NOVEL DISEASE MECHANISMS AND EFFECTS OF GENE THERAPY IN MOUSE MODELS OF NEONATAL MITOCHONDRIAL COMPLEX III DEFICIENCY

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UNIVERSITY OF HELSINKI

DISSERTATIONES UNIVERSITATIS HELSINGIENSIS | 175 2025

Stem Cells and Metabolism Research program, Finnish Doctoral Programme in Oral Sciences & Faculty of Medicine
Folkhälsan Research Center
University of Helsinki
Dissertationes Universitatis Helsingiensis 175/2025

Novel Disease Mechanisms and Effects of Gene Therapy in Mouse Models of Neonatal Mitochondrial Complex III Deficiency

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Academic dissertation
To be presented for public discussion with the permission of
the Faculty of Medicine of the University of Helsinki,
at Lecture Hall 2, Biomedicum 1, Helsinki,
on the 16th of May 2022, at 1 o'clock

Helsinki | Finland | 2025

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Publisher: Helsingin yliopisto
Series: Dissertationes Universitatis Helsingiensis 175/2025

ISBN 978-952-84-0946-5 (print)
ISBN 978-952-84-0945-8 (online)
ISSN 2954-2898 (print)
ISSN 2954-2952 (online)
PunaMusta, Joensuu 2025

"সত্যিকারের পরীক্ষাগার হল মন, যেখানে আমরা বিভ্রমের আড়ালে সত্যের নিয়ম উন্মোচন করি।"

(The true laboratory is the mind, where behind illusions we uncover the laws of truth)

জগদীশ চন্দ্র বসু

Jagadish Chandra Bose

Renowned Bengali scientist, inventor, and
pioneer of wireless communication and plant physiology

ABSTRACT

Mitochondrial disorders represent a diverse group of monogenic metabolic conditions, often characterized by impaired electron transport chain (ETC), also known as the respiratory chain function. These disorders present a wide range of clinical manifestations, including myