. Pivotal role of CIV in mitochondrial bioenergetics prompted us to investigate how developmentally switched subunits contribute to the CIV dynamics.

A.



B.



C.

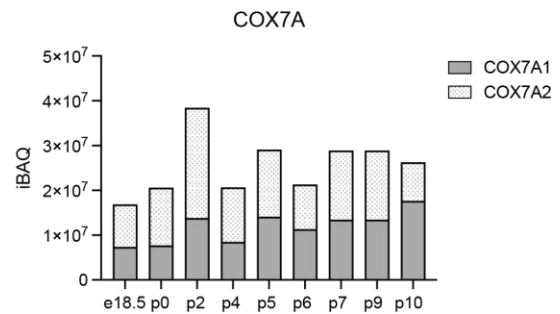


Figure 3.29. Developmentally regulated subunit COX6A is responsible for CIV shift in size. A. Heatmap of relative abundance for CIV subunits at indicated timepoints. Subunits with isoforms labeled in red font; B. iBAQ values for COX6A isoforms, checkered – embryonal isoform, dark grey – adult isoform; C. iBAQ values for COX7A isoforms, checkered – embryonal isoform, dark grey – adult isoform.

Resolving further the structure and composition changes in CIV that happen at birth, we analyzed all subunits identified for CIV, including developmentally regulated isoforms (Figure 3.29A). We could confirm that at E18.5 and P0 the nuclear encoded subunit COX6A nor its isoform can be detected in any CIV structures (Figure 3.29A). However, at P2 we detect both isoforms, coinciding with appearance of full-size free CIV. Further on, COX6A isoforms show a clear switch from COX6A1, prevalent at P2, to COX6A2, prevalent from P7 (Figure 3.29.B). Other structures of CIV (RC and CIV_n) do not show a change in size and migration pattern and we did not detect this subunit in any of these structures (Figure 3.29A). However, for COX7A our data shows that

both isoforms can be detected throughout postnatal development in all structures (Figure 3.29C).

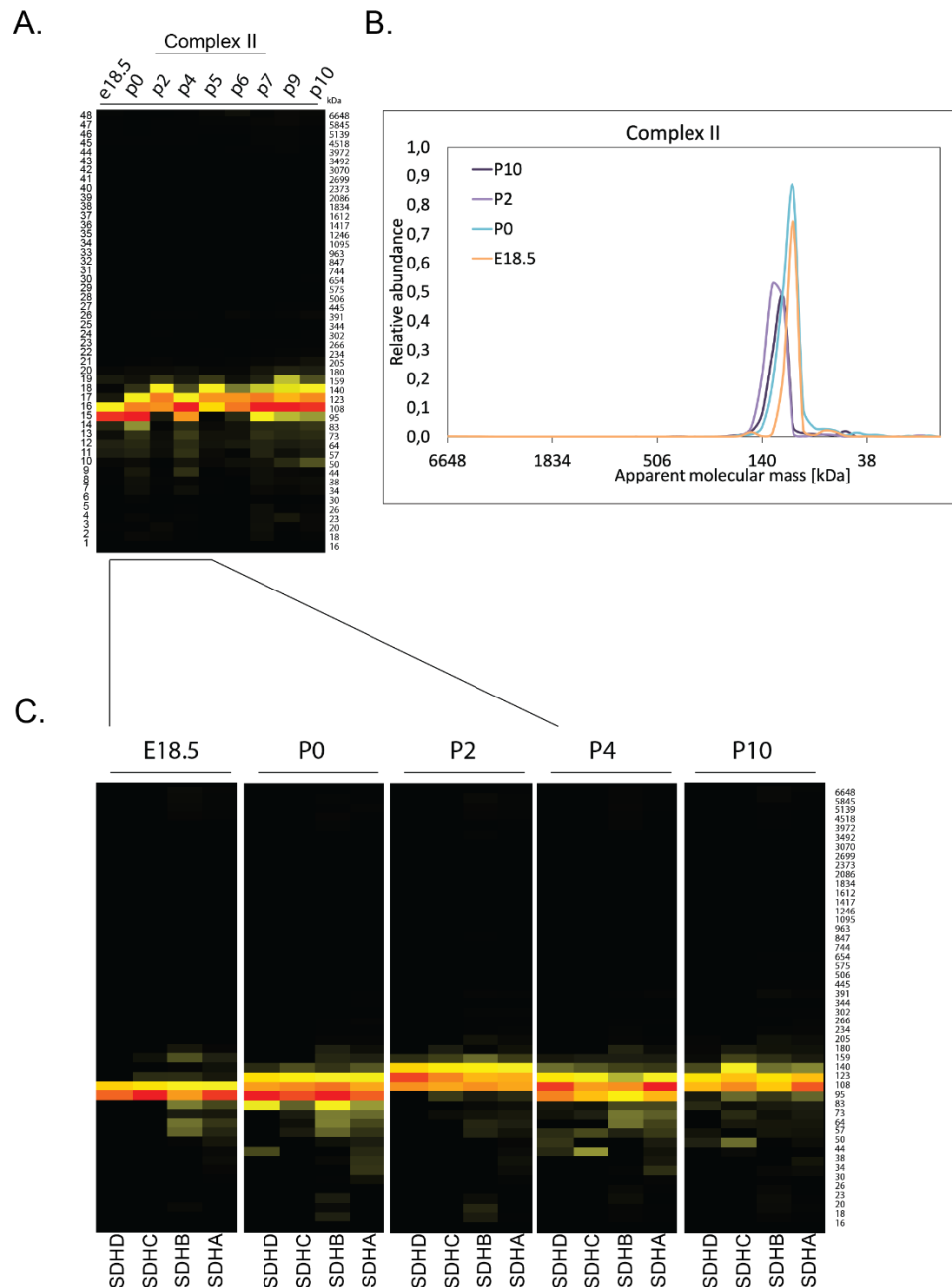


Figure 3.30. Complex II dynamics during early postnatal heart development. A. Time resolved heatmap of CII mean relative abundance; B. Line graphs for relative abundance peak for CII at indicated timepoints; C. Relative abundance of each CII subunit at indicated timepoints.

CII changes in size are also detected for the earliest timepoints, and just before birth (Figure 3.30). We also detect higher abundance for CII at lower size at E18.5 and P2 than at P2 or P10 (Figure 3.30B). SDHA complex has 4 subunits, and they are all detected in all timepoints during development (Figure 3.30C), indicating that the shift in size could be a consequence of scaffold or assembly proteins, or posttranslational modifications to CII subunits.

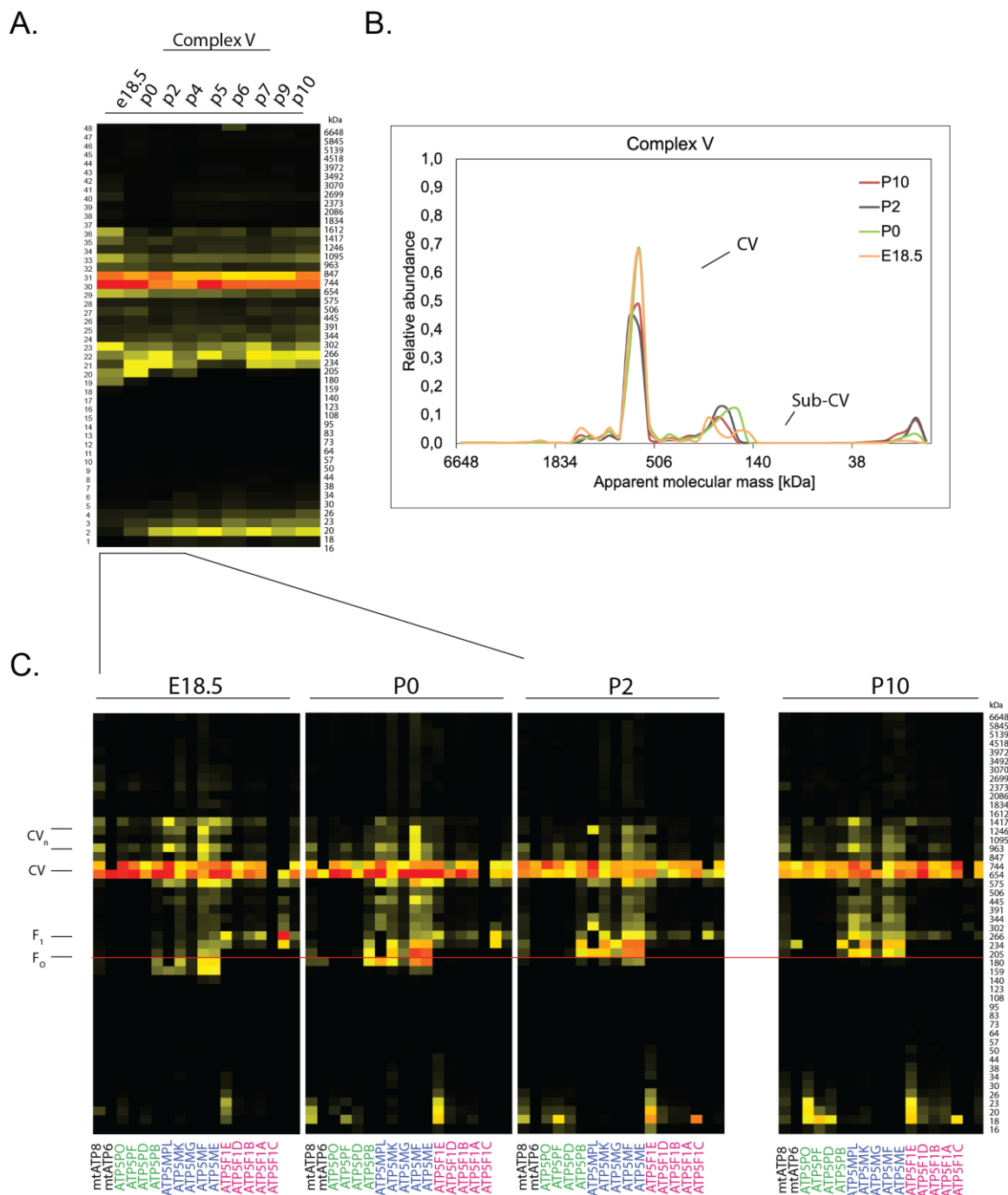


Figure 3.31. Complex V dynamics during postnatal heart development. **A.** Time-resolved heatmap of CV mean relative abundance with indicated CV structures; **B.** Line graphs for relative abundance peak for CV structures at indicated timepoints; **C.** Relative abundance of each CV subunit. Peripheral stalk subunits in green; membrane component labeled in blue; F1 component labeled in pink.

CV shows two distinct peaks in most timepoints (Figure 3.31A). Only at E18.5 we see two additional peaks, one at 200kDa and one at 266kDa (Figure 3.31B). We can also identify dimer of CV and higher CV structures (Figure 3.31C). Resolving CV complexome by subunits reveals two structures accumulated at E18.5 to be F1 and FO components (Figure 3.31C). FO component is the membrane part, and its size shift changes with incorporation of membrane subunit g (ATP5MG) at P2 (Figure 3.31C).

F1 component shows the highest peak and abundance at E18.5 (Figure 3.31B). It is present in the P0 and P2 timepoints, while at p10 its almost completely absent (Figure 3.31C).

3.6 Time-resolved metabolome of postnatal heart development

With the changes described for OXPHOS complexes CII, CIV and CV and the changes in abundance for all OXPHOS structures, we wanted to investigate corresponding changes to metabolism during development. We performed metabolomics analysis on the same timepoints as complexome profiling, to determine how changes in OXPHOS correlate to metabolic changes and maturation of cardiomyocytes.

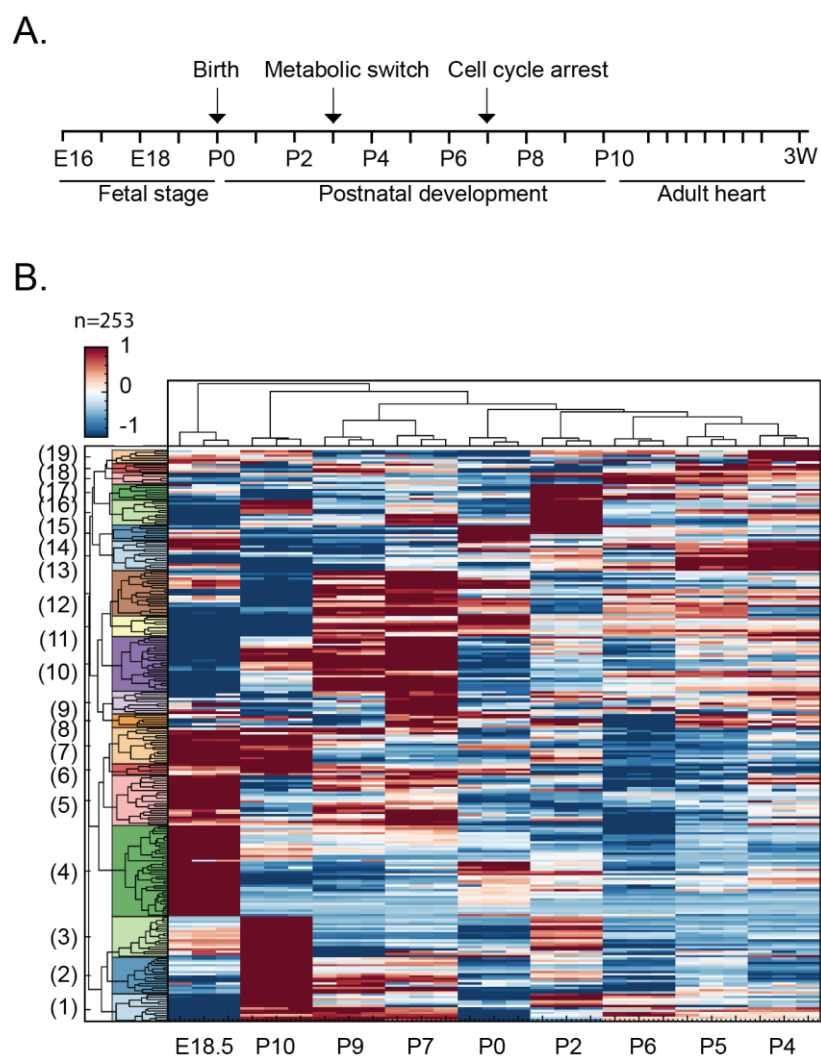


Figure 3.32. Metabolic changes during postnatal development. **A.** Timescale diagram of heart postnatal development and key events; **B.** Hierarchical clustering analysis of metabolomics time series for postnatal heart development (n=3).

We could identify 253 metabolites for 9 timepoints (Figure 3.32B). Hierarchical clustering analysis reveals 19 distinct clusters for metabolites and shows E18.5 to be most distinct in metabolic profile, followed by P10. P7 and P9 cluster together, and so

do P5 and P4, indicating very little differences in metabolic profile between these timepoints (Figure 3.32B). While E18.5 is the most distinct metabolic profile, P0 and P2 have distinct changes to cluster separately as well. For this reason, we decided to further describe changes at birth, at P2 and at final timepoint, p10.

3.6.1 Metabolic changes at birth

Since E18.5 showed a distinct metabolic profile compared to other timepoints, we analyzed changes that happen at birth by comparing P0 to E18.5. Most accumulated metabolites include carnosine and carnitines, indicating changes to fatty acid metabolism (Figure 3.33A). Most depleted metabolites after birth are kynurenine and intermediates of nucleotide synthesis such as uridine, uracil, and xanthosine (Figure 3.33A). At P0 we see most changes to nucleotide metabolism and CoA biosynthesis as they significantly decrease at birth (Figure 3.33B), indicating their importance during embryonic development. Histidine and arginine metabolism are among most upregulated metabolic pathways at birth, together with changes to fatty acid metabolism (Figure 3.33B). As we could see on our hierarchical clustering analysis, clusters 4, 5 and 7 show metabolites specifically highly accumulated at e18.5. To investigate this further, we looked at metabolites that show strongest peak at e18.5 (Figure 3.33C). Most of the metabolites confirm our enrichment analysis (Figure 3.33B), as they belong to nucleotide metabolism and aromatic amino acid metabolism.

It is well established that heart failure leads to reactivation of fetal gene expression program and leads to reversal of oxidative to glycolytic metabolism. To see how similar immature cardiomyocytes are to cardiomyopathy, we compared the proteome of COX10^{MKO} mice who developed mitochondrial cardiomyopathy, and immature cardiomyocytes. We found an overlap of 42 proteins, 172 proteins were still specific for mitochondrial cardiomyopathy, and 84 were specific for immature cardiomyocytes, indicating a significant similarity in proteomic profile (Figure 3.33D).

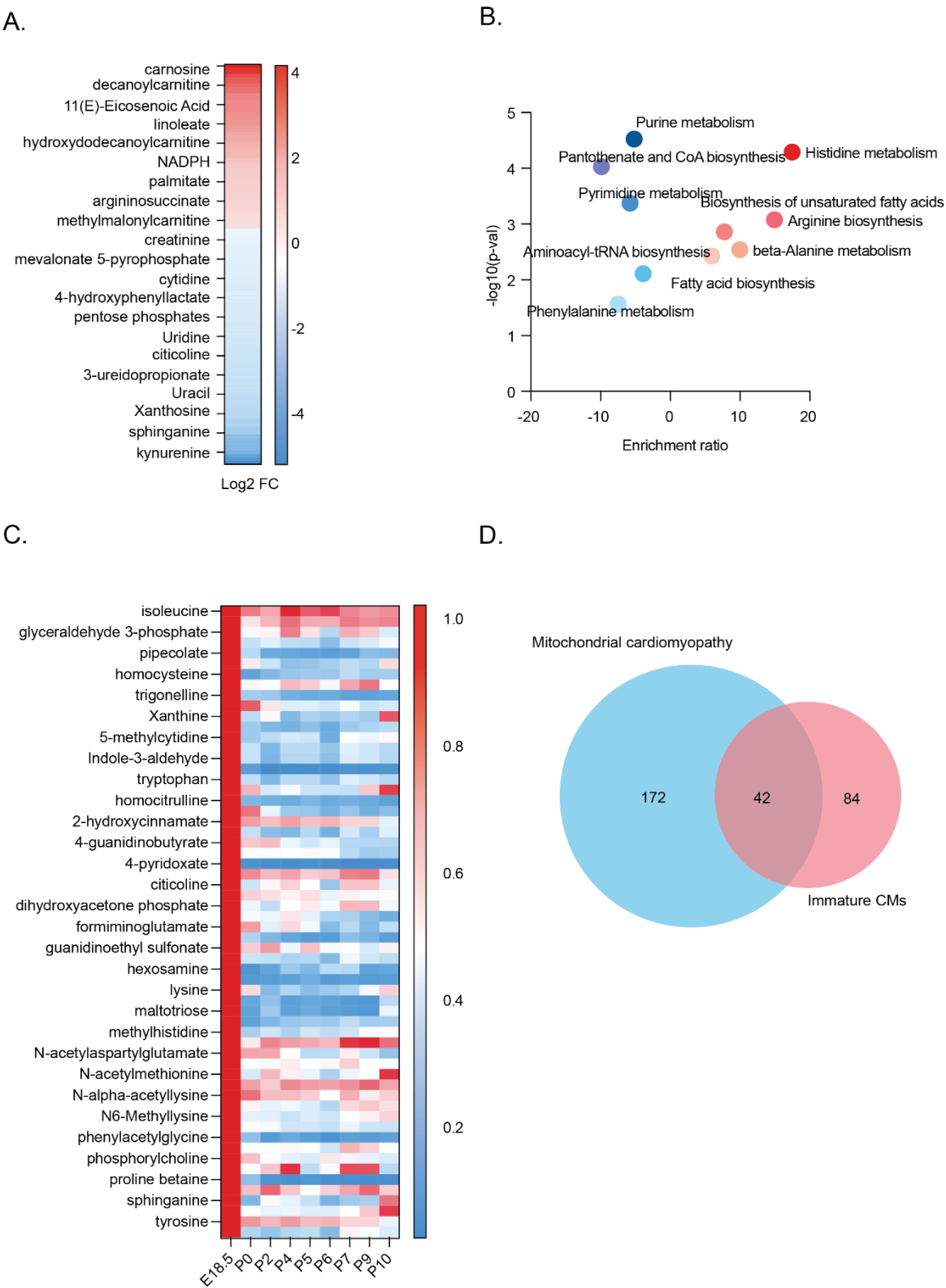


Figure 3.33. Metabolic changes at birth. **A.** Fold changes for metabolites significantly changed at birth. **B.** MetaboAnalyst enrichment analysis of metabolites significantly changed at birth (P0 to E18.5) (Student’s t-test, p-val < 0,05) **C.** Heat map of metabolites that are most abundant at E18.5. **D.** Venn diagram of common identified proteins between COX10^{MKO} and immature cardiomyocytes at P0.

3.6.2 Metabolic changes at P2

To follow what happens in the first couple days after birth and how first steps of metabolic maturation and heart growth might be regulated, we compared P2, a timepoint when we see a stabilization in OXPHOS complexes as well (Figure 3.27), to P0.

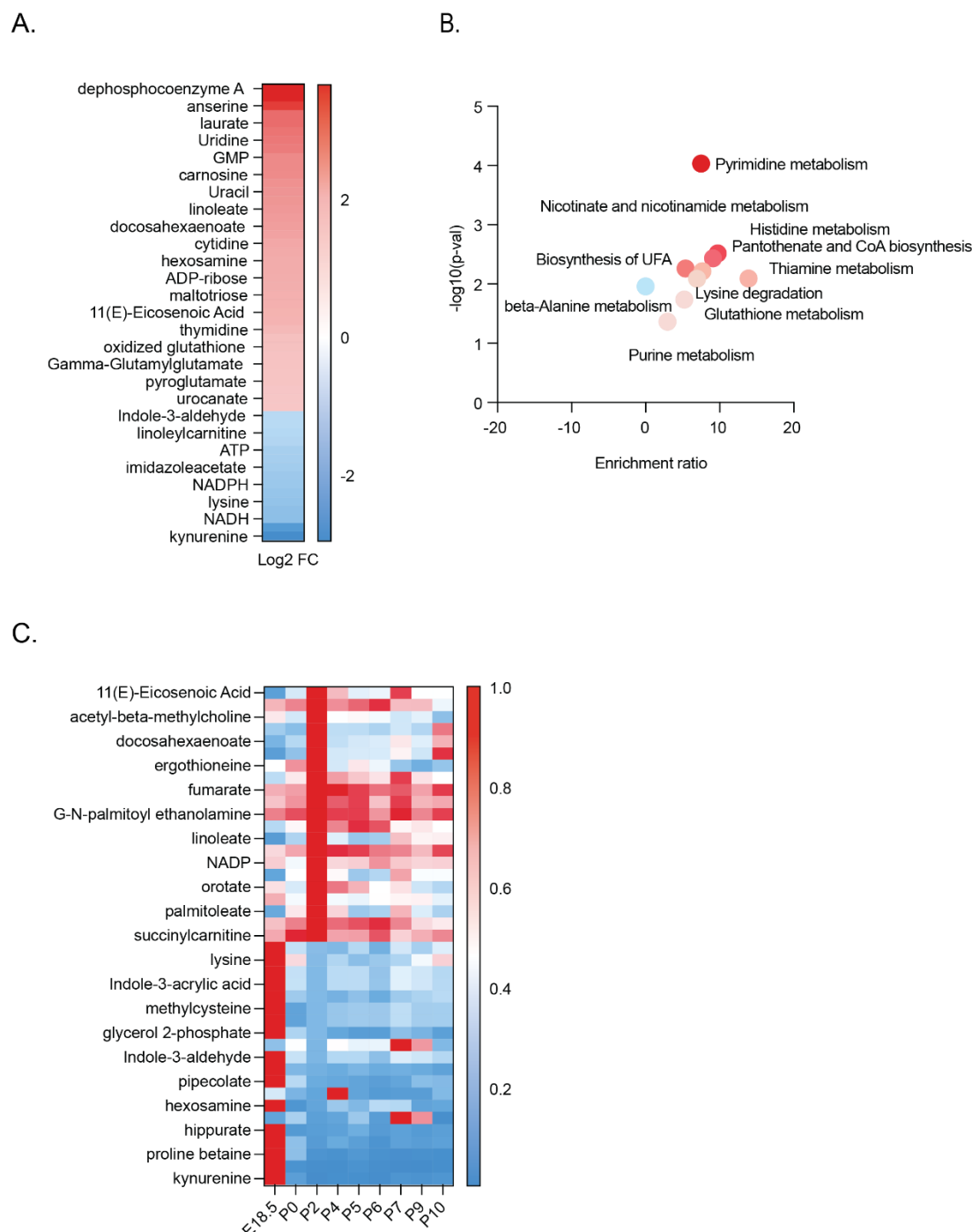


Figure 3.34. Metabolic changes at P2. **A.** Log2 fold changes for metabolites significantly changed from P0 to P2. **B.** MetaboAnalyst enrichment analysis of metabolites significantly changed at P2 (P2 to P0) (Student's t-test, $p\text{-val} < 0,05$) **C.** Heat map of metabolites that are most abundant and most depleted at P2.

At P2 we see an increase of metabolites involved in nucleotide metabolism (Figure 3.34A) and pyrimidine metabolism as one of the most enriched pathways (Figure

3.34B). Additionally, we see changes in nicotinate and nicotinamide metabolism, glutathione metabolism and an upregulation of CoA biosynthesis, all indicative of an increase of OXPHOS activity and maintenance of redox balance (Figure 3.34B).

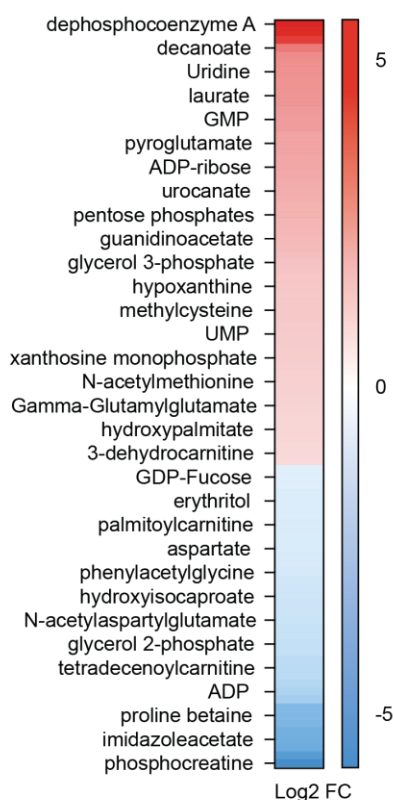
Metabolites that show highest and lowest peak at p2 mostly recapitulate enrichment analysis (Figure 3.34C). We can conclude the following metabolites show complete reversal compared to E18.5 – pipecolate, proline betaine, kynurenine, hippurate and hexosamine (Figure 3.34C).

3.6.3 Metabolic changes at P10

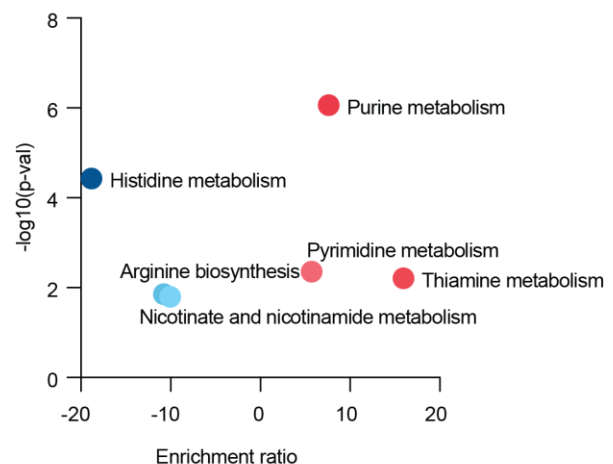
At P10 the most prominent metabolites are involved in thiamine and nucleotide metabolism (Figure 35A), showing that changes to nucleotide metabolism are consistent up until and including P10 (Figure 3.34B)

What is specific for P10 is the synthesis and increase of thiamine monophosphate, a cofactor of key enzymes in pentose-phosphate pathway (PPP), BCAA metabolism and TCA cycle Figure 35), indicating that these pathways are remodeled in late postnatal heart development, beyond P10.

A.



B.



C.

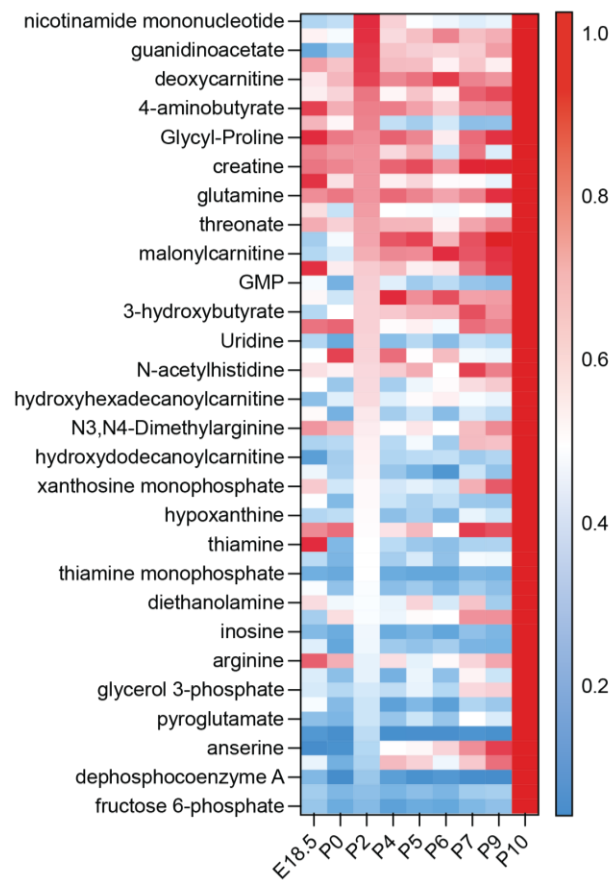


Figure 3.35. Heart metabolic profile at p10. **A.** Log2 fold changes for metabolites significantly changed from p0 to p10. **B.** MetaboAnalyst enrichment analysis of metabolites significantly changed at p10 (p10 to p0) **C.** Heat map of metabolites that are most abundant at p10.

3.6.4 Metabolic maturation of cardiomyocytes after P10

Since we could detect some of the targets of ISR in our P10 control animals, such as enzymes involved in serine and proline metabolism, while the 3 weeks control animals did not show any signal (Figure 3.17), we speculated that metabolic maturation of cardiomyocytes does not end at P10. Further on, metabolome analysis of P10 animals shows an increase in cofactor synthesis for enzymes of TCA cycle, BCAA metabolism and PPP (Figure 35). To confirm this, we analyzed WT hearts at multiple timepoints on WB for some known ISR targets (Figure 3.36A). We could identify SHMT2 (serine and 1C metabolism) and ALDH18a1 (P5CS - proline metabolism) to be active during postnatal development (Figure 3.36A). We could detect a clear signal for both SHMT2 and ALDH18a1 up until and including p10 WT hearts while at 3.5 weeks and at 8 weeks the signal is completely absent (Figure 3.36A). Additionally, there is no evidence of involvement of ISR or increase in phosphorylation of eIF2a (Figure 3.36A).

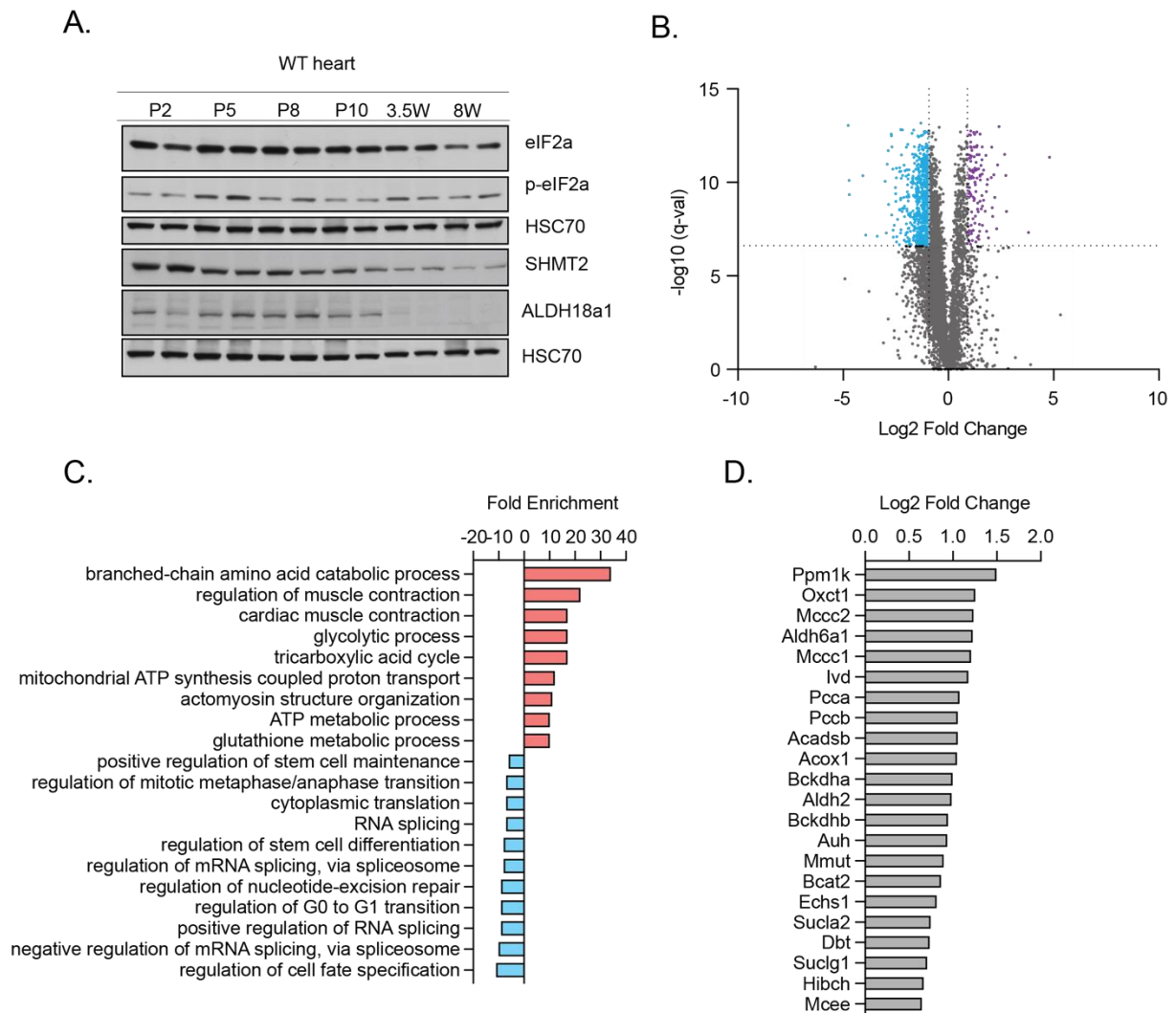


Figure 3.36. Metabolic maturation of cardiomyocytes continues past P10. **A.** Western blot of whole heart lysate for P2, P5, P8, P10, 3 weeks and 8 weeks old wild-type mice; **B.** Comparative data analysis of proteomics data from 3 weeks and P10 mouse heart as a volcano plot ($n=4$, $q\text{-value}=0.05$); **C.** Enrichment analysis corresponding to proteomics data set from B; **D.** Log_2 fold change for enzymes differentially upregulated in 3 weeks old versus P10 hearts.

To decipher which other changes happen from P10 to 3 weeks in mouse hearts, we compared proteomics data from p10 and 3 weeks wild type animals and could confirm extensive changes to the heart proteome even after P10 (Figure 3.365D). To annotate which metabolic pathways change at 3 weeks in comparison to 10 days, we performed GO Term pathway enrichment analysis, and among the highest hits we identify upregulation in branched chain amino acid catabolism, glucose metabolism, TCA cycle and OXPHOS (Figure 3.36C). Moreover, BCAA catabolism enzymes are among significantly upregulated proteins at 3 weeks (Figure 3.36D). Together with changes identified at P10, we can conclude that these pathways are necessary for later stages of postnatal maturation of cardiomyocytes, and that complete catabolism of BCAAs happens after P10, together with changes in mitochondrial 1C metabolism.

4 Discussion

Our results indicate that COX7C and CIV might be implicated in maturation of cardiomyocytes during postnatal development, since the loss of COX7C led to CIV deficiency and subsequent defects in nucleotide metabolism, glucose and BCAA metabolism remodelling of the developing heart. Further on, while loss of CIV in COX7C and COX10 mice seem to be comparable, the defect and extent of ISR activation differs between the models, and is dependent on the status of maturation of the heart. Loss of CIV in adult cardiomyocytes seems to lead to an extensive proteome and metabolome remodelling, and activation of compensatory mechanisms that would maintain heart function until loss of CIV cannot be tolerated anymore. However, loss of CIV during postnatal development leads to a fast-progressing cardiomyopathy, with very modest changes to metabolome and proteome, again indicating the importance of functional CIV during this development window.

Our results on OXPHOS dynamics during postnatal development show that CIV goes through extensive remodelling, with replacement of some nuclear encoded subunits with their corresponding heart-specific isoform. Additionally, composition of CIV seems to change with an addition of subunit COX6A that is induced at birth to form fully assembled CIV. Metabolic profiling of postnatal development seems to indicate that metabolic maturation of the heart coincides with maturation of CIV. Corroborating these findings is the observation that immature cardiomyocytes have a metabolic profile similar to that of mitochondrial cardiomyopathy caused by loss of CIV. Other than CIV, CII and CV both show changes in their structure during postnatal development, showing that more than one complex of OXPHOS goes through maturation and is dynamically regulated in this period. Key metabolic pathways that seem to be important for postnatal development include purine and pyrimidine metabolism, metabolism of amino acids such as histidine, lysine, and cofactor synthesis pathways such as thiamine and nicotine metabolism.

4.1 Development-specific differences in phenotype of CIV deficiency

Comparing to other mitochondrial dysfunction models that also lead to CIV deficiency such as DARS2 (Dogan et al., 2014a) and LRPPRC (Mootha et al., 2003) and arrest development already at E6.5, COX7C KO mice display a relatively mild phenotype and develop past organogenesis. Only visible difference is the size of the embryos. Further on, we could not recover any living mice, indicating that the mice die between E15.5 and E18.5. This relatively mild and less severe phenotype of whole-body depletion of COX7C and subsequent CIV deficiency could be explained by reliability of embryos on OXPHOS. Oxidative phosphorylation is low until E10.5 and increases only between E10.5-E14.5 (Cox and Gunberg, 1972). Even with the increase in OXPHOS, it is known that embryos are in a state of relative hypoxia. Oxygen tension in human alveolar arterioles is around 100mm, while for systemic tissues is between 40 and 20mm Hg (Webster and Abela, 2007). Values for fetal arterial blood pO₂ range from 30 to 20mm Hg (Lawrence et al., 2008, Reynolds et al., 1996, Mitchell and Van Kainen, 1992), suggesting that normal fetal oxygen is close to that of human systemic

tissues. Fetal heart has a higher tolerance to hypoxia and generally enhanced ability to increase glycolytic flux (Ascuitto and Ross-Ascuitto, 1996), potentially handling bioenergetic OXPHOS defects better. Considering that CIV loss we detected at E13.5 is severe, where only 20% of CIV activity could be detected, these embryos show an effect only on growth. Additionally, most of the mitochondrial defects that are essential for early fetal development and present with arrest in before organogenesis, at E6,5 - E8,5 affect mtDNA and mitochondrial gene expression rather than bioenergetics, such as already mentioned LRPPRC and DARS2, TWINKLE (Milenkovic et al., 2013), TFAM (Larsson et al., 1998), POLRMT (Kuhl et al., 2016) KO mice. In line with this observation, and surprisingly, isolated CIV deficiency of COX7C KO mice can be considered a mitochondrial defect that does not affect early stages of fetal development.

While we observed this relatively mild phenotype of a whole-body mitochondrial dysfunction caused by loss of COX7C, heart-specific depletion leads to a severe progressive cardiomyopathy (Figure 3.3) with early markers present already at P5 (Figure 3.12). It is one of the most severe heart specific models reported today, with models such as COX10, LRPPRC, DARS2 presenting with much longer lifespans (Ahola et al., 2022, Ruzzenente et al., 2012, Dogan et al., 2014b).

As mentioned before, COX7C has been identified as one of the long-lived proteins of OXPHOS, with a half-life of 6 days (Krishna et al., 2021). In our COX7C^{MKO} we could detect very low levels of COX7C at P10 (Figure 3.6). Its levels corresponded to residual CIV, indicating its importance in CIV stability. COX7C role in stability of CIV has been demonstrated in C2C12 cell system, with an emphasis on developmental status of the cells. Depletion of *Cox7c* transcript in 8-day differentiated myotubes did not affect levels of CIV. COX7C longevity provided protection to myotubes from CIV defects and maintain activity of CIV. The same was absent in dividing and differentiating myoblasts, which lost *Cox7c* levels immediately upon knockdown (Krishna et al., 2021). Early postnatal cardiomyocytes are differentiating cells, still proliferative and gradually switch from glycolytic to oxidative metabolism for energy production (Mollova et al., 2013). Thus, loss of COX7C in early postnatal cardiomyocytes leads to a fast destabilisation of CIV, same as in the case of myoblasts. Furthermore, increased reliance on OXPHOS in the first days after birth could aggravate the heart phenotype, leading to heart failure in first weeks of life. Together with recent reports from Suomalainen group indicate that differentiating cardiomyocytes are highly dependent on functional OXPHOS to be able to transform into adult cardiomyocytes (Landoni et al., 2022). Comparing to other CIV heart deficiency mouse models, loss of CIV in adult heart does not lead to such a fast progressing phenotype, indicating that the differentiation status of cardiomyocytes has a role in severity of COX7C^{MKO} phenotype.

4.2 Loss of COX7C affects stability and assembly of CIV

Our BN-PAGE analysis of embryo and heart mitochondria clearly shows an isolated CIV deficiency with a loss of activity in both models for COX7C (Figure 3.2 and Figure 3.5). Since we could still detect some remaining CIV activity and signal in

heart mitochondria, we investigated the composition and assembly of mitochondrial complexes in COX7C^{MKO}. To follow changes in CIV in COX7C^{MKO} hearts we performed a time-resolved complexome profiling analysis at three timepoints: P2, P5 and P10 (Figure 3.6). Our complexomics data shows that loss of CIV happens progressively from P2 to P10, where remaining CIV is very low, at about 20% (Figure 3.6). Remaining CIV correlates to COX7C levels, leaving very little expectation of functional subassemblies of CIV lacking COX7C. Still, mitochondrial protein synthesis is intact, indicating that *de-novo* synthesis of mitochondrial encoded subunits COX1, COX2 and COX3 of CIV is still ongoing. Since COX1 is the earliest module to assemble, we looked at components of COX1 module assembly, such as COX4 and COX5 subunits. Formation of the COX1 module during assembly is dependent on association to COX4 subunit, which happens post-translationally (Richter-Dennerlein et al., 2016). We detected accumulation COX4I1 and COX5B in COX7C^{MKO} mice at P10 in our complexomics data (Figure 3.9). Proteomics data shows a deficiency in all identified CIV subunits, indicating that the accumulation cannot be an upregulation of said subunits, but a change in their migration pattern due to not being incorporated into CIV. Further on, these subunits are tightly bound to COX1 in assembled CIV, so their accumulation is most likely not due to their detachment from existing CIV. It is more probable that these are signs of efforts to assemble new CIV. However, we could not see any stable subassembly complexes containing the core subunits that could contribute to CIV activity (Figure 3.6). This indicates that CIV assembly might be initiated, and that the assembly intermediates formed in the process are transient and unstable. A study on stability and turnover of CIV upon loss of COX7C during postnatal development would reveal further information. Steady state protein turnover can be determined by introduction of stable isotope labels followed by protein sample analysis by mass spectrometer in a method called Pulse-Silac (Fierro-Monti et al., 2013). This approach can be achieved *in-vivo* (Savas et al., 2016) by supplying animals with 15N-enriched food. Mitochondrial import of radiolabeled subunits and their incorporation into CIV can be followed on isolated mitochondria of COX7C^{MKO}. Identifying which factors contribute to assembly and stability of CIV during this crucial timeframe in postnatal heart development might help postpone onset of phenotype of CIV deficiency thus rescuing the early cardiac phenotype and prolonging life expectancy of these mice.

4.3 Developmental state of the heart dictates severity of mitochondrial cardiomyopathy

In our COX7C^{MKO}, we lose CIV progressively during postnatal development of the heart, so our next hypothesis is that early fetal development (COX7C KO embryos) and fully matured hearts (COX10^{MKO}) handle loss of CIV better than differentiating cardiomyocytes. While we still detect CIV and COX7C in P10, the decrease in amount is 75-80% comparing to the control animals (Figure 3.6). CIV deficiency models reported so far show that even as low as 20% of remaining CIV activity can sustain heart energy demands in mice, but this was mostly shown for models that lose CIV

after postnatal development, such as COX6A2 mice (Radford et al., 2002). To our knowledge, COX7C^{MKO} is the first mouse model of CIV deficiency reported to develop during early postnatal heart development, making our mouse model a very good resource to study mitochondrial involvement in cell differentiation and heart development. Recent report shows that increased mtDNA replication and processivity shows similar detrimental effect to postnatal heart development (Landoni et al., 2022). The mice show early postnatal growth defects with life expectancy of 9 days, characteristics of dilated cardiomyopathy, with delayed maturation of cardiomyocytes evident by defective nuclear polyploidization (Landoni et al., 2022). Other than the heart, neither organ showed significant changes at P7 pointing to the heart as the primary failing organ (Landoni et al., 2022). This is the only other report showing arrest in cardiomyocyte maturation during postnatal development, showing how mitochondrial function and involvement in differentiation needs to be studied further.

An answer to why postnatal development is this sensitive to mitochondrial function might be in differential capability to activate compensatory response to heart failure. In COX10 mice, heart enlargement is caused by dilated cardiomyopathy (Ahola et al., 2022) and heart tissue remodeling together with metabolic alterations such as switching to fetal metabolism and gene expression compensates some of the cardiac dysfunction caused by loss of COX10. Since adult heart failure is followed by a switch to fetal gene expression, many fetal isoforms for proteins of the contractile machinery are re-expressed and lead to remodeling of heart structure. Coincidentally, remodeling of the proteome and metabolome indicates that these changes are an orchestrated physiological response to mitochondrial dysfunction (Figure 3.14).

While the hearts are enlarged in COX7C^{MKO} mice, not all mice develop severely enlarged hearts, as shown by the heart-to-body weight measurements (Figure 3.3). Still, they do not survive past two weeks of age. In comparison to COX10^{MKO}, changes to the proteome and metabolome are not as extensive. Contradictory as it is, to have such a severe and early phenotype with little changes to the metabolome and proteome, it indicates that the extensity of changes to the proteome reinforces cardiac remodeling, and absence of changes on proteome and metabolome might indicate an inability to counter the cardiac defect. Cardiomyopathy is, at its core, a compensatory process to hearts failure to contract and function orderly. Absence of strong proteome and metabolome changes in response to mitochondrial dysfunction in COX7C mice indicates that these hearts cannot develop compensatory mechanisms for heart failure as efficiently as COX10 mice, and thus, die early and suddenly. However, we studied the steady state levels for all of our omics analysis performed, so an additional contributor to the severity of COX7C^{MKO} could be the flux of specific metabolites, energy production, and even protein turnover rates for CIV.

As was mentioned already, a characteristic of fetal tissues is an increased metabolic flexibility, especially for the fetal heart, where short bouts of low oxygen (hypoxia) that usually impede OXPHOS function, are essential for proper heart development. Hypoxia has a physiological and developmental role for the heart, through HIF and VEGF signaling, and the tolerance of the fetal heart to increased

glycolysis makes it less dependent of OXPHOS for energy production, explaining the COX7C KO phenotype in embryos. This characteristic is true to an extent for early postnatal hearts that are still differentiating. It's been demonstrated by tracing F-labeled 2-deoxy-2-fluoroglucose (FDG) in fetal and postnatal hearts that glucose uptake significantly decreases from E10 to P7, with P7 mice hearts taking up 0,05% of what E10 hearts do (Nakano et al., 2011). Even though increased glycolytic flux is the key characteristic of metabolic flexibility in embryos, prolonged glucose uptake can have adverse effects on the developing heart. Experiments done on human embryonic stem cell derived cardiomyocytes (hESC-CM) show that prolonged glucose uptake in differentiating cardiomyocytes maintains proliferation and inhibits their maturation (Nakano et al., 2017). Pro-mitotic and anti-maturation effect of prolonged glucose metabolism is mediated by nucleotide synthesis through pentose phosphate pathway (Nakano et al., 2017). Coincidentally, in heart failure a general compensatory response to reduction in OXPHOS is an upregulation of glycolytic pathways (Lopaschuk et al., 2021), seen in both our models of CIV deficiency. One of the common changes between two models is increase of glycolysis and serine biosynthesis from glucose, changes in nucleotide synthesis and amino acid metabolism (Figure 3.19).

Hence, CIV dysfunction in adult hearts would lead to change in substrate utilization for energy production from OXPHOS to glycolysis, further compensated by a change in the structure of contractile machinery. The same defect in differentiating cardiomyocytes could affect maturation status and proliferation. This could be an underlying cause of the early and sudden phenotype of COX7C mice and provide explanation for the difference between two CIV deficiency models.

Moreover, as mentioned already, protein turnover might have an important role in the severity of phenotype. Since COX7C as a structural subunit, and COX10 as an assembly factor might have different half-life in cardiomyocytes, loss of CIV in the two models might follow two different timescales. As an assembly factor, COX10 could be involved in assembling more than one CIV, while COX7C amount is directly reflective of CIV levels.

4.4 ISR in COX7C and COX10

Our data indicates that integrated stress response seems to vary depending on whether it is activated in differentiating cells or in terminally differentiated tissue. Activation of ISR happens through differing axes, and via different triggers, such as heme depletion, amino acid starvation, viral infection, proteostasis disruption (Mick et al., 2020), with remodelling of 1C metabolism and activation of trans-sulphuration pathway to produce GSH and prevent lipid peroxidation and ferroptosis (Ahola et al., 2022). COX7C mice, however, show remodelling of 1C metabolism as well, but it corresponds more towards SAH production and methylation (Figure 3.22). In adult tissues such as kidney and liver, 1C metabolism serves the production of creatine and choline, while in heart folate metabolism serves to maintain nucleotide and amino acid homeostasis (Ducker and Rabinowitz, 2017).

It has also been reported that both human and mouse embryonic stem cells show elevated levels of phosphorylated eIF2 α , which decreases upon differentiation (Friend et al., 2015). Phosphorylation of eIF2 α is maintained through growth factors, which promotes expression of *Nanog* and *c-Myc* in murine ESCs (Friend et al., 2015). ATF4 shares most of its target genes with c-MYC, CHOP and CEBP (Tameire et al., 2019, Cohen et al., 2015, Han et al., 2013) indicating that a different stress pathway, without major involvement of ATF4 might be active in differentiating cells.

ATF4 functions as a homodimer and a heterodimer, and its dimerising partners contribute further to the variability in ATF4 related responses. While c-MYC and ATF4 seem to work in coordination in both cell stress and cancer (suomalainen paper), other transcription factors of the ATF/CEBP family of transcription factors act as dimerising partners for ATF4, including CHOP, CEBPB, ATF3 and many more (Neill and Masson, 2023). In our COX7C mice, we detected high levels of CEBPB (Fig. 13), a known dimerising partner of ATF4 (Lopez et al., 2007, Thiaville et al., 2008, Cohen et al., 2015). ATF4-CEBPB heterodimers were implicated in differentiation of human adipocytes, where ATF4 requires CEBPB for genomic binding and activation of a transcriptional program in naïve early differentiating pre-adipocytes (Cohen et al., 2015). Transcription of CEBPB can be activated in response to amino acid deficiency (Chen et al. 2005, Lee et al. 2008, Sikalidis et al. 2011), and ATF4 bound to an enhancer downstream of the CEBPB coding region (Chen et al. 2005) increases transcription of CEBPB approximately 4-fold (Chen et al. 2005, Lee et al. 2008, Sikalidis et al. 2011). Moreover, methylation of CGAT|GCAA sequence promotes binding of ATF4/CEBPB heterodimers to DNA (Mann et al., 2013). Since this is one of the proteins in COX7C^{MKO} hearts that is not present in COX10, we hypothesise that involvement of CEBPB might be contributing to the difference in ISR response between the two models. Assuming that p-eIF2 α and CEBPB are involved in differentiation of cardiomyocytes and cell fate decision, it is possible that further activation of ISR due to mitochondrial dysfunction might be prolonging the immature status of cardiomyocytes, leading to pathological changes in the heart and early neonatal death of mice. Whether overactivation of ISR and related metabolic pathways are a contributing factor in pathology of COX7C is unsure, since these pathways have never been investigated in depth during postnatal development of the heart, nor has OXPHOS function been contributed to the process so far.

4.5 Complexomics of postnatal heart development

Most surprising change in OXPHOS concerns the structure of CIV. Since CIV is the only OXPHOS complex with tissue specific isoforms, the role of CIV remodelling has been under spotlight for a while. As mentioned before, subunits with heart specific isoforms are COX6A, COX7A and COX8. Heart isoform for COX6A is COX6A2 (COX6AH), and for COX7A is the COX7A1 (COX7AH) isoform, while the ubiquitously expressed subunits are COX6A1 (COX6AL) and COX7A2 (COX7AL) (Bonne et al., 1993, Taanman et al., 1993). In heart and skeletal muscle of the mouse, it has been shown that replacement of the ubiquitous isoform of COX6A and COX7A with the heart

isoform happens gradually until 28 days postnatally (Parsons et al., 1996, Boczonadi et al., 2015), when the heart isoform completely takes over and persists for the rest of the mouse lifespan. More recently, a study on mitochondrial network dynamics during muscle postnatal development shows that COX6A1 to COX6A2 isoform switch happens by P42, while COX7A2 to COX7A1 switch could not be observed. Although the liver isoform decreases by 1.6 fold and heart isoform increases by 2.4 fold, finally showed that 88% of total COX7A was the liver isoform at P42 (Kim et al., 2019). Our data on the heart corroborates this finding, as we could show that both isoforms COX7A are present in CIV during postnatal development (Figure 3.28). If there is ever a complete switch between isoforms, it happens in the later stages when the heart is mature. This observation indicates that there is a certain level of heterogeneity in CIV composition; CIV with each isoform exists at the same time. Role of COX7A has not been investigated thoroughly yet, but its importance might lay in modulating activity of CIV in adult heart rather than being involved in regulation of CIV during postnatal development.

Surprisingly, our complexome profiling data shows COX6A subunit to be present in CIV structure only at P2 (Figure 3.29). Whether this subunit contributes to CIV structure in earlier timepoints, or if we managed to capture remodelling of CIV in a span of couple days after birth remains unclear. Nonetheless, the shift in size we observe for CIV at P2 could be explained by the insertion of this subunit. While both isoforms of COX6A are present at P2, ubiquitous subunit is the more abundant one. Additionally, the isoform switch for COX6A can be clearly observed during postnatal development, as we see an increase of COX6A2 over COX6A1 at P7 (Figure 3.29). The fact that we see a signal for this subunit at the timepoint when we see fully assembled CIV supports the idea that COX6A is incorporated into CIV holoenzyme, making the exchange of isoforms mechanistically based on incorporation rather than *de-novo* assembly. Further on, yeast COX6A is not necessary for assembly of the holoenzyme but has a function in modulating CIV activity (Taanman and Capaldi, 1993).

The concept of direct incorporation of nuclear subunits added in the last stages of CIV assembly has been proposed before, namely for COX6A, COX6B and COX7A (Lazarou et al., 2009). The proposed mechanism employs a late state intermediate complex containing COX6A and COX7A with ancillary factors that could be incorporated into holoenzyme primarily, but also to CIV within SC structures.

A body of research investigating the function of COX6A exists. Loss of COX6A2 isoform in the heart lead to a decrease in myocardial diastolic performance, despite unchanged ATP levels (Radford et al., 2002). CIV levels and activity decreased to 23% of the control animals upon loss of COX6A2, and while control animals contained a mixture of CIV containing either COX6A2 or the ubiquitous COX6A1, the KO mice showed an increase in COX6A1 subunit (Radford et al., 2002). While showing a compensatory mechanism to the loss of COX6A2, by its ubiquitously expressed isoform, it also indicates that heart isoform does not contribute to postnatal heart development (Radford et al., 2002). Since the goal of the study was to conclude on

COX6A2 role in heart function, and not postnatal development, the study does not comment on half-life of COX6A2 or when the loss of subunit happens. It is only shown that at 28 days, loss of COX6A2 is evident (Radford et al., 2002), indicating that loss of this isoform in their model could be happening outside the critical window of early postnatal development, which in our data would be first couple days.

Function of COX6A subunit and its isoforms might lay in modulating CIV activity to meet the demands of the differentiating tissue. It can be regarded as maturation of CIV, referring to a change in activity or capacity of CIV necessary to supply the tissue with growing bioenergetic demands. For example, COX6A isoforms could be regulating the proton pumping activity of CIV, as shown in experiments done on bovine reconstituted CIV (Lee and Kadenbach, 2001). In comparison to COX6A2 isoform, COX6A1-containing CIV showed decreased proton pumping activity with addition of free palmitate (Lee and Kadenbach, 2001). Additionally, COX6A2 isoform has an ADP-binding site shown to contribute to allosteric activation of reconstituted CIV (Anthony et al., 1993). An increase in proton pumping and a possibility to modulate CIV via ADP/ATP ratio could regulate energy metabolism of the growing and developing heart. While the exchange in isoforms and their respective role in CIV function have been researched, potential existence of CIV without COX6A subunit could add another layer of regulation of CIV function. Remaking the whole complex could be both energy demanding and damaging to a differentiating cell, emphasising necessity for increased complex stability and quality control.

Other than CIV changes, we saw an interesting migration pattern for CII (Figure 3.30) and for CV components (Figure 3.30). For changes in CII, we could not identify a clear reasoning since all components of the fully assembled CII are present. On the other hand, CV clearly showed an increase in assembly of the Fo component from birth to P10, and a reverse effect can be seen for free F1 component, showing most abundance at E18.5 and disappearing by P10. To our knowledge this is the first report of regulation of CV assembly by birth. There are reports that oxygen availability has a regulatory role in expression of CV subunits, such as ATP5ME during differentiation of myoblasts in C2C12 model system (Levy and Kelly, 1997), indicating that normoxia might be leading to an upregulation of assembly of Fo subunits.

While our complexomics data brings many new information, optimisation of the method would be a priority. We performed our analysis on 500ug of heart mitochondria isolated from pulled hearts for each timepoint to obtain enough material. Additionally, processing time on mass spectrometer for all 9 timepoints is very long since each timepoint provided with 48 samples, therefore 48 MS runs per timepoint. For earliest timepoints, such as E18.5 we pulled as many as 10 embryo hearts. To perform this experiment in more replicates it would entail much more processing power and time, and many more animals. Therefore, confirming our findings in specific timepoints following this analysis would be the best compromise, having animal experiment ethics and general time and financial effectiveness in mind. To confirm our observations, since they mostly happen in the first couple days after birth, we aim to confirm our findings by BN-PAGE analysis for E18.5-P5 timepoints only. An eligible strategy to

investigate how isoforms switch in CIV would be to import radioactively labelled isoforms into isolated mitochondria from E18.5 hearts and try to observe a shift in size for CIV. Further on, establishing a cell culture model for cardiomyocytes where we could follow their differentiation *in-vitro* could prove helpful in investigating these regulatory mechanisms and functional significance to cell differentiation. Currently, C2C12 cell system is used for investigating differentiating cells, but has proven to be subpar in investigating isoform switch for CIV (Ye et al., 2023). Changes we observed at birth to OXPHOS complexes of the heart indicate that, together with maturation of heart metabolism and cellular structure, mitochondrial maturation includes changes to OXPHOS complexes and their composition, assembly and/or distribution within the inner mitochondrial membrane.

4.6 Metabolic maturation during postnatal heart development

Our metabolome analysis of postnatal hearts highlights the immaturity of metabolism in E18.5 and the drastic change that happens at birth, with changes to components of Further on, in days after birth, key metabolic pathways that change are

As we could identify most changes in the earliest timepoints, we observed how metabolic profiles of hearts at those timepoints change. Firstly, with birth, mice switch from hypoxic embryonic environment utilising mostly glucose for energy production, to normoxia and high fat diet in mother's milk. Maternally supplied fatty acids bind retinoid X receptors and activate expression of fatty acid metabolism components and lipid handling (Paredes et al., 2023). With oxidation of lipids to provide energy, dependence on OXPHOS increases in comparison to embryonic metabolism, and it is expected that changes in OXPHOS activity should follow. Looking at metabolic profile of E18.5 hearts, we noticed an overlap in metabolites we identify upon mitochondrial dysfunction in the heart (Figure 3.32). Among the metabolites that overlap we would highlight dependence of E18.5 hearts on amino acid degradation, aminoacyl-tRNA biosynthesis and nucleotide metabolism. Key marker metabolites of ISR also show high abundance in E18.5 hearts, such as pipecolate, homocysteine, homocitrulline and proline betaine (Figure 3.32). While they decrease gradually after birth, we still see some of the pathways active in later timepoints, as shown with ISR targets (Figure 3.35).

Our results demonstrate that known targets of ISR commonly used as markers, are present in P10 WT animals, while completely absent in 3-week-old WT mice (Figure 3.17). Although we could detect p-eIF2a in these hearts, we could not show any significant change during postnatal development (Figure 3.35). Presence of SHMT2 and ALDH18A1 in control mice from P2 to P10 indicates that metabolic pathways we see in ISR might contribute to postnatal development of the heart. Due to this fact, analysis of COX7C^{MKO} hearts could be skewed; we might not find significance because the increase on protein level for targets of ISR could be an overactivation of the metabolic pathways already active during development. Additionally, this might make COX10 phenotype look more severe in comparison. Further on, overactivation of there pathways could be contributing to immature status

of cardiomyocytes. Since we show that immature cardiomyocytes at E18.5 show similarities with mitochondrial cardiomyopathy, it is not far fetched to assume that maintained activity of these pathways could impede metabolic maturation of cardiomyocytes.

One other common change between COX7C^{MKO} and COX10^{MKO} mice, includes changes to BCAA metabolism. BCAA metabolites that are accumulated are C5, C5:1, C5-OH carnitine and tiglyl-carnitine, all of them a byproduct of BCAA metabolism rewiring that cannot be processed further. It has recently been shown that these metabolites accumulate upon inhibition of BCAT in cancer cells (Sciacovelli et al., 2022). Inhibition of BCAT led to rewiring of BCAA metabolism towards aspartate synthesis and nucleotide metabolism, specifically pyrimidine synthesis (Sciacovelli et al., 2022). BCAT is responsible for the transamination reaction which converts BCAAs to their corresponding keto acids, producing glutamate in the reaction (Papathanassiou et al., 2017). Thus, one of the main outputs of BCAA metabolism and BCAT transamination reaction is glutamate. Further on, glutamate can be used to make α -ketoglutarate and subsequently, aspartate through ASNS, providing a necessary nitrogen source for pyrimidine synthesis. BCAAs are the second most abundant nitrogen source after glutamine. Moreover, a role for BCAA catabolism in liver cancer cells has also been reported, where an increased flux of BCAAs rescues cancer cells from glutamine starvation conditions, through O-GlcNAcylation of the main regulatory protein PPM1K, which in normal conditions is necessary for activity of BCKDH complex through dephosphorylation (Yang et al., 2022). They report an increase in cancer proliferation and progression, while inhibition of BCAA catabolism leads to cell cycle arrest in G2/M phase (Yang et al., 2022). Involvement of defective BCAA catabolism in heart failure has also been reported before, with downregulation of PPM1K as the main regulatory point (Li et al., 2017). Both of our CIV deficiency models show an accumulation of C5 carnitines that are a byproduct of incomplete BCAA catabolism (Figure 3.19 and Figure 3.20). Further on, PPM1K is downregulated in proteomics data sets in both COX7C^{MKO} and COX10^{MKO} (Figure 3.13Figure 3.15), indicating that rewiring of BCAA catabolism is a common strategy in CIV deficiency and mitochondrial cardiomyopathy.

Since most reports connect BCAA catabolism alterations and pyrimidine synthesis, we also looked at pyrimidine synthesis in our COX7C^{MKO} mice. We saw an increase in orotate and N-carbamoyl aspartate, and some of the products of pyrimidine synthesis (Figure 3.21). Rate limiting enzyme of pyrimidine synthesis is DHODH complex (Amos et al., 2023). DHODH is responsible for conversion of dihydroorotate to orotate, transferring electrons to the Q-pool, linking its activity to the electron transport chain (Amos et al., 2023). Since our data does not show any changes to DHODH and any of the components of the electron transport chain other than CIV, it would be important to analyse the flux of pyrimidine synthesis. Additionally, we see an upregulation of cytosolic salvage pathway for pyrimidine synthesis, indicating that the nucleotide pool in the cytosol is affected by a failure to export precursors for nucleotide synthesis out of mitochondria.

Further on, one of the clearest observations in our analysis of postnatal heart development relates to BCAA metabolism. Throughout early postnatal development in WT mice we do not detect much change in BCAA metabolism (Figure 3.33 - Figure 3.35). In the earliest timepoints we can detect accumulation of metabolic products of incomplete BCAA catabolism, like in COX7C and COX10 mice, indicating increased dependence on BCAA as nitrogen and carbon sources. This observation indicates that immature cardiomyocytes might be more sensitive to mitochondrial dysfunction and further increase BCAA remodelling in need for alternative nitrogen sources, explaining how detrimental mitochondrial disfunction during this period could be.

Conversely, our enrichment data comparing proteomics of 3 weeks to P10 shows upregulation of pathways involved in BCAA degradation (Figure 3.36). Looking closer at the proteomics data, we see a stark increase in almost all enzymes involved in BCAA catabolism at 3 weeks (Figure 3.36). Furthermore, one of the main metabolic changes at P10 is an increase in thiamine metabolism, from which a coenzyme for BCAA catabolism enzyme is produced (Figure 3.35). With a strong increase in key enzymes in BCAA catabolism, and an increase in TCA cycle and OXPHOS, we can conclude that BCAA catabolism is fully functional only after p10, and a complete oxidation of BCAAs is possible in late postnatal development and in the adult heart.

With remodelling of OXPHOS as we describe it, it is possible that embryonic heart maintains a metabolic state which compensates for OXPHOS maturation. It is interesting that the strategy is very similar with ISR remodelling upon mitochondrial dysfunction, and it seems to be maintained well into postnatal development. It would be of interest to the field to pinpoint what aspect of metabolic remodelling upon mitochondrial dysfunction is stress activated and pathological, and what has a role in determining and maintaining a certain cellular status. Conversely, ISR might not be a pathway evolutionarily conserved to answer to stress and could in fact be in place to buffer and maintain cellular plasticity when needed. Additionally, with our COX7C^{MKO} model we could see how detrimental overactivation of these pathways is, and how sensitivity to mitochondrial dysfunction relates to the phenotype of the cell.

4.7 Summary

Taken together, our study has shed light on involvement of mitochondrial CIV in cell fate and differentiation, by mediating metabolic remodelling of glucose metabolism, nucleotide synthesis and BCAA catabolism. In postnatal heart development, loss of CIV lead to early onset cardiomyopathy, fatal by P10. Cause of such early and fatal phenotype might lay in disruption of maturation of cardiomyocytes, a process that entails switch from glycolytic to oxidative metabolism. By disrupting CIV during this window, glycolytic metabolism is prolonged and rewired, potentially blocking maturation of cardiomyocytes and maintaining them in an immature state, unable to produce energy for the growing and changing heart structure. Further on, our data indicate that changes in pyrimidine synthesis might be at the core of dysrupted maturation of cardiomyocytes, together with innability to fully oxidise BCAAs. While we can speculate that both glucose metabolism changes and BCAA metabolism rewiring might be an attempt to provide the cell with essential building blocks for growth and

differentiation such as glutamate and aspartate, further studies on metabolic flux in postnatal heart development are needed.

Additionally, our time resolved data from wild-type heart development corroborates the idea that these exact metabolic pathways might be essential for fine-tuning the metabolism of differentiating cells, with nucleotide metabolism and amino acid metabolism changing dynamically with birth, and BCAA metabolism maturation identified at later stages of postnatal development. Dynamic changes in composition of OXPHOS complexes CII, CIV and CV could be providing a regulatory aspect in differentiation and cell fate regulation in heart development that is insufficiently explored.

This study aimed to shed light to involvement of OXPHOS and mitochondrial function in differentiation and maturation of cardiomyocytes. The results produced in this study have and will further on, be useful in elucidating molecular mechanisms in which mitochondria enable integration of cellular cues and condition a response that lead to changes in cellular phenotype.

5 References

- ACIN-PEREZ, R., FERNANDEZ-SILVA, P., PELEATO, M. L., PEREZ-MARTOS, A. & ENRIQUEZ, J. A. 2008. Respiratory active mitochondrial supercomplexes. *Mol Cell*, 32, 529-39.
- AHOLA, S., RIVERA MEJIAS, P., HERMANS, S., CHANDRAGIRI, S., GIAVALISCO, P., NOLTE, H. & LANGER, T. 2022. OMA1-mediated integrated stress response protects against ferroptosis in mitochondrial cardiomyopathy. *Cell Metab*, 34, 1875-1891 e7.
- ALGIRE, M. A., MAAG, D. & LORSCH, J. R. 2005. Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation. *Mol Cell*, 20, 251-62.
- AMERI, K. & HARRIS, A. L. 2008. Activating transcription factor 4. *Int J Biochem Cell Biol*, 40, 14-21.
- ANTHONY, G., REIMANN, A. & KADENBACH, B. 1993. Tissue-specific regulation of bovine heart cytochrome-c oxidase activity by ADP via interaction with subunit VIa. *Proc Natl Acad Sci U S A*, 90, 1652-6.
- ASCUITTO, R. J. & ROSS-ASCUITTO, N. T. 1996. Substrate metabolism in the developing heart. *Semin Perinatol*, 20, 542-63.
- BEN-SAHRA, I., HOXHAI, G., RICOULT, S. J. H., ASARA, J. M. & MANNING, B. D. 2016. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science*, 351, 728-733.
- BOCZONADI, V., GIUNTA, M., LANE, M., TULINIUS, M., SCHARA, U. & HORVATH, R. 2015. Investigating the role of the physiological isoform switch of cytochrome c oxidase subunits in reversible mitochondrial disease. *Int J Biochem Cell Biol*, 63, 32-40.
- BONNE, G., SEIBEL, P., POSSEKEL, S., MARSAC, C. & KADENBACH, B. 1993. Expression of human cytochrome c oxidase subunits during fetal development. *Eur J Biochem*, 217, 1099-107.
- BRISCHIGLIARO, M. & ZEVIANI, M. 2021. Cytochrome c oxidase deficiency. *Biochim Biophys Acta Bioenerg*, 1862, 148335.
- COHEN, D. M., WON, K. J., NGUYEN, N., LAZAR, M. A., CHEN, C. S. & STEGER, D. J. 2015. ATF4 licenses C/EBPbeta activity in human mesenchymal stem cells primed for adipogenesis. *Elife*, 4, e06821.
- COX, S. J. & GUNBERG, D. L. 1972. Metabolite utilization by isolated embryonic rat hearts in vitro. *J Embryol Exp Morphol*, 28, 235-45.
- DANG, C., GOTTSCHLING, M., MANNING, K., O'CURRAIN, E., SCHNEIDER, S., STERRY, W., STOCKFLETH, E. & NINDL, I. 2006. Identification of dysregulated genes in cutaneous squamous cell carcinoma. *Oncology reports*, 16, 513-519.
- DEY, M., CAO, C., DAR, A. C., TAMURA, T., OZATO, K., SICHERI, F. & DEVER, T. E. 2005. Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell*, 122, 901-13.
- DIRKX, E., DA COSTA MARTINS, P. A. & DE WINDT, L. J. 2013. Regulation of fetal gene expression in heart failure. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1832, 2414-2424.
- DOGAN, S. A., PUJOL, C., MAITI, P., KUKAT, A., WANG, S., HERMANS, S., SENFT, K., WIBOM, R., RUGARLI, E. I. & TRIFUNOVIC, A. 2014. Tissue-specific loss of DARS2 activates stress responses independently of respiratory chain deficiency in the heart. *Cell Metab*, 19, 458-69.

- DONNELLY, N., GORMAN, A. M., GUPTA, S. & SAMALI, A. 2013. The eIF2 α kinases: their structures and functions. *Cell Mol Life Sci*, 70, 3493-511.
- DUCKER, G. S. & RABINOWITZ, J. D. 2017. One-Carbon Metabolism in Health and Disease. *Cell Metab*, 25, 27-42.
- EL-MASRY, O. S. 2023. Gene expression profile and presentation of novel gene variants of COX7B and COX7C in a cohort of patients with chronic myeloid leukemia. *Electronic Journal of General Medicine*, 20.
- FESSLER, E., ECKL, E. M., SCHMITT, S., MANCILLA, I. A., MEYER-BENDER, M. F., HANF, M., PHILIPPOU-MASSIER, J., KREBS, S., ZISCHKA, H. & JAE, L. T. 2020. A pathway coordinated by DELE1 relays mitochondrial stress to the cytosol. *Nature*, 579, 433-437.
- FIERRO-MONTI, I., RACLE, J., HERNANDEZ, C., WARIDEL, P., HATZIMANIKATIS, V. & QUADRONI, M. 2013. A novel pulse-chase SILAC strategy measures changes in protein decay and synthesis rates induced by perturbation of proteostasis with an Hsp90 inhibitor. *PLoS One*, 8, e80423.
- FRIEND, K., BROOKS, H. A., PROPSON, N. E., THOMSON, J. A. & KIMBLE, J. 2015. Embryonic Stem Cell Growth Factors Regulate eIF2 α Phosphorylation. *PLoS One*, 10, e0139076.
- GARCIA, M. A., MEURS, E. F. & ESTEBAN, M. 2007. The dsRNA protein kinase PKR: virus and cell control. *Biochimie*, 89, 799-811.
- GUO, X., AVILES, G., LIU, Y., TIAN, R., UNGER, B. A., LIN, Y. T., WIITA, A. P., XU, K., CORREIA, M. A. & KAMPMANN, M. 2020. Mitochondrial stress is relayed to the cytosol by an OMA1-DELE1-HRI pathway. *Nature*, 579, 427-432.
- HAI, T. & CURRAN, T. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A*, 88, 3720-4.
- HAN, A. P., YU, C., LU, L., FUJIWARA, Y., BROWNE, C., CHIN, G., FLEMING, M., LEBOULCH, P., ORKIN, S. H. & CHEN, J. J. 2001. Heme-regulated eIF2 α kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J*, 20, 6909-18.
- HAN, J., BACK, S. H., HUR, J., LIN, Y. H., GILDERSLEEVE, R., SHAN, J., YUAN, C. L., KROKOWSKI, D., WANG, S., HATZOGLOU, M., KILBERG, M. S., SARTOR, M. A. & KAUFMAN, R. J. 2013. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol*, 15, 481-90.
- HARDING, H. P., ORDONEZ, A., ALLEN, F., PARTS, L., INGLIS, A. J., WILLIAMS, R. L. & RON, D. 2019. The ribosomal P-stalk couples amino acid starvation to GCN2 activation in mammalian cells. *Elife*, 8.
- HINNEBUSCH, A. G. 1997. Translational regulation of yeast GCN4. A window on factors that control initiator-tRNA binding to the ribosome. *J Biol Chem*, 272, 21661-4.
- HOLMGREN, D., WAHLANDER, H., ERIKSSON, B., OLDFORS, A., HOLME, E. & TULINIUS, M. 2003. Cardiomyopathy in children with mitochondrial disease: clinical course and cardiological findings. *European heart journal*, 24, 280-288.
- HU, H., TIAN, M., DING, C. & YU, S. 2018. The C/EBP Homologous Protein (CHOP) Transcription Factor Functions in Endoplasmic Reticulum Stress-Induced Apoptosis and Microbial Infection. *Front Immunol*, 9, 3083.
- HUGGINS, C. J., MAYEKAR, M. K., MARTIN, N., SAYLOR, K. L., GONIT, M., JAILWALA, P., KASOJI, M., HAINES, D. C., QUIÑONES, O. A. & JOHNSON, P. F. 2016. C/EBP γ Is a Critical Regulator of Cellular Stress Response

- Networks through Heterodimerization with ATF4. *Molecular and Cellular Biology*, 36, 693-713.
- INGLES, J., GOLDSTEIN, J., THAXTON, C., CALESHU, C., CORTY, E. W., CROWLEY, S. B., DOUGHERTY, K., HARRISON, S. M., MCGLAUGHON, J. & MILKO, L. V. 2019. Evaluating the clinical validity of hypertrophic cardiomyopathy genes. *Circulation: Genomic and Precision Medicine*, 12, e002460.
- INGLIS, A. J., MASSON, G. R., SHAO, S., PERISIC, O., MCLAUGHLIN, S. H., HEGDE, R. S. & WILLIAMS, R. L. 2019. Activation of GCN2 by the ribosomal P-stalk. *Proc Natl Acad Sci U S A*, 116, 4946-4954.
- JOUSSE, C., OYADOMARI, S., NOVOA, I., LU, P., ZHANG, Y., HARDING, H. P. & RON, D. 2003. Inhibition of a constitutive translation initiation factor 2alpha phosphatase, CReP, promotes survival of stressed cells. *J Cell Biol*, 163, 767-775.
- KASPAR, S., OERTLIN, C., SZCZEPANOWSKA, K., KUKAT, A., SENFT, K., LUCAS, C., BRODESSER, S., HATZOGLOU, M., LARSSON, O., TOPISIROVIC, I. & TRIFUNOVIC, A. 2021. Adaptation to mitochondrial stress requires CHOP-directed tuning of ISR. *Sci Adv*, 7.
- KENNER, L. R., ANAND, A. A., NGUYEN, H. C., MYASNIKOV, A. G., KLOSE, C. J., MCGEEVER, L. A., TSAI, J. C., MILLER-VEDAM, L. E., WALTER, P. & FROST, A. 2019. eIF2B-catalyzed nucleotide exchange and phosphoregulation by the integrated stress response. *Science*, 364, 491-495.
- KIM, Y., YANG, D. S., KATTI, P. & GLANCY, B. 2019. Protein composition of the muscle mitochondrial reticulum during postnatal development. *J Physiol*, 597, 2707-2727.
- KRISHNA, S., ARROJO, E. D. R., CAPITANIO, J. S., RAMACHANDRA, R., ELLISMAN, M. & HETZER, M. W. 2021. Identification of long-lived proteins in the mitochondria reveals increased stability of the electron transport chain. *Dev Cell*, 56, 2952-2965 e9.
- KUHL, I., MIRANDA, M., POSSE, V., MILENKOVIC, D., MOURIER, A., SIIRA, S. J., BONEKAMP, N. A., NEUMANN, U., FILIPOVSKA, A., POLOSA, P. L., GUSTAFSSON, C. M. & LARSSON, N. G. 2016. POLRMT regulates the switch between replication primer formation and gene expression of mammalian mtDNA. *Sci Adv*, 2, e1600963.
- LANDONI, J. C., LAALO, T., GOFFART, S., KIVELÄ, R., SKUBE, K., NIEMINEN, A. I., WICKSTRÖM, S. A., STEWART, J. & SUOMALAINEN, A. 2022. Overactive mitochondrial DNA replisome causes neonatal heart failure via ferroptosis. *bioRxiv*, 2022.04.04.485133.
- LARSSON, N.-G., WANG, J., WILHELMSSON, H., OLDFORS, A., RUSTIN, P., LEWANDOSKI, M., BARSH, G. S. & CLAYTON, D. A. 1998. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature Genetics*, 18, 231-236.
- LAWRENCE, J., XIAO, D., XUE, Q., REJALI, M., YANG, S. & ZHANG, L. 2008. Prenatal nicotine exposure increases heart susceptibility to ischemia/reperfusion injury in adult offspring. *J Pharmacol Exp Ther*, 324, 331-41.
- LAZAROU, M., SMITH, S. M., THORBURN, D. R., RYAN, M. T. & MCKENZIE, M. 2009. Assembly of nuclear DNA-encoded subunits into mitochondrial complex IV, and their preferential integration into supercomplex forms in patient mitochondria. *FEBS J*, 276, 6701-13.

- LEE, I. & KADENBACH, B. 2001. Palmitate decreases proton pumping of liver-type cytochrome c oxidase. *Eur J Biochem*, 268, 6329-34.
- LEE, Y. Y., CEVALLOS, R. C. & JAN, E. 2009. An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2 α phosphorylation. *J Biol Chem*, 284, 6661-73.
- LEHMAN, J. J., BARGER, P. M., KOVACS, A., SAFFITZ, J. E., MEDEIROS, D. M. & KELLY, D. P. 2000. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest*, 106, 847-56.
- LEVY, F. H. & KELLY, D. P. 1997. Regulation of ATP synthase subunit e gene expression by hypoxia: cell differentiation stage-specific control. *Am J Physiol*, 272, C457-65.
- LI, L., TAO, G., HILL, M. C., ZHANG, M., MORIKAWA, Y. & MARTIN, J. F. 2018. Pitx2 maintains mitochondrial function during regeneration to prevent myocardial fat deposition. *Development*, 145.
- LI, T., ZHANG, Z., KOLWICZ, S. C., JR., ABELL, L., ROE, N. D., KIM, M., ZHOU, B., CAO, Y., RITTERHOFF, J., GU, H., RAFTERY, D., SUN, H. & TIAN, R. 2017. Defective Branched-Chain Amino Acid Catabolism Disrupts Glucose Metabolism and Sensitizes the Heart to Ischemia-Reperfusion Injury. *Cell Metab*, 25, 374-385.
- LOPASCHUK, G. D., KARWI, Q. G., TIAN, R., WENDE, A. R. & ABEL, E. D. 2021. Cardiac Energy Metabolism in Heart Failure. *Circ Res*, 128, 1487-1513.
- LOPEZ, A. B., WANG, C., HUANG, C. C., YAMAN, I., LI, Y., CHAKRAVARTY, K., JOHNSON, P. F., CHIANG, C. M., SNIDER, M. D., WEK, R. C. & HATZOGLOU, M. 2007. A feedback transcriptional mechanism controls the level of the arginine/lysine transporter cat-1 during amino acid starvation. *Biochem J*, 402, 163-73.
- LU, P. D., HARDING, H. P. & RON, D. 2004. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol*, 167, 27-33.
- LYONS, G. E., MUHLEBACH, S., MOSER, A., MASOOD, R., PATERSON, B. M., BUCKINGHAM, M. E. & PERRIARD, J. C. 1991. Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. *Development*, 113, 1017-29.
- MANN, I. K., CHATTERJEE, R., ZHAO, J., HE, X., WEIRAUCH, M. T., HUGHES, T. R. & VINSON, C. 2013. CG methylated microarrays identify a novel methylated sequence bound by the CEBPB|ATF4 heterodimer that is active in vivo. *Genome Res*, 23, 988-97.
- MILENKOVIC, D., MATIC, S., KUHL, I., RUZZENENTE, B., FREYER, C., JEMT, E., PARK, C. B., FALKENBERG, M. & LARSSON, N. G. 2013. TWINKLE is an essential mitochondrial helicase required for synthesis of nascent D-loop strands and complete mtDNA replication. *Hum Mol Genet*, 22, 1983-93.
- MISRA, J., HOLMES, M. J., E, T. M., LANGEVIN, M., KIM, H. G., CARLSON, K. R., WATFORD, M., DONG, X. C., ANTHONY, T. G. & WEK, R. C. 2021. Discordant regulation of eIF2 kinase GCN2 and mTORC1 during nutrient stress. *Nucleic Acids Res*, 49, 5726-5742.
- MITCHELL, J. A. & VAN KAINEN, B. R. 1992. Effects of alcohol on intrauterine oxygen tension in the rat. *Alcohol Clin Exp Res*, 16, 308-10.
- MOLLOVA, M., BERSELL, K., WALSH, S., SAVLA, J., DAS, L. T., PARK, S. Y., SILBERSTEIN, L. E., DOS REMEDIOS, C. G., GRAHAM, D., COLAN, S. &

- KUHN, B. 2013. Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc Natl Acad Sci U S A*, 110, 1446-51.
- MOOTHA, V. K., LEPAGE, P., MILLER, K., BUNKENBORG, J., REICH, M., HJERRILD, M., DELMONTE, T., VILLENEUVE, A., SLADEK, R., XU, F., MITCHELL, G. A., MORIN, C., MANN, M., HUDSON, T. J., ROBINSON, B., RIOUX, J. D. & LANDER, E. S. 2003. Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc Natl Acad Sci U S A*, 100, 605-10.
- MORRIS, D. R. & GEBALLE, A. P. 2000. Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol*, 20, 8635-42.
- NAKANO, A., TSUJI, D., MIKI, H., CUI, Q., EL SAYED, S. M., IKEGAME, A., ODA, A., AMOU, H., NAKAMURA, S., HARADA, T., FUJII, S., KAGAWA, K., TAKEUCHI, K., SAKAI, A., OZAKI, S., OKANO, K., NAKAMURA, T., ITOH, K., MATSUMOTO, T. & ABE, M. 2011. Glycolysis inhibition inactivates ABC transporters to restore drug sensitivity in malignant cells. *PLoS One*, 6, e27222.
- NAKANO, H., MINAMI, I., BRAAS, D., PAPPOE, H., WU, X., SAGADEVAN, A., VERGNES, L., FU, K., MORSELLI, M., DUNHAM, C., DING, X., STIEG, A. Z., GIMZEWSKI, J. K., PELLEGRINI, M., CLARK, P. M., REUE, K., LUSIS, A. J., RIBALET, B., KURDISTANI, S. K., CHRISTOFK, H., NAKATSUJI, N. & NAKANO, A. 2017. Glucose inhibits cardiac muscle maturation through nucleotide biosynthesis. *Elife*, 6.
- NEILL, G. & MASSON, G. R. 2023. A stay of execution: ATF4 regulation and potential outcomes for the integrated stress response. *Front Mol Neurosci*, 16, 1112253.
- NOVOA, I., ZENG, H., HARDING, H. P. & RON, D. 2001. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol*, 153, 1011-22.
- OHOKA, N., YOSHII, S., HATTORI, T., ONOZAKI, K. & HAYASHI, H. 2005. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J*, 24, 1243-55.
- PAKOS-ZEBRUCKA, K., KORYGA, I., MNICH, K., LJUJIC, M., SAMALI, A. & GORMAN, A. M. 2016. The integrated stress response. *EMBO Rep*, 17, 1374-1395.
- PALAM, L. R., BAIRD, T. D. & WEK, R. C. 2011. Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. *J Biol Chem*, 286, 10939-49.
- PAPATHANASSIU, A. E., KO, J. H., IMPRIALOU, M., BAGNATI, M., SRIVASTAVA, P. K., VU, H. A., CUCCHI, D., MCADOO, S. P., ANANIEVA, E. A., MAURO, C. & BEHMOARAS, J. 2017. BCAT1 controls metabolic reprogramming in activated human macrophages and is associated with inflammatory diseases. *Nat Commun*, 8, 16040.
- PAREDES, A., JUSTO-MENDEZ, R., JIMENEZ-BLASCO, D., NUNEZ, V., CALERO, I., VILLALBA-ORERO, M., ALEGRE-MARTI, A., FISCHER, T., GRADILLAS, A., SANT'ANNA, V. A. R., WERE, F., HUANG, Z., HERNANSANZ-AGUSTIN, P., CONTRERAS, C., MARTINEZ, F., CAMAFEITA, E., VAZQUEZ, J., RUIZ-CABELLO, J., AREA-GOMEZ, E., SANCHEZ-CABO, F., TREUTER, E., BOLANOS, J. P., ESTEBANEZ-PERPINA, E., RUPEREZ, F. J., BARBAS, C., ENRIQUEZ, J. A. & RICOTE, M. 2023. Author Correction: gamma-Linolenic acid in maternal milk drives cardiac metabolic maturation. *Nature*, 619, E24.
- PARSONS, W. J., WILLIAMS, R. S., SHELTON, J. M., LUO, Y., KESSLER, D. J. & RICHARDSON, J. A. 1996. Developmental regulation of cytochrome oxidase

- subunit VIa isoforms in cardiac and skeletal muscle. *Am J Physiol*, 270, H567-74.
- RADFORD, N. B., WAN, B., RICHMAN, A., SZCZEPANIAK, L. S., LI, J. L., LI, K., PFEIFFER, K., SCHAGGER, H., GARRY, D. J. & MOREADITH, R. W. 2002. Cardiac dysfunction in mice lacking cytochrome-c oxidase subunit VIaH. *Am J Physiol Heart Circ Physiol*, 282, H726-33.
- REYNOLDS, J. D., PENNING, D. H., DEXTER, F., ATKINS, B., HRDY, J., PODUSKA, D. & BRIEN, J. F. 1996. Ethanol increases uterine blood flow and fetal arterial blood oxygen tension in the near-term pregnant ewe. *Alcohol*, 13, 251-6.
- RICHTER-DENNERLEIN, R., OELJEKLAUS, S., LORENZI, I., RONSOR, C., BARETH, B., SCHENDZIELORZ, A. B., WANG, C., WARSCHEID, B., REHLING, P. & DENNERLEIN, S. 2016. Mitochondrial Protein Synthesis Adapts to Influx of Nuclear-Encoded Protein. *Cell*, 167, 471-483 e10.
- RIMBAUD, S., GARNIER, A. & VENTURA-CLAPIER, R. 2009. Mitochondrial biogenesis in cardiac pathophysiology. *Pharmacol Rep*, 61, 131-8.
- SAVAS, J. N., PARK, S. K. & YATES, J. R., 3RD 2016. Proteomic Analysis of Protein Turnover by Metabolic Whole Rodent Pulse-Chase Isotopic Labeling and Shotgun Mass Spectrometry Analysis. *Methods Mol Biol*, 1410, 293-304.
- SCHÄGGER, H. & VON JAGOW, G. 1991. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Analytical biochemistry*, 199, 223-231.
- SCIACOVELLI, M., DUGOURD, A., JIMENEZ, L. V., YANG, M., NIKITOPOULOU, E., COSTA, A. S. H., TRONCI, L., CARAFFINI, V., RODRIGUES, P., SCHMIDT, C., RYAN, D. G., YOUNG, T., ZECCHINI, V. R., ROSSI, S. H., MASSIE, C., LOHOFF, C., MASID, M., HATZIMANIKATIS, V., KUPPE, C., VON KRIEGSHEIM, A., KRAMANN, R., GNANAPRAGASAM, V., WARREN, A. Y., STEWART, G. D., EREZ, A., VANHARANTA, S., SAEZ-RODRIGUEZ, J. & FREZZA, C. 2022. Dynamic partitioning of branched-chain amino acids-derived nitrogen supports renal cancer progression. *Nat Commun*, 13, 7830.
- SPINELLI, J. B., ROSEN, P. C., SPRENGER, H. G., PUSZYNSKA, A. M., MANN, J. L., ROESSLER, J. M., CANGELOSI, A. L., HENNE, A., CONDON, K. J., ZHANG, T., KUNCHOK, T., LEWIS, C. A., CHANDEL, N. S. & SABATINI, D. M. 2021. Fumarate is a terminal electron acceptor in the mammalian electron transport chain. *Science*, 374, 1227-1237.
- STANLEY, W. C., RECCHIA, F. A. & LOPASCHUK, G. D. 2005. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev*, 85, 1093-129.
- TAANMAN, J. W. & CAPALDI, R. A. 1993. Subunit VIa of yeast cytochrome c oxidase is not necessary for assembly of the enzyme complex but modulates the enzyme activity. Isolation and characterization of the nuclear-coded gene. *J Biol Chem*, 268, 18754-61.
- TAANMAN, J. W., HALL, R. E., TANG, C., MARUSICH, M. F., KENNAWAY, N. G. & CAPALDI, R. A. 1993. Tissue distribution of cytochrome c oxidase isoforms in mammals. Characterization with monoclonal and polyclonal antibodies. *Biochim Biophys Acta*, 1225, 95-100.
- TAMEIRE, F., VERGINADIS, II, LELI, N. M., POLTE, C., CONN, C. S., OJHA, R., SALINAS, C. S., CHINGA, F., MONROY, A. M., FU, W., WANG, P., KOSSENKOV, A., YE, J., AMARAVADI, R. K., IGNATOVA, Z., FUCHS, S. Y., DIEHL, J. A., RUGGERO, D. & KOUMENIS, C. 2019. Author Correction: ATF4 couples MYC-dependent translational activity to bioenergetic demands during tumour progression. *Nat Cell Biol*, 21, 1052.

- TAO, G., KAHR, P. C., MORIKAWA, Y., ZHANG, M., RAHMANI, M., HEALLEN, T. R., LI, L., SUN, Z., OLSON, E. N., AMENDT, B. A. & MARTIN, J. F. 2016. Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. *Nature*, 534, 119-23.
- THIAVILLE, M. M., DUDENHAUSEN, E. E., ZHONG, C., PAN, Y. X. & KILBERG, M. S. 2008. Deprivation of protein or amino acid induces C/EBP β synthesis and binding to amino acid response elements, but its action is not an absolute requirement for enhanced transcription. *Biochem J*, 410, 473-84.
- VATTEM, K. M. & WEK, R. C. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A*, 101, 11269-74.
- VERCELLINO, I. & SAZANOV, L. A. 2022. The assembly, regulation and function of the mitochondrial respiratory chain. *Nat Rev Mol Cell Biol*, 23, 141-161.
- WANG, P., LI, J., TAO, J. & SHA, B. 2018. The luminal domain of the ER stress sensor protein PERK binds misfolded proteins and thereby triggers PERK oligomerization. *J Biol Chem*, 293, 4110-4121.
- WANG, X., WANG, L.-T. & YU, B. 2022. UBE2D1 and COX7C as Potential Biomarkers of Diabetes-Related Sepsis. *BioMed Research International*, 2022, 9463717.
- WEBSTER, W. S. & ABELA, D. 2007. The effect of hypoxia in development. *Birth Defects Res C Embryo Today*, 81, 215-28.
- WEK, R. C. 2018. Role of eIF2 α Kinases in Translational Control and Adaptation to Cellular Stress. *Cold Spring Harb Perspect Biol*, 10.
- WITTIG, I., BRAUN, H. P. & SCHAGGER, H. 2006. Blue native PAGE. *Nat Protoc*, 1, 418-28.
- WITTIG, I. & MALACARNE, P. F. 2021. Complexome Profiling: Assembly and Remodeling of Protein Complexes. *Int J Mol Sci*, 22.
- WU, B., CHEN, S., ZHUANG, L. & ZENG, J. 2020. The expression level of COX7C associates with venous thromboembolism in colon cancer patients. *Clinical and Experimental Medicine*, 20, 527-533.
- XIAO, F. & GUO, F. 2022. Impacts of essential amino acids on energy balance. *Mol Metab*, 57, 101393.
- YANG, D., LIU, H., CAI, Y., LU, K., ZHONG, X., XING, S., SONG, W., ZHANG, Y., YE, L., ZHU, X., WANG, T., ZHANG, P., LI, S. T., FENG, J., JIA, W., ZHANG, H. & GAO, P. 2022. Branched-chain amino acid catabolism breaks glutamine addiction to sustain hepatocellular carcinoma progression. *Cell Rep*, 41, 111691.
- YE, X., XIE, Y., SHI, Y., WANG, B., HAN, X., ZHOU, X., PAN, K., WANG, M. & FANG, H. 2023. Switching ubiquitous and muscle-specific isoforms of mitochondrial respiratory complex IV in skeletal muscle fine-tunes complex IV activity. *FASEB J*, 37, e22891.
- ZHOU, D., PALAM, L. R., JIANG, L., NARASIMHAN, J., STASCHKE, K. A. & WEK, R. C. 2008. Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. *J Biol Chem*, 283, 7064-73.
- ZONG, S., WU, M., GU, J., LIU, T., GUO, R. & YANG, M. 2018. Structure of the intact 14-subunit human cytochrome c oxidase. *Cell Res*, 28, 1026-1034.
- ABBAS, Y. M., WU, D., BUELER, S. A., ROBINSON, C. V. & RUBINSTEIN, J. L. 2020. Structure of V-ATPase from the mammalian brain. *Science*, 367, 1240-1246.
- ACIN-PEREZ, R., FERNANDEZ-SILVA, P., PELEATO, M. L., PEREZ-MARTOS, A. & ENRIQUEZ, J. A. 2008. Respiratory active mitochondrial supercomplexes. *Mol Cell*, 32, 529-39.

- AHOLA, S., RIVERA MEJIAS, P., HERMANS, S., CHANDRAGIRI, S., GIAVALISCO, P., NOLTE, H. & LANGER, T. 2022. OMA1-mediated integrated stress response protects against ferroptosis in mitochondrial cardiomyopathy. *Cell Metab*, 34, 1875-1891 e7.
- ALCÁZAR-FABRA, M., RODRÍGUEZ-SÁNCHEZ, F., TREVISSON, E. & BREA-CALVO, G. 2021. Primary Coenzyme Q deficiencies: A literature review and online platform of clinical features to uncover genotype-phenotype correlations. *Free Radical Biology and Medicine*, 167, 141-180.
- ALGIRE, M. A., MAAG, D. & LORSCH, J. R. 2005. Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation. *Mol Cell*, 20, 251-62.
- AMERI, K. & HARRIS, A. L. 2008. Activating transcription factor 4. *Int J Biochem Cell Biol*, 40, 14-21.
- AMOS, A., AMOS, A., WU, L. & XIA, H. 2023. The Warburg effect modulates DHODH role in ferroptosis: a review. *Cell Commun Signal*, 21, 100.
- ANDERSON, S., BANKIER, A. T., BARRELL, B. G., DE BRUIJN, M. H., COULSON, A. R., DROUIN, J., EPERON, I. C., NIERLICH, D. P., ROE, B. A., SANGER, F., SCHREIER, P. H., SMITH, A. J., STADEN, R. & YOUNG, I. G. 1981. Sequence and organization of the human mitochondrial genome. *Nature*, 290, 457-65.
- ANTHONY, G., REIMANN, A. & KADENBACH, B. 1993. Tissue-specific regulation of bovine heart cytochrome-c oxidase activity by ADP via interaction with subunit VIa. *Proc Natl Acad Sci U S A*, 90, 1652-6.
- ASCUITTO, R. J. & ROSS-ASCUITTO, N. T. 1996. Substrate metabolism in the developing heart. *Semin Perinatol*, 20, 542-63.
- BABCOCK, G. T. & WIKSTROM, M. 1992. Oxygen activation and the conservation of energy in cell respiration. *Nature*, 356, 301-9.
- BALSA, E., SOUSTEK, M. S., THOMAS, A., COGLIATI, S., GARCIA-POYATOS, C., MARTIN-GARCIA, E., JEDRYCHOWSKI, M., GYGI, S. P., ENRIQUEZ, J. A. & PUIGSERVER, P. 2019. ER and Nutrient Stress Promote Assembly of Respiratory Chain Supercomplexes through the PERK-eIF2alpha Axis. *Mol Cell*, 74, 877-890 e6.
- BARADARAN, R., BERRISFORD, J. M., MINHAS, G. S. & SAZANOV, L. A. 2013. Crystal structure of the entire respiratory complex I. *Nature*, 494, 443-8.
- BEN-SAHRA, I., HOXHAJ, G., RICOULT, S. J. H., ASARA, J. M. & MANNING, B. D. 2016. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science*, 351, 728-733.
- BENSLEY, J. G., STACY, V. K., DE MATTEO, R., HARDING, R. & BLACK, M. J. 2010. Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease. *Eur Heart J*, 31, 2058-66.
- BERTAGNOLLI, M., HUYPARD, F., CLOUTIER, A., ANSTEY, Z., HUOT-MARCHAND, J. E., FALLAHA, C., PARADIS, P., SCHIFFRIN, E. L., DEBLOIS, D. & NUYT, A. M. 2014. Transient neonatal high oxygen exposure leads to early adult cardiac dysfunction, remodeling, and activation of the renin-angiotensin system. *Hypertension*, 63, 143-50.
- BESSMAN, S. P. & GEIGER, P. J. 1981. Transport of energy in muscle: the phosphorylcreatine shuttle. *Science*, 211, 448-52.
- BIRRELL, J. A., KING, M. S. & HIRST, J. 2011. A ternary mechanism for NADH oxidation by positively charged electron acceptors, catalyzed at the flavin site in respiratory complex I. *FEBS Lett*, 585, 2318-22.

- BLAUT, M., WHITTAKER, K., VALDOVINOS, A., ACKRELL, B. A., GUNSALUS, R. P. & CECCHINI, G. 1989. Fumarate reductase mutants of *Escherichia coli* that lack covalently bound flavin. *J Biol Chem*, 264, 13599-604.
- BOCZONADI, V., GIUNTA, M., LANE, M., TULINIUS, M., SCHARA, U. & HORVATH, R. 2015. Investigating the role of the physiological isoform switch of cytochrome c oxidase subunits in reversible mitochondrial disease. *Int J Biochem Cell Biol*, 63, 32-40.
- BONNE, G., SEIBEL, P., POSSEKEL, S., MARSAC, C. & KADENBACH, B. 1993. Expression of human cytochrome c oxidase subunits during fetal development. *Eur J Biochem*, 217, 1099-107.
- BRISCHIGLIARO, M. & ZEVIANI, M. 2021. Cytochrome c oxidase deficiency. *Biochim Biophys Acta Bioenerg*, 1862, 148335.
- BROOKS, S. P. J. & STOREY, K. B. 1991. Where is the glycolytic complex? A critical evaluation of present data from muscle tissue. *FEBS Letters*, 278, 135-138.
- BUCKINGHAM, M., MEILHAC, S. & ZAFFRAN, S. 2005. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet*, 6, 826-35.
- CARROLL, J., HE, J., DING, S., FEARNLEY, I. M. & WALKER, J. E. 2021. TMEM70 and TMEM242 help to assemble the rotor ring of human ATP synthase and interact with assembly factors for complex I. *Proc Natl Acad Sci U S A*, 118.
- CHAKRABARTY, R. P. & CHANDEL, N. S. 2022. Beyond ATP, new roles of mitochondria. *Biochem (Lond)*, 44, 2-8.
- CHAVEZ, J. D., LEE, C. F., CAUDAL, A., KELLER, A., TIAN, R. & BRUCE, J. E. 2018. Chemical Crosslinking Mass Spectrometry Analysis of Protein Conformations and Supercomplexes in Heart Tissue. *Cell Syst*, 6, 136-141 e5.
- CHO, Y. M., KWON, S., PAK, Y. K., SEOL, H. W., CHOI, Y. M., PARK, D. J., PARK, K. S. & LEE, H. K. 2006. Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun*, 348, 1472-8.
- CHOMYN, A., CLEETER, M. W., RAGAN, C. I., RILEY, M., DOOLITTLE, R. F. & ATTARDI, G. 1986. URF6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. *Science*, 234, 614-8.
- CHOMYN, A., MARIOTTINI, P., CLEETER, M. W., RAGAN, C. I., MATSUNO-YAGI, A., HATEFI, Y., DOOLITTLE, R. F. & ATTARDI, G. 1985. Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. *Nature*, 314, 592-7.
- CHUNG, S., ARRELL, D. K., FAUSTINO, R. S., TERZIC, A. & DZEJA, P. P. 2010. Glycolytic network restructuring integral to the energetics of embryonic stem cell cardiac differentiation. *J Mol Cell Cardiol*, 48, 725-34.
- CHUNG, S., DZEJA, P. P., FAUSTINO, R. S., PEREZ-TERZIC, C., BEHFAR, A. & TERZIC, A. 2007. Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells. *Nat Clin Pract Cardiovasc Med*, 4 Suppl 1, S60-7.
- CLISSOLD, P. & COLE, R. J. 1973. Regulation of ribosomal RNA synthesis during mammalian myogenesis in culture. *Exp Cell Res*, 80, 159-69.
- COHEN, D. M., WON, K. J., NGUYEN, N., LAZAR, M. A., CHEN, C. S. & STEGER, D. J. 2015. ATF4 licenses C/EBP β activity in human mesenchymal stem cells primed for adipogenesis. *Elife*, 4, e06821.
- COSTA-MATTIOLI, M. & WALTER, P. 2020. The integrated stress response: From mechanism to disease. *Science*, 368.
- COX, S. J. & GUNBERG, D. L. 1972. Metabolite utilization by isolated embryonic rat hearts in vitro. *J Embryol Exp Morphol*, 28, 235-45.

- CUNATOVA, K., REGUERA, D. P., HOUSTEK, J., MRACEK, T. & PECINA, P. 2020. Role of cytochrome c oxidase nuclear-encoded subunits in health and disease. *Physiol Res*, 69, 947-965.
- DANG, C., GOTTSCHLING, M., MANNING, K., O'CURRAIN, E., SCHNEIDER, S., STERRY, W., STOCKFLETH, E. & NINDL, I. 2006. Identification of dysregulated genes in cutaneous squamous cell carcinoma. *Oncology reports*, 16, 513-519.
- DEY, M., CAO, C., DAR, A. C., TAMURA, T., OZATO, K., SICHERI, F. & DEVER, T. E. 2005. Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell*, 122, 901-13.
- DIRKX, E., DA COSTA MARTINS, P. A. & DE WINDT, L. J. 2013. Regulation of fetal gene expression in heart failure. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1832, 2414-2424.
- DOGAN, S. A., PUJOL, C., MAITI, P., KUKAT, A., WANG, S., HERMANS, S., SENFT, K., WIBOM, R., RUGARLI, E. I. & TRIFUNOVIC, A. 2014a. Tissue-specific loss of DARS2 activates stress responses independently of respiratory chain deficiency in the heart. *Cell Metab*, 19, 458-69.
- DOGAN, S. A., PUJOL, C., MAITI, P., KUKAT, A., WANG, S., HERMANS, S., SENFT, K., WIBOM, R., RUGARLI, E. I. & TRIFUNOVIC, A. 2014b. Tissue-specific loss of DARS2 activates stress responses independently of respiratory chain deficiency in the heart. *Cell metabolism*, 19, 458-469.
- DONNELLY, N., GORMAN, A. M., GUPTA, S. & SAMALI, A. 2013. The eIF2alpha kinases: their structures and functions. *Cell Mol Life Sci*, 70, 3493-511.
- DUCKER, G. S. & RABINOWITZ, J. D. 2017. One-Carbon Metabolism in Health and Disease. *Cell Metab*, 25, 27-42.
- EL-MASRY, O. S. 2023. Gene expression profile and presentation of novel gene variants of COX7B and COX7C in a cohort of patients with chronic myeloid leukemia. *Electronic Journal of General Medicine*, 20.
- EWART, G. D., ZHANG, Y. Z. & CAPALDI, R. A. 1991. Switching of bovine cytochrome c oxidase subunit VIa isoforms in skeletal muscle during development. *FEBS Lett*, 292, 79-84.
- FEDOR, J. G. & HIRST, J. 2018. Mitochondrial Supercomplexes Do Not Enhance Catalysis by Quinone Channeling. *Cell Metab*, 28, 525-531 e4.
- FESSLER, E., ECKL, E. M., SCHMITT, S., MANCILLA, I. A., MEYER-BENDER, M. F., HANF, M., PHILIPPOU-MASSIER, J., KREBS, S., ZISCHKA, H. & JAE, L. T. 2020. A pathway coordinated by DELE1 relays mitochondrial stress to the cytosol. *Nature*, 579, 433-437.
- FIEDORCZUK, K., LETTS, J. A., DEGLIESPOSTI, G., KASZUBA, K., SKEHEL, M. & SAZANOV, L. A. 2016. Atomic structure of the entire mammalian mitochondrial complex I. *Nature*, 538, 406-410.
- FIERRO-MONTI, I., RACLE, J., HERNANDEZ, C., WARIDEL, P., HATZIMANIKATIS, V. & QUADRONI, M. 2013. A novel pulse-chase SILAC strategy measures changes in protein decay and synthesis rates induced by perturbation of proteostasis with an Hsp90 inhibitor. *PLoS One*, 8, e80423.
- FISCHER, A., TEN HOVE, M., SEBAG-MONTEFIORE, L., WAGNER, H., CLARKE, K., WATKINS, H., LYGATE, C. A. & NEUBAUER, S. 2010. Changes in creatine transporter function during cardiac maturation in the rat. *BMC Dev Biol*, 10, 70.
- FISHMAN, M. C. & CHIEN, K. R. 1997. Fashioning the vertebrate heart: earliest embryonic decisions. *Development*, 124, 2099-117.
- FOGLIA, M. J. & POSS, K. D. 2016. Building and re-building the heart by cardiomyocyte proliferation. *Development*, 143, 729-40.

- FORMOSA, L. E., MUELLNER-WONG, L., RELJIC, B., SHARPE, A. J., JACKSON, T. D., BEILHARZ, T. H., STOJANOVSKI, D., LAZAROU, M., STROUD, D. A. & RYAN, M. T. 2020. Dissecting the Roles of Mitochondrial Complex I Intermediate Assembly Complex Factors in the Biogenesis of Complex I. *Cell Rep*, 31, 107541.
- FRANGINI, M., FRANZOLIN, E., CHEMELLO, F., LAVEDER, P., ROMUALDI, C., BIANCHI, V. & RAMPAZZO, C. 2013. Synthesis of mitochondrial DNA precursors during myogenesis, an analysis in purified C2C12 myotubes. *J Biol Chem*, 288, 5624-35.
- FRIEND, K., BROOKS, H. A., PROPSON, N. E., THOMSON, J. A. & KIMBLE, J. 2015. Embryonic Stem Cell Growth Factors Regulate eIF2alpha Phosphorylation. *PLoS One*, 10, e0139076.
- GAO, X., WEN, X., ESSER, L., QUINN, B., YU, L., YU, C. A. & XIA, D. 2003. Structural basis for the quinone reduction in the bc1 complex: a comparative analysis of crystal structures of mitochondrial cytochrome bc1 with bound substrate and inhibitors at the Qi site. *Biochemistry*, 42, 9067-80.
- GARCIA, M. A., MEURS, E. F. & ESTEBAN, M. 2007. The dsRNA protein kinase PKR: virus and cell control. *Biochimie*, 89, 799-811.
- GARRY, D. J. & OLSON, E. N. 2006. A common progenitor at the heart of development. *Cell*, 127, 1101-4.
- GIRARD, J., FERRE, P., PEGORIER, J. P. & DUEE, P. H. 1992. Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. *Physiol Rev*, 72, 507-62.
- GLATZ, J. F. & VEERKAMP, J. H. 1982. Postnatal development of palmitate oxidation and mitochondrial enzyme activities in rat cardiac and skeletal muscle. *Biochim Biophys Acta*, 711, 327-35.
- GONG, G., SONG, M., CSORDAS, G., KELLY, D. P., MATKOVICH, S. J. & DORN, G. W., 2ND 2015. Parkin-mediated mitophagy directs perinatal cardiac metabolic maturation in mice. *Science*, 350, aad2459.
- GROSSMAN, L. I. & LOMAX, M. I. 1997. Nuclear genes for cytochrome c oxidase. *Biochim Biophys Acta*, 1352, 174-92.
- GU, J., WU, M., GUO, R., YAN, K., LEI, J., GAO, N. & YANG, M. 2016. The architecture of the mammalian respirasome. *Nature*, 537, 639-43.
- GUERRERO-CASTILLO, S., BAERTLING, F., KOWNATZKI, D., WESSELS, H. J., ARNOLD, S., BRANDT, U. & NIJTMANS, L. 2017. The Assembly Pathway of Mitochondrial Respiratory Chain Complex I. *Cell Metab*, 25, 128-139.
- GUO, X., AVILES, G., LIU, Y., TIAN, R., UNGER, B. A., LIN, Y. T., WIITA, A. P., XU, K., CORREIA, M. A. & KAMPMANN, M. 2020. Mitochondrial stress is relayed to the cytosol by an OMA1-DELE1-HRI pathway. *Nature*, 579, 427-432.
- HAI, T. & CURRAN, T. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A*, 88, 3720-4.
- HALLMAN, M. 1971. Changes in mitochondrial respiratory chain proteins during perinatal development. Evidence of the importance of environmental oxygen tension. *Biochim Biophys Acta*, 253, 360-72.
- HAN, A. P., YU, C., LU, L., FUJIWARA, Y., BROWNE, C., CHIN, G., FLEMING, M., LEBOULCH, P., ORKIN, S. H. & CHEN, J. J. 2001. Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J*, 20, 6909-18.
- HAN, J., BACK, S. H., HUR, J., LIN, Y. H., GILDERSLEEVE, R., SHAN, J., YUAN, C. L., KROKOWSKI, D., WANG, S., HATZOGLOU, M., KILBERG, M. S.,

- SARTOR, M. A. & KAUFMAN, R. J. 2013. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol*, 15, 481-90.
- HARDING, H. P., ORDONEZ, A., ALLEN, F., PARTS, L., INGLIS, A. J., WILLIAMS, R. L. & RON, D. 2019. The ribosomal P-stalk couples amino acid starvation to GCN2 activation in mammalian cells. *Elife*, 8.
- HARDING, H. P., ZHANG, Y. & RON, D. 1999. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature*, 397, 271-4.
- HARDING, H. P., ZHANG, Y., ZENG, H., NOVOA, I., LU, P. D., CALFON, M., SADRI, N., YUN, C., POPKO, B., PAULES, R., STOJDL, D. F., BELL, J. C., HETTMANN, T., LEIDEN, J. M. & RON, D. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell*, 11, 619-33.
- HEYMANN, M. A., IWAMOTO, H. S. & RUDOLPH, A. M. 1981. Factors affecting changes in the neonatal systemic circulation. *Annu Rev Physiol*, 43, 371-83.
- HINNEBUSCH, A. G. 1997. Translational regulation of yeast GCN4. A window on factors that control initiator-tRNA binding to the ribosome. *J Biol Chem*, 272, 21661-4.
- HOEIJMAKERS, J. H. 2009. DNA damage, aging, and cancer. *N Engl J Med*, 361, 1475-85.
- HOERTER, J. A., KUZNETSOV, A. & VENTURA-CLAPIER, R. 1991. Functional development of the creatine kinase system in perinatal rabbit heart. *Circ Res*, 69, 665-76.
- HOLMGREN, D., WAHLANDER, H., ERIKSSON, B., OLDFORS, A., HOLME, E. & TULINIUS, M. 2003. Cardiomyopathy in children with mitochondrial disease: clinical course and cardiological findings. *European heart journal*, 24, 280-288.
- HU, H., TIAN, M., DING, C. & YU, S. 2018. The C/EBP Homologous Protein (CHOP) Transcription Factor Functions in Endoplasmic Reticulum Stress-Induced Apoptosis and Microbial Infection. *Front Immunol*, 9, 3083.
- HUGGINS, C. J., MAYEKAR, M. K., MARTIN, N., SAYLOR, K. L., GONIT, M., JAILWALA, P., KASOJI, M., HAINES, D. C., QUIÑONES, O. A. & JOHNSON, P. F. 2016. C/EBP γ Is a Critical Regulator of Cellular Stress Response Networks through Heterodimerization with ATF4. *Molecular and Cellular Biology*, 36, 693-713.
- HUTTEMANN, M., JARADAT, S. & GROSSMAN, L. I. 2003. Cytochrome c oxidase of mammals contains a testes-specific isoform of subunit VIb--the counterpart to testes-specific cytochrome c? *Mol Reprod Dev*, 66, 8-16.
- HUTTEMANN, M., KADENBACH, B. & GROSSMAN, L. I. 2001. Mammalian subunit IV isoforms of cytochrome c oxidase. *Gene*, 267, 111-23.
- IKENISHI, A., OKAYAMA, H., IWAMOTO, N., YOSHITOME, S., TANE, S., NAKAMURA, K., OBAYASHI, T., HAYASHI, T. & TAKEUCHI, T. 2012. Cell cycle regulation in mouse heart during embryonic and postnatal stages. *Dev Growth Differ*, 54, 731-8.
- INGLES, J., GOLDSTEIN, J., THAXTON, C., CALESHU, C., CORTY, E. W., CROWLEY, S. B., DOUGHERTY, K., HARRISON, S. M., MCGLAUGHON, J. & MILKO, L. V. 2019. Evaluating the clinical validity of hypertrophic cardiomyopathy genes. *Circulation: Genomic and Precision Medicine*, 12, e002460.
- INGLIS, A. J., MASSON, G. R., SHAO, S., PERISIC, O., MCLAUGHLIN, S. H., HEGDE, R. S. & WILLIAMS, R. L. 2019. Activation of GCN2 by the ribosomal P-stalk. *Proc Natl Acad Sci U S A*, 116, 4946-4954.

- ITOI, T. & LOPASCHUK, G. D. 1993. The contribution of glycolysis, glucose oxidation, lactate oxidation, and fatty acid oxidation to ATP production in isolated biventricular working hearts from 2-week-old rabbits. *Pediatr Res*, 34, 735-41.
- IWATA, S., OSTERMEIER, C., LUDWIG, B. & MICHEL, H. 1995. Structure at 2.8 Å resolution of cytochrome c oxidase from *Paracoccus denitrificans*. *Nature*, 376, 660-9.
- JONES, A. J., BLAZA, J. N., VARGHESE, F. & HIRST, J. 2017. Respiratory Complex I in *Bos taurus* and *Paracoccus denitrificans* Pumps Four Protons across the Membrane for Every NADH Oxidized. *J Biol Chem*, 292, 4987-4995.
- JOUSSE, C., OYADOMARI, S., NOVOA, I., LU, P., ZHANG, Y., HARDING, H. P. & RON, D. 2003. Inhibition of a constitutive translation initiation factor 2α phosphatase, CReP, promotes survival of stressed cells. *J Cell Biol*, 163, 767-75.
- KALPAGE, H. A., BAZYLIANSKA, V., RECANATI, M. A., FITE, A., LIU, J., WAN, J., MANTENA, N., MALEK, M. H., PODGORSKI, I., HEATH, E. I., VAISHNAV, A., EDWARDS, B. F., GROSSMAN, L. I., SANDERSON, T. H., LEE, I. & HUTTEMANN, M. 2019. Tissue-specific regulation of cytochrome c by post-translational modifications: respiration, the mitochondrial membrane potential, ROS, and apoptosis. *FASEB J*, 33, 1540-1553.
- KANNT, A., SOULIMANE, T., BUSE, G., BECKER, A., BAMBERG, E. & MICHEL, H. 1998. Electrical current generation and proton pumping catalyzed by the ba3-type cytochrome c oxidase from *Thermus thermophilus*. *FEBS Lett*, 434, 17-22.
- KASHIWAGI, K., YOKOYAMA, T., NISHIMOTO, M., TAKAHASHI, M., SAKAMOTO, A., YONEMOCHI, M., SHIROUZU, M. & ITO, T. 2019. Structural basis for eIF2B inhibition in integrated stress response. *Science*, 364, 495-499.
- KASPAR, S., OERTLIN, C., SZCZEPANOWSKA, K., KUKAT, A., SENFT, K., LUCAS, C., BRODESSER, S., HATZOGLOU, M., LARSSON, O., TOPISIROVIC, I. & TRIFUNOVIC, A. 2021. Adaptation to mitochondrial stress requires CHOP-directed tuning of ISR. *Sci Adv*, 7.
- KELLY, R. G., BUCKINGHAM, M. E. & MOORMAN, A. F. 2014. Heart fields and cardiac morphogenesis. *Cold Spring Harb Perspect Med*, 4.
- KENNER, L. R., ANAND, A. A., NGUYEN, H. C., MYASNIKOV, A. G., KLOSE, C. J., MCGEEVER, L. A., TSAI, J. C., MILLER-VEDAM, L. E., WALTER, P. & FROST, A. 2019. eIF2B-catalyzed nucleotide exchange and phosphoregulation by the integrated stress response. *Science*, 364, 491-495.
- KHAN, N. A., NIKKANEN, J., YATSUGA, S., JACKSON, C., WANG, L., PRADHAN, S., KIVELA, R., PESSIA, A., VELAGAPUDI, V. & SUOMALAINEN, A. 2017. mTORC1 Regulates Mitochondrial Integrated Stress Response and Mitochondrial Myopathy Progression. *Cell Metab*, 26, 419-428 e5.
- KIM, Y., YANG, D. S., KATTI, P. & GLANCY, B. 2019. Protein composition of the muscle mitochondrial reticulum during postnatal development. *J Physiol*, 597, 2707-2727.
- KRISHNA, S., ARROJO, E. D. R., CAPITANIO, J. S., RAMACHANDRA, R., ELLISMAN, M. & HETZER, M. W. 2021. Identification of long-lived proteins in the mitochondria reveals increased stability of the electron transport chain. *Dev Cell*, 56, 2952-2965 e9.
- KUHL, I., MIRANDA, M., ATANASSOV, I., KUZNETSOVA, I., HINZE, Y., MOURIER, A., FILIPOVSKA, A. & LARSSON, N. G. 2017. Transcriptomic and proteomic landscape of mitochondrial dysfunction reveals secondary coenzyme Q deficiency in mammals. *Elife*, 6.

- KUHL, I., MIRANDA, M., POSSE, V., MILENKOVIC, D., MOURIER, A., SIIRA, S. J., BONEKAMP, N. A., NEUMANN, U., FILIPOVSKA, A., POLOSA, P. L., GUSTAFSSON, C. M. & LARSSON, N. G. 2016. POLRMT regulates the switch between replication primer formation and gene expression of mammalian mtDNA. *Sci Adv*, 2, e1600963.
- KÜHLBRANDT, W. 2019. Structure and Mechanisms of F-Type ATP Synthases. *Annual Review of Biochemistry*, 88, 515-549.
- KUKAT, C., DAVIES, K. M., WURM, C. A., SPAHR, H., BONEKAMP, N. A., KUHL, I., JOOS, F., POLOSA, P. L., PARK, C. B., POSSE, V., FALKENBERG, M., JAKOBS, S., KÜHLBRANDT, W. & LARSSON, N. G. 2015. Cross-strand binding of TFAM to a single mtDNA molecule forms the mitochondrial nucleoid. *Proc Natl Acad Sci U S A*, 112, 11288-93.
- LAI, L., WANG, M., MARTIN, O. J., LEONE, T. C., VEGA, R. B., HAN, X. & KELLY, D. P. 2014. A role for peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) in the regulation of cardiac mitochondrial phospholipid biosynthesis. *J Biol Chem*, 289, 2250-9.
- LANDONI, J. C., LAALO, T., GOFFART, S., KIVELÄ, R., SKUBE, K., NIEMINEN, A. I., WICKSTRÖM, S. A., STEWART, J. & SUOMALAINEN, A. 2022. Overactive mitochondrial DNA replisome causes neonatal heart failure via ferroptosis. *bioRxiv*, 2022.04.04.485133.
- LAPUENTE-BRUN, E., MORENO-LOSHUERTOS, R., ACIN-PEREZ, R., LATORRE-PELLICER, A., COLAS, C., Balsa, E., PERALES-CLEMENTE, E., QUIROS, P. M., CALVO, E., RODRIGUEZ-HERNANDEZ, M. A., NAVAS, P., CRUZ, R., CARRACEDO, A., LOPEZ-OTIN, C., PEREZ-MARTOS, A., FERNANDEZ-SILVA, P., FERNANDEZ-VIZARRA, E. & ENRIQUEZ, J. A. 2013. Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. *Science*, 340, 1567-70.
- LARSON, D. E., XIE, W., GLIBETIC, M., O'MAHONY, D., SELLS, B. H. & ROTHBLUM, L. I. 1993. Coordinated decreases in rRNA gene transcription factors and rRNA synthesis during muscle cell differentiation. *Proc Natl Acad Sci U S A*, 90, 7933-6.
- LARSSON, N.-G., WANG, J., WILHELMSSON, H., OLDFORS, A., RUSTIN, P., LEWANDOSKI, M., BARSH, G. S. & CLAYTON, D. A. 1998. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature Genetics*, 18, 231-236.
- LAWRENCE, J., XIAO, D., XUE, Q., REJALI, M., YANG, S. & ZHANG, L. 2008. Prenatal nicotine exposure increases heart susceptibility to ischemia/reperfusion injury in adult offspring. *J Pharmacol Exp Ther*, 324, 331-41.
- LAZAROU, M., SMITH, S. M., THORBURN, D. R., RYAN, M. T. & MCKENZIE, M. 2009. Assembly of nuclear DNA-encoded subunits into mitochondrial complex IV, and their preferential integration into supercomplex forms in patient mitochondria. *FEBS J*, 276, 6701-13.
- LEE, I. & KADENBACH, B. 2001. Palmitate decreases proton pumping of liver-type cytochrome c oxidase. *Eur J Biochem*, 268, 6329-34.
- LEE, Y. Y., CEVALLOS, R. C. & JAN, E. 2009. An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2alpha phosphorylation. *J Biol Chem*, 284, 6661-73.
- LEHMAN, J. J., BARGER, P. M., KOVACS, A., SAFFITZ, J. E., MEDEIROS, D. M. & KELLY, D. P. 2000. Peroxisome proliferator-activated receptor gamma

- coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest*, 106, 847-56.
- LEONE, V. & FARALDO-GOMEZ, J. D. 2016. Structure and mechanism of the ATP synthase membrane motor inferred from quantitative integrative modeling. *J Gen Physiol*, 148, 441-457.
- LESCROART, F., CHABAB, S., LIN, X., RULANDS, S., PAULISSEN, C., RODOLOSSE, A., AUER, H., ACHOURI, Y., DUBOIS, C., BONDUE, A., SIMONS, B. D. & BLANPAIN, C. 2014. Early lineage restriction in temporally distinct populations of Mesp1 progenitors during mammalian heart development. *Nat Cell Biol*, 16, 829-40.
- LETTIS, J. A., FIEDORCZUK, K. & SAZANOV, L. A. 2016. The architecture of respiratory supercomplexes. *Nature*, 537, 644-648.
- LEVY, F. H. & KELLY, D. P. 1997. Regulation of ATP synthase subunit e gene expression by hypoxia: cell differentiation stage-specific control. *Am J Physiol*, 272, C457-65.
- LI, L., TAO, G., HILL, M. C., ZHANG, M., MORIKAWA, Y. & MARTIN, J. F. 2018. Pitx2 maintains mitochondrial function during regeneration to prevent myocardial fat deposition. *Development*, 145.
- LI, T., ZHANG, Z., KOLWICZ, S. C., JR., ABELL, L., ROE, N. D., KIM, M., ZHOU, B., CAO, Y., RITTERHOFF, J., GU, H., RAFTERY, D., SUN, H. & TIAN, R. 2017. Defective Branched-Chain Amino Acid Catabolism Disrupts Glucose Metabolism and Sensitizes the Heart to Ischemia-Reperfusion Injury. *Cell Metab*, 25, 374-385.
- LIU, F., LOSSL, P., RABBITS, B. M., BALABAN, R. S. & HECK, A. J. R. 2018. The interactome of intact mitochondria by cross-linking mass spectrometry provides evidence for coexisting respiratory supercomplexes. *Mol Cell Proteomics*, 17, 216-232.
- LIU, J., FU, X. & CHANG, Z. 2016. A reciprocating motion-driven rotation mechanism for the ATP synthase. *Sci China Life Sci*, 59, 44-8.
- LOBO-JARNE, T., PEREZ-PEREZ, R., FONTANESI, F., TIMON-GOMEZ, A., WITTIG, I., PENAS, A., SERRANO-LORENZO, P., GARCIA-CONSUEGRA, I., ARENAS, J., MARTIN, M. A., BARRIENTOS, A. & UGALDE, C. 2020. Multiple pathways coordinate assembly of human mitochondrial complex IV and stabilization of respiratory supercomplexes. *EMBO J*, 39, e103912.
- LOPASCHUK, G. D. & JASWAL, J. S. 2010. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. *J Cardiovasc Pharmacol*, 56, 130-40.
- LOPASCHUK, G. D., KARWI, Q. G., TIAN, R., WENDE, A. R. & ABEL, E. D. 2021. Cardiac Energy Metabolism in Heart Failure. *Circ Res*, 128, 1487-1513.
- LOPASCHUK, G. D. & SPAFFORD, M. A. 1990. Energy substrate utilization by isolated working hearts from newborn rabbits. *Am J Physiol*, 258, H1274-80.
- LOPEZ, A. B., WANG, C., HUANG, C. C., YAMAN, I., LI, Y., CHAKRAVARTY, K., JOHNSON, P. F., CHIANG, C. M., SNIDER, M. D., WEK, R. C. & HATZOGLOU, M. 2007. A feedback transcriptional mechanism controls the level of the arginine/lysine transporter cat-1 during amino acid starvation. *Biochem J*, 402, 163-73.
- LU, P. D., HARDING, H. P. & RON, D. 2004. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol*, 167, 27-33.

- LUNT, S. Y. & VANDER HEIDEN, M. G. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol*, 27, 441-64.
- LYONS, G. E., MUHLEBACH, S., MOSER, A., MASOOD, R., PATERSON, B. M., BUCKINGHAM, M. E. & PERRIARD, J. C. 1991. Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. *Development*, 113, 1017-29.
- MANASEK, F. J. 1968. Mitosis in developing cardiac muscle. *J Cell Biol*, 37, 191-6.
- MANN, I. K., CHATTERJEE, R., ZHAO, J., HE, X., WEIRAUCH, M. T., HUGHES, T. R. & VINSON, C. 2013. CG methylated microarrays identify a novel methylated sequence bound by the CEBPB|ATF4 heterodimer that is active in vivo. *Genome Res*, 23, 988-97.
- MARTIN, O. J., LAI, L., SOUNDARAPANDIAN, M. M., LEONE, T. C., ZORZANO, A., KELLER, M. P., ATTIE, A. D., MUOIO, D. M. & KELLY, D. P. 2014. A role for peroxisome proliferator-activated receptor gamma coactivator-1 in the control of mitochondrial dynamics during postnatal cardiac growth. *Circ Res*, 114, 626-36.
- MERCER, T. R., NEPH, S., DINGER, M. E., CRAWFORD, J., SMITH, M. A., SHEARWOOD, A. M., HAUGEN, E., BRACKEN, C. P., RACKHAM, O., STAMATOYANNOPOULOS, J. A., FILIPOVSKA, A. & MATTICK, J. S. 2011. The human mitochondrial transcriptome. *Cell*, 146, 645-58.
- MICK, D. U., DENNERLEIN, S., WIESE, H., REINHOLD, R., PACHEU-GRAU, D., LORENZI, I., SASARMAN, F., WERAARPACHAI, W., SHOUBRIDGE, E. A., WARSCHIED, B. & REHLING, P. 2012. MITRAC links mitochondrial protein translocation to respiratory-chain assembly and translational regulation. *Cell*, 151, 1528-41.
- MICK, E., TITOV, D. V., SKINNER, O. S., SHARMA, R., JOURDAIN, A. A. & MOOTHA, V. K. 2020. Distinct mitochondrial defects trigger the integrated stress response depending on the metabolic state of the cell. *Elife*, 9.
- MILENKOVIC, D., MATIC, S., KUHL, I., RUZZENENTE, B., FREYER, C., JEMT, E., PARK, C. B., FALKENBERG, M. & LARSSON, N. G. 2013. TWINKLE is an essential mitochondrial helicase required for synthesis of nascent D-loop strands and complete mtDNA replication. *Hum Mol Genet*, 22, 1983-93.
- MILENKOVIC, D., MISIC, J., HEVLER, J. F., MOLINIE, T., CHUNG, I., ATANASSOV, I., LI, X., FILOGRANA, R., MESAROS, A., MOURIER, A., HECK, A. J. R., HIRST, J. & LARSSON, N. G. 2023. Preserved respiratory chain capacity and physiology in mice with profoundly reduced levels of mitochondrial respirasomes. *Cell Metab*.
- MISRA, J., HOLMES, M. J., E, T. M., LANGEVIN, M., KIM, H. G., CARLSON, K. R., WATFORD, M., DONG, X. C., ANTHONY, T. G. & WEK, R. C. 2021. Discordant regulation of eIF2 kinase GCN2 and mTORC1 during nutrient stress. *Nucleic Acids Res*, 49, 5726-5742.
- MITCHELL, J. A. & VAN KAINEN, B. R. 1992. Effects of alcohol on intrauterine oxygen tension in the rat. *Alcohol Clin Exp Res*, 16, 308-10.
- MITCHELL, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*, 191, 144-8.
- MITCHELL, P. 1976. Possible molecular mechanisms of the protonmotive function of cytochrome systems. *J Theor Biol*, 62, 327-67.
- MOLINIE, T., COUGOUILLES, E., DAVID, C., CAHOREAU, E., PORTAIS, J. C. & MOURIER, A. 2022. MDH2 produced OAA is a metabolic switch rewiring the

- fuelling of respiratory chain and TCA cycle. *Biochim Biophys Acta Bioenerg*, 1863, 148532.
- MOLLOVA, M., BERSELL, K., WALSH, S., SAVLA, J., DAS, L. T., PARK, S. Y., SILBERSTEIN, L. E., DOS REMEDIOS, C. G., GRAHAM, D., COLAN, S. & KUHN, B. 2013. Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc Natl Acad Sci U S A*, 110, 1446-51.
- MONTOYA, J., CHRISTIANSON, T., LEVENS, D., RABINOWITZ, M. & ATTARDI, G. 1982. Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. *Proc Natl Acad Sci U S A*, 79, 7195-9.
- MOOSAVI, B., BERRY, E. A., ZHU, X. L., YANG, W. C. & YANG, G. F. 2019. The assembly of succinate dehydrogenase: a key enzyme in bioenergetics. *Cell Mol Life Sci*, 76, 4023-4042.
- MOOTHA, V. K., LEPAGE, P., MILLER, K., BUNKENBORG, J., REICH, M., HJERRILD, M., DELMONTE, T., VILLENEUVE, A., SLADEK, R., XU, F., MITCHELL, G. A., MORIN, C., MANN, M., HUDSON, T. J., ROBINSON, B., RIOUX, J. D. & LANDER, E. S. 2003. Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc Natl Acad Sci U S A*, 100, 605-10.
- MORRIS, D. R. & GEBALLE, A. P. 2000. Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol*, 20, 8635-42.
- NAKANO, A., TSUJI, D., MIKI, H., CUI, Q., EL SAYED, S. M., IKEGAME, A., ODA, A., AMOU, H., NAKAMURA, S., HARADA, T., FUJII, S., KAGAWA, K., TAKEUCHI, K., SAKAI, A., OZAKI, S., OKANO, K., NAKAMURA, T., ITOH, K., MATSUMOTO, T. & ABE, M. 2011. Glycolysis inhibition inactivates ABC transporters to restore drug sensitivity in malignant cells. *PLoS One*, 6, e27222.
- NAKANO, H., MINAMI, I., BRAAS, D., PAPPOE, H., WU, X., SAGADEVAN, A., VERGNES, L., FU, K., MORSELLI, M., DUNHAM, C., DING, X., STIEG, A. Z., GIMZEWSKI, J. K., PELLEGRINI, M., CLARK, P. M., REUE, K., LUSIS, A. J., RIBALET, B., KURDISTANI, S. K., CHRISTOFK, H., NAKATSUJI, N. & NAKANO, A. 2017. Glucose inhibits cardiac muscle maturation through nucleotide biosynthesis. *Elife*, 6.
- NEILL, G. & MASSON, G. R. 2023. A stay of execution: ATF4 regulation and potential outcomes for the integrated stress response. *Front Mol Neurosci*, 16, 1112253.
- NIJTMANS, L. G., TAANMAN, J. W., MUIJSERS, A. O., SPEIJER, D. & VAN DEN BOGERT, C. 1998. Assembly of cytochrome-c oxidase in cultured human cells. *Eur J Biochem*, 254, 389-94.
- NIKKANEN, J., FORSSTROM, S., EURO, L., PAETAU, I., KOHNZ, R. A., WANG, L., CHILOV, D., VIINAMAKI, J., ROIVAINEN, A., MARJAMAKI, P., LILJENBACK, H., AHOLA, S., BUZKOVA, J., TERZIOGLU, M., KHAN, N. A., PIRNES-KARHU, S., PAETAU, A., LONNQVIST, T., SAJANTILA, A., ISOHANNI, P., TYYNISMAA, H., NOMURA, D. K., BATTERSBY, B. J., VELAGAPUDI, V., CARROLL, C. J. & SUOMALAINEN, A. 2016. Mitochondrial DNA Replication Defects Disturb Cellular dNTP Pools and Remodel One-Carbon Metabolism. *Cell Metab*, 23, 635-48.
- NOVOA, I., ZENG, H., HARDING, H. P. & RON, D. 2001. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol*, 153, 1011-22.
- OHOKA, N., YOSHII, S., HATTORI, T., ONOZAKI, K. & HAYASHI, H. 2005. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J*, 24, 1243-55.

- PAKOS-ZEBRUCKA, K., KORYGA, I., MNICH, K., LJUJIC, M., SAMALI, A. & GORMAN, A. M. 2016. The integrated stress response. *EMBO Rep*, 17, 1374-1395.
- PALAM, L. R., BAIRD, T. D. & WEK, R. C. 2011. Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. *J Biol Chem*, 286, 10939-49.
- PAPANICOLAOU, K. N., KIKUCHI, R., NGOH, G. A., COUGHLAN, K. A., DOMINGUEZ, I., STANLEY, W. C. & WALSH, K. 2012. Mitofusins 1 and 2 are essential for postnatal metabolic remodeling in heart. *Circ Res*, 111, 1012-26.
- PAPATHANASSIU, A. E., KO, J. H., IMPRIALOU, M., BAGNATI, M., SRIVASTAVA, P. K., VU, H. A., CUCCHI, D., MCADOO, S. P., ANANIEVA, E. A., MAURO, C. & BEHMOARAS, J. 2017. BCAT1 controls metabolic reprogramming in activated human macrophages and is associated with inflammatory diseases. *Nat Commun*, 8, 16040.
- PEREDES, A., JUSTO-MENDEZ, R., JIMENEZ-BLASCO, D., NUNEZ, V., CALERO, I., VILLALBA-ORERO, M., ALEGRE-MARTI, A., FISCHER, T., GRADILLAS, A., SANT'ANNA, V. A. R., WERE, F., HUANG, Z., HERNANSANZ-AGUSTIN, P., CONTRERAS, C., MARTINEZ, F., CAMAFEITA, E., VAZQUEZ, J., RUIZ-CABELLO, J., AREA-GOMEZ, E., SANCHEZ-CABO, F., TREUTER, E., BOLANOS, J. P., ESTEBANEZ-PERPINA, E., RUPEREZ, F. J., BARBAS, C., ENRIQUEZ, J. A. & RICOTE, M. 2023. Author Correction: gamma-Linolenic acid in maternal milk drives cardiac metabolic maturation. *Nature*, 619, E24.
- PARSONS, W. J., WILLIAMS, R. S., SHELTON, J. M., LUO, Y., KESSLER, D. J. & RICHARDSON, J. A. 1996. Developmental regulation of cytochrome oxidase subunit VIa isoforms in cardiac and skeletal muscle. *Am J Physiol*, 270, H567-74.
- PIQUEREAU, J., NOVOTOVA, M., FORTIN, D., GARNIER, A., VENTURA-CLAPIER, R., VEKSLER, V. & JOUBERT, F. 2010. Postnatal development of mouse heart: formation of energetic microdomains. *J Physiol*, 588, 2443-54.
- PIQUEREAU, J. & VENTURA-CLAPIER, R. 2018. Maturation of Cardiac Energy Metabolism During Perinatal Development. *Front Physiol*, 9, 959.
- POHJOISMAKI, J. L., BOETTGER, T., LIU, Z., GOFFART, S., SZIBOR, M. & BRAUN, T. 2012. Oxidative stress during mitochondrial biogenesis compromises mtDNA integrity in growing hearts and induces a global DNA repair response. *Nucleic Acids Res*, 40, 6595-607.
- POLINGER, I. S. 1973. Growth and DNA synthesis in embryonic chick heart cells, in vivo and in vitro. *Exp Cell Res*, 76, 253-62.
- PROTASONI, M., PEREZ-PEREZ, R., LOBO-JARNE, T., HARBOUR, M. E., DING, S., PENAS, A., DIAZ, F., MORAES, C. T., FEARNLEY, I. M., ZEVIANI, M., UGALDE, C. & FERNANDEZ-VIZARRA, E. 2020. Respiratory supercomplexes act as a platform for complex III-mediated maturation of human mitochondrial complexes I and IV. *EMBO J*, 39, e102817.
- PUENTE, B. N., KIMURA, W., MURALIDHAR, S. A., MOON, J., AMATRUDA, J. F., PHELPS, K. L., GRINSFELDER, D., ROTHERMEL, B. A., CHEN, R., GARCIA, J. A., SANTOS, C. X., THET, S., MORI, E., KINTER, M. T., RINDLER, P. M., ZACCHIGNA, S., MUKHERJEE, S., CHEN, D. J., MAHMOUD, A. I., GIACCA, M., RABINOVITCH, P. S., AROUMOUGAME, A., SHAH, A. M., SZWEDA, L. I. & SADEK, H. A. 2014. The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell*, 157, 565-79.

- RADFORD, N. B., WAN, B., RICHMAN, A., SZCZEPANIAK, L. S., LI, J. L., LI, K., PFEIFFER, K., SCHAGGER, H., GARRY, D. J. & MOREADITH, R. W. 2002. Cardiac dysfunction in mice lacking cytochrome-c oxidase subunit VIaH. *Am J Physiol Heart Circ Physiol*, 282, H726-33.
- RASHEED, M. & TARJAN, G. 2018. Succinate Dehydrogenase Complex: An Updated Review. *Arch Pathol Lab Med*, 142, 1564-1570.
- REYNOLDS, J. D., PENNING, D. H., DEXTER, F., ATKINS, B., HRDY, J., PODUSKA, D. & BRIEN, J. F. 1996. Ethanol increases uterine blood flow and fetal arterial blood oxygen tension in the near-term pregnant ewe. *Alcohol*, 13, 251-6.
- RICHTER-DENNERLEIN, R., OELJEKLAUS, S., LORENZI, I., RONSOR, C., BARETH, B., SCHENDZIELORZ, A. B., WANG, C., WARSCHEID, B., REHLING, P. & DENNERLEIN, S. 2016. Mitochondrial Protein Synthesis Adapts to Influx of Nuclear-Encoded Protein. *Cell*, 167, 471-483 e10.
- RIEGER, B., SHALAEVA, D. N., SOHNEL, A. C., KOHL, W., DUWE, P., MULKIDJANIAN, A. Y. & BUSCH, K. B. 2017. Lifetime imaging of GFP at CoxVIIIa reports respiratory supercomplex assembly in live cells. *Sci Rep*, 7, 46055.
- RIMBAUD, S., GARNIER, A. & VENTURA-CLAPIER, R. 2009. Mitochondrial biogenesis in cardiac pathophysiology. *Pharmacol Rep*, 61, 131-8.
- ROHRICHT, H., PRZYBYLA-TOSCANO, J., FORNER, J., BOUSSARDON, C., KEECH, O., ROUHIER, N. & MEYER, E. H. 2023. Mitochondrial ferredoxin-like is essential for forming complex I-containing supercomplexes in Arabidopsis. *Plant Physiol*, 191, 2170-2184.
- ROTTENBERG, H. 2022. The accelerated evolution of human cytochrome c oxidase - Selection for reduced rate and proton pumping efficiency? *Biochim Biophys Acta Bioenerg*, 1863, 148595.
- RUDOLPH, A. M. 1979. Fetal and neonatal pulmonary circulation. *Annu Rev Physiol*, 41, 383-95.
- RUDOLPH, A. M. & HEYMAN, M. A. 1974. Fetal and neonatal circulation and respiration. *Annu Rev Physiol*, 36, 187-207.
- RUPRECHT, J., IWATA, S., ROTHERY, R. A., WEINER, J. H., MAKLASHINA, E. & CECCHINI, G. 2011. Perturbation of the quinone-binding site of complex II alters the electronic properties of the proximal [3Fe-4S] iron-sulfur cluster. *J Biol Chem*, 286, 12756-65.
- RUZZENENTE, B., METODIEV, M. D., WREDENBERG, A., BRATIC, A., PARK, C. B., CÁMARA, Y., MILENKOVIC, D., ZICKERMANN, V., WIBOM, R. & HULTENBY, K. 2012. LRPPRC is necessary for polyadenylation and coordination of translation of mitochondrial mRNAs. *The EMBO journal*, 31, 443-456.
- SAKS, V., BERAUD, N. & WALLIMANN, T. 2008. Metabolic compartmentation - a system level property of muscle cells: real problems of diffusion in living cells. *Int J Mol Sci*, 9, 751-767.
- SAKS, V., DZEJA, P., SCHLATTNER, U., VENDELIN, M., TERZIC, A. & WALLIMANN, T. 2006. Cardiac system bioenergetics: metabolic basis of the Frank-Starling law. *J Physiol*, 571, 253-73.
- SAKS, V. A., BELIKOVA, Y. O. & KUZNETSOV, A. V. 1991. In vivo regulation of mitochondrial respiration in cardiomyocytes: specific restrictions for intracellular diffusion of ADP. *Biochim Biophys Acta*, 1074, 302-11.
- SAVAS, J. N., PARK, S. K. & YATES, J. R., 3RD 2016. Proteomic Analysis of Protein Turnover by Metabolic Whole Rodent Pulse-Chase Isotopic Labeling and Shotgun Mass Spectrometry Analysis. *Methods Mol Biol*, 1410, 293-304.

- SAZANOV, L. A. 2015. A giant molecular proton pump: structure and mechanism of respiratory complex I. *Nat Rev Mol Cell Biol*, 16, 375-88.
- SCHÄGGER, H. & PFEIFFER, K. 2000. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *The EMBO Journal*, 19, 1777-1783.
- SCHÄGGER, H. & VON JAGOW, G. 1991. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Analytical biochemistry*, 199, 223-231.
- SCIACOVELLI, M., DUGOURD, A., JIMENEZ, L. V., YANG, M., NIKITOPOULOU, E., COSTA, A. S. H., TRONCI, L., CARAFFINI, V., RODRIGUES, P., SCHMIDT, C., RYAN, D. G., YOUNG, T., ZECCHINI, V. R., ROSSI, S. H., MASSIE, C., LOHOFF, C., MASID, M., HATZIMANIKATIS, V., KUPPE, C., VON KRIEGSHEIM, A., KRAMANN, R., GNANAPRAGASAM, V., WARREN, A. Y., STEWART, G. D., EREZ, A., VANHARANTA, S., SAEZ-RODRIGUEZ, J. & FREZZA, C. 2022. Dynamic partitioning of branched-chain amino acids-derived nitrogen supports renal cancer progression. *Nat Commun*, 13, 7830.
- SHINZAWA-ITOH, K., AOYAMA, H., MURAMOTO, K., TERADA, H., KURAUCHI, T., TADEHARA, Y., YAMASAKI, A., SUGIMURA, T., KURONO, S., TSUJIMOTO, K., MIZUSHIMA, T., YAMASHITA, E., TSUKIHARA, T. & YOSHIKAWA, S. 2007. Structures and physiological roles of 13 integral lipids of bovine heart cytochrome c oxidase. *EMBO J*, 26, 1713-25.
- SINKLER, C. A., KALPAGE, H., SHAY, J., LEE, I., MALEK, M. H., GROSSMAN, L. I. & HUTTEMANN, M. 2017. Tissue- and Condition-Specific Isoforms of Mammalian Cytochrome c Oxidase Subunits: From Function to Human Disease. *Oxid Med Cell Longev*, 2017, 1534056.
- SOONPAA, M. H. & FIELD, L. J. 1998. Survey of studies examining mammalian cardiomyocyte DNA synthesis. *Circ Res*, 83, 15-26.
- SOUSA, J. S., MILLS, D. J., VONCK, J. & KUHLBRANDT, W. 2016. Functional asymmetry and electron flow in the bovine respirasome. *Elife*, 5.
- ST JOHN, J. C., RAMALHO-SANTOS, J., GRAY, H. L., PETROSKO, P., RAWE, V. Y., NAVARA, C. S., SIMERLY, C. R. & SCHATTEN, G. P. 2005. The expression of mitochondrial DNA transcription factors during early cardiomyocyte in vitro differentiation from human embryonic stem cells. *Cloning Stem Cells*, 7, 141-53.
- STANLEY, W. C., RECCHIA, F. A. & LOPASCHUK, G. D. 2005. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev*, 85, 1093-129.
- STROUD, D. A., SURGENOR, E. E., FORMOSA, L. E., RELJIC, B., FRAZIER, A. E., DIBLEY, M. G., OSELLAME, L. D., STAIT, T., BEILHARZ, T. H., THORBURN, D. R., SALIM, A. & RYAN, M. T. 2016. Accessory subunits are integral for assembly and function of human mitochondrial complex I. *Nature*, 538, 123-126.
- SUN, F., HUO, X., ZHAI, Y., WANG, A., XU, J., SU, D., BARTLAM, M. & RAO, Z. 2005. Crystal structure of mitochondrial respiratory membrane protein complex II. *Cell*, 121, 1043-57.
- SUOMALAINEN, A. & BATTERSBY, B. J. 2018. Mitochondrial diseases: the contribution of organelle stress responses to pathology. *Nat Rev Mol Cell Biol*, 19, 77-92.
- TAANMAN, J. W. & CAPALDI, R. A. 1993. Subunit VIa of yeast cytochrome c oxidase is not necessary for assembly of the enzyme complex but modulates the enzyme activity. Isolation and characterization of the nuclear-coded gene. *J Biol Chem*, 268, 18754-61.

- TAANMAN, J. W., HALL, R. E., TANG, C., MARUSICH, M. F., KENNAWAY, N. G. & CAPALDI, R. A. 1993. Tissue distribution of cytochrome c oxidase isoforms in mammals. Characterization with monoclonal and polyclonal antibodies. *Biochim Biophys Acta*, 1225, 95-100.
- TAEGTMEYER, H., SEN, S. & VELA, D. 2010. Return to the fetal gene program: a suggested metabolic link to gene expression in the heart. *Ann N Y Acad Sci*, 1188, 191-8.
- TALVAS, J., OBLED, A., FAFOURNOUX, P. & MORDIER, S. 2006. Regulation of protein synthesis by leucine starvation involves distinct mechanisms in mouse C2C12 myoblasts and myotubes. *J Nutr*, 136, 1466-71.
- TAMEIRE, F., VERGINADIS, II, LELI, N. M., POLTE, C., CONN, C. S., OJHA, R., SALINAS, C. S., CHINGA, F., MONROY, A. M., FU, W., WANG, P., KOSSENKOV, A., YE, J., AMARAVADI, R. K., IGNATOVA, Z., FUCHS, S. Y., DIEHL, J. A., RUGGERO, D. & KOUMENIS, C. 2019. Author Correction: ATF4 couples MYC-dependent translational activity to bioenergetic demands during tumour progression. *Nat Cell Biol*, 21, 1052.
- TAN, B. G., MUTTI, C. D., SHI, Y., XIE, X., ZHU, X., SILVA-PINHEIRO, P., MENDER, K. E., DIAZ-MALDONADO, H., WEI, W., NICHOLLS, T. J., CHINNERY, P. F., MINCZUK, M., FALKENBERG, M. & GUSTAFSSON, C. M. 2022. The human mitochondrial genome contains a second light strand promoter. *Mol Cell*, 82, 3646-3660 e9.
- TAN, C. M. J. & LEWANDOWSKI, A. J. 2020. The Transitional Heart: From Early Embryonic and Fetal Development to Neonatal Life. *Fetal Diagn Ther*, 47, 373-386.
- TANG, J. X., THOMPSON, K., TAYLOR, R. W. & OLAHOVA, M. 2020. Mitochondrial OXPHOS Biogenesis: Co-Regulation of Protein Synthesis, Import, and Assembly Pathways. *Int J Mol Sci*, 21.
- TAO, G., KAHR, P. C., MORIKAWA, Y., ZHANG, M., RAHMANI, M., HEALLEN, T. R., LI, L., SUN, Z., OLSON, E. N., AMENDT, B. A. & MARTIN, J. F. 2016. Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. *Nature*, 534, 119-23.
- THIAVILLE, M. M., DUDENHAUSEN, E. E., ZHONG, C., PAN, Y. X. & KILBERG, M. S. 2008. Deprivation of protein or amino acid induces C/EBPbeta synthesis and binding to amino acid response elements, but its action is not an absolute requirement for enhanced transcription. *Biochem J*, 410, 473-84.
- TIMON-GOMEZ, A., NYVLTOVA, E., ABRIATA, L. A., VILA, A. J., HOSLER, J. & BARRIENTOS, A. 2018. Mitochondrial cytochrome c oxidase biogenesis: Recent developments. *Semin Cell Dev Biol*, 76, 163-178.
- TRUMPOWER, B. L. 1990. The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc1 complex. *J Biol Chem*, 265, 11409-12.
- TYYNISMAA, H., CARROLL, C. J., RAIMUNDO, N., AHOLA-ERKKILA, S., WENZ, T., RUHANEN, H., GUSE, K., HEMMINKI, A., PELTOLA-MJOSUND, K. E., TULKKI, V., ORESIC, M., MORAES, C. T., PIETILAINEN, K., HOVATTA, I. & SUOMALAINEN, A. 2010. Mitochondrial myopathy induces a starvation-like response. *Hum Mol Genet*, 19, 3948-58.
- VAN BEEUMEN, J. J., VAN KUILENBURG, A. B. P., VAN BUN, S., VAN DEN BOGERT, C., TAGER, J. M. & MUIJSERS, A. O. 1990. Demonstration of two isoforms of subunit VIIa of cytochrome c oxidase from human skeletal muscle. *FEBS Letters*, 263, 213-216.

- VATTEM, K. M. & WEK, R. C. 2004. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc Natl Acad Sci U S A*, 101, 11269-74.
- VENTURA-CLAPIER, R., GARNIER, A., VEKSLER, V. & JOUBERT, F. 2011. Bioenergetics of the failing heart. *Biochim Biophys Acta*, 1813, 1360-72.
- VENTURA-CLAPIER, R., KUZNETSOV, A., VEKSLER, V., BOEHM, E. & ANFLOUS, K. 1998. Functional coupling of creatine kinases in muscles: species and tissue specificity. *Mol Cell Biochem*, 184, 231-47.
- VERCELLINO, I. & SAZANOV, L. A. 2022. The assembly, regulation and function of the mitochondrial respiratory chain. *Nat Rev Mol Cell Biol*, 23, 141-161.
- VIDONI, S., HARBOUR, M. E., GUERRERO-CASTILLO, S., SIGNES, A., DING, S., FEARNLEY, I. M., TAYLOR, R. W., TIRANTI, V., ARNOLD, S., FERNANDEZ-VIZARRA, E. & ZEVIANI, M. 2017. MR-1S Interacts with PET100 and PET117 in Module-Based Assembly of Human Cytochrome c Oxidase. *Cell Rep*, 18, 1727-1738.
- WAI, T., GARCIA-PRIETO, J., BAKER, M. J., MERKWIRTH, C., BENIT, P., RUSTIN, P., RUPEREZ, F. J., BARBAS, C., IBANEZ, B. & LANGER, T. 2015. Imbalanced OPA1 processing and mitochondrial fragmentation cause heart failure in mice. *Science*, 350, aad0116.
- WANG, L., KO, K. W., LUCCHINETTI, E., ZHANG, L., TROXLER, H., HERSBERGER, M., OMAR, M. A., POSSE DE CHAVES, E. I., LOPASCHUK, G. D., CLANACHAN, A. S. & ZAUGG, M. 2010. Metabolic profiling of hearts exposed to sevoflurane and propofol reveals distinct regulation of fatty acid and glucose oxidation: CD36 and pyruvate dehydrogenase as key regulators in anesthetic-induced fuel shift. *Anesthesiology*, 113, 541-51.
- WANG, P., LI, J., TAO, J. & SHA, B. 2018. The luminal domain of the ER stress sensor protein PERK binds misfolded proteins and thereby triggers PERK oligomerization. *J Biol Chem*, 293, 4110-4121.
- WANG, X., WANG, L.-T. & YU, B. 2022. *UBE2D1* and *COX7C* as Potential Biomarkers of Diabetes-Related Sepsis. *BioMed Research International*, 2022, 9463717.
- WANG, Y. & HEKIMI, S. 2016. Understanding Ubiquinone. *Trends Cell Biol*, 26, 367-378.
- WEBSTER, W. S. & ABELA, D. 2007. The effect of hypoxia in development. *Birth Defects Res C Embryo Today*, 81, 215-28.
- WEK, R. C. 2018. Role of eIF2alpha Kinases in Translational Control and Adaptation to Cellular Stress. *Cold Spring Harb Perspect Biol*, 10.
- WERAARPACHAI, W., ANTONICKA, H., SASARMAN, F., SEEGER, J., SCHRANK, B., KOLESAR, J. E., LOCHMULLER, H., CHEVRETTE, M., KAUFMAN, B. A., HORVATH, R. & SHOUBRIDGE, E. A. 2009. Mutation in TACO1, encoding a translational activator of COX I, results in cytochrome c oxidase deficiency and late-onset Leigh syndrome. *Nat Genet*, 41, 833-7.
- WITTIG, I., BRAUN, H. P. & SCHAGGER, H. 2006. Blue native PAGE. *Nat Protoc*, 1, 418-28.
- WITTIG, I. & MALACARNE, P. F. 2021. Complexome Profiling: Assembly and Remodeling of Protein Complexes. *Int J Mol Sci*, 22.
- WU, B., CHEN, S., ZHUANG, L. & ZENG, J. 2020. The expression level of COX7C associates with venous thromboembolism in colon cancer patients. *Clinical and Experimental Medicine*, 20, 527-533.

- XIA, D., ESSER, L., TANG, W. K., ZHOU, F., ZHOU, Y., YU, L. & YU, C. A. 2013. Structural analysis of cytochrome bc₁ complexes: implications to the mechanism of function. *Biochim Biophys Acta*, 1827, 1278-94.
- XIA, D., YU, C. A., KIM, H., XIA, J. Z., KACHURIN, A. M., ZHANG, L., YU, L. & DEISENHOFER, J. 1997. Crystal structure of the cytochrome bc₁ complex from bovine heart mitochondria. *Science*, 277, 60-6.
- XIAO, F. & GUO, F. 2022. Impacts of essential amino acids on energy balance. *Mol Metab*, 57, 101393.
- YAFFE, D. & SAXEL, O. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature*, 270, 725-7.
- YANG, D., LIU, H., CAI, Y., LU, K., ZHONG, X., XING, S., SONG, W., ZHANG, Y., YE, L., ZHU, X., WANG, T., ZHANG, P., LI, S. T., FENG, J., JIA, W., ZHANG, H. & GAO, P. 2022. Branched-chain amino acid catabolism breaks glutamine addiction to sustain hepatocellular carcinoma progression. *Cell Rep*, 41, 111691.
- YE, X., XIE, Y., SHI, Y., WANG, B., HAN, X., ZHOU, X., PAN, K., WANG, M. & FANG, H. 2023. Switching ubiquitous and muscle-specific isoforms of mitochondrial respiratory complex IV in skeletal muscle fine-tunes complex IV activity. *FASEB J*, 37, e22891.
- YOSHIKAWA, S., MURAMOTO, K. & SHINZAWA-ITOH, K. 2012. Reaction mechanism of mammalian mitochondrial cytochrome c oxidase. *Adv Exp Med Biol*, 748, 215-36.
- YOSHIKAWA, S., SHINZAWA-ITOH, K. & TSUKIHARA, T. 1998. Crystal structure of bovine heart cytochrome c oxidase at 2.8 Å resolution. *J Bioenerg Biomembr*, 30, 7-14.
- ZHOU, D., PALAM, L. R., JIANG, L., NARASIMHAN, J., STASCHKE, K. A. & WEK, R. C. 2008. Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. *J Biol Chem*, 283, 7064-73.
- ZHU, J., VINOTHKUMAR, K. R. & HIRST, J. 2016. Structure of mammalian respiratory complex I. *Nature*, 536, 354-358.
- ZONG, S., GU, J., LIU, T., GUO, R., WU, M. & YANG, M. 2018a. UQCRFS1N assembles mitochondrial respiratory complex-III into an asymmetric 21-subunit dimer. *Protein Cell*, 9, 586-591.
- ZONG, S., WU, M., GU, J., LIU, T., GUO, R. & YANG, M. 2018b. Structure of the intact 14-subunit human cytochrome c oxidase. *Cell Res*, 28, 1026-1034.

Acknowledgements

I would like to express my biggest gratitude to **Prof. Aleksandra Trifunovic**, for mentoring me and teaching me how to be a scientist. Firstly, thank you for showing me kindness and patience, and showing me that having empathy goes before any scientific achievements. Thank you for giving me an opportunity to become what I always dreamed of, a part of the scientific community on such a high level. Thank you for the advice, criticism, comforting words and all the snacks. Thank you for having a place for me and allowing me to grow both personally and academically. I will be forever grateful.

Further on, I would like to thank **Prof. Dr. Stefan Rozenkranz** and **Prof. Marcus Krüger** for their time and advice as my thesis advisor committee. I would also like to extend my gratitude to **Prof. Ana Garcia Saez** and **Prof. Jan Riemer** for taking the time to review my thesis and be part of my examination board. I am looking forward to discussing science with you in the future as well.

I would like to specifically thank the coordinator of the CCRC graduate program, which I was a part of, and my personal close friend **Dr. Monika Schlosser**. From the first day and the broken Elsa cup, to today you have provided support and care like no one else could. I am forever grateful and happy to have you in my life.

Thank you to **Trifunovic lab members**, past and present, for creating a constructive working environment that enabled me to grow as a scientist. Special thank you to **Matthijs** and **Hien** for being my best friends throughout the years. I will always remember all the coffee dates, the late night working hours, or the early working hours, and never ending conversations. Hien, you have been my sister in arm through out this phase of life, and I can never thank you enough for including me in your life. Cannot and will not imagine a life without you. Thank you **Alex**, for all the advice and coffee dates full of conversation, support and understanding and for being my scientific idol. Thank you **Aca**, for being my friend and scientific companion in the last two years in the lab, and for the science filled lunch and coffee dates.

Special thank you to **Marijana**, an amazing friend and older sister, for showing me what life is all about. I maybe would have saved my liver, but I would have been blind without you. Thank you to **Cristiana, Mariana, Andreas** and **Vincent** for giving me so much happiness in the last four years. The adventures of the hanging gardens will forever be remembered. Or forever not remembered. Love you all.

Finally, my last and biggest gratitude I extend towards my family. To my parents, **Snezana** and **Aleksandar**: there are no words to describe how grateful I am to be your daughter. You have shaped me in and out, and prepared me to be who I am today. To my mother who has unintentionally made a clone version of herself, I love you. To my father, who has unconditionally supported me and found ways to understand me, I love you.

To my sister, **Jasna**, and my brother, **Uros**, both my soulmates, thank you for all the understanding, listening and support. Thank you for keeping me in your minds and hearts even though I was so far away. I love you forever, and I love you the most.

Declaration for the doctoral thesis (dissertation)
according to the doctoral regulations published 12th March 2020

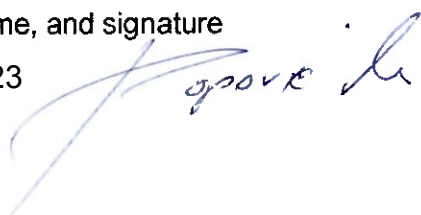
Non-official English translation of the "Erklärung zur Dissertation"
(The German version must be included in the doctoral thesis)

"I hereby declare that I have completed the present dissertation independently and without the use of any aids or literature other than those referred to. All passages that have been taken, either literally or in sense, from published and unpublished works, are marked as such. I declare that this dissertation has not been submitted to any other faculty or university; that - apart from the partial publications and included articles and manuscripts listed below - it has not yet been published, and that I will not publish the dissertation before completing my doctorate without the permission of the PhD Committee. I am aware of the terms of the doctoral regulations. In addition, I hereby declare that I am aware of the "Regulations for Safeguarding Good Scientific Practice and Dealing with Scientific Misconduct" of the University of Cologne, and that I have observed them during the work on the thesis project and the written doctoral thesis. I hereby commit myself to observe and implement the guidelines mentioned there in all scientific activities. I assure that the submitted electronic version is identical to the submitted printed version".

Partial publications of the thesis:

Date, name, and signature

02.09.2023

A handwritten signature in blue ink, appearing to be 'S. J. J. J.', written over the printed text 'Date, name, and signature'.

Erklärung zur Dissertation
gemäß der Promotionsordnung vom 12. März 2020

Diese Erklärung muss in der Dissertation enthalten sein.
(This version must be included in the doctoral thesis)

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.“

Teilpublikationen:

Datum, Name und Unterschrift

02.09.2023

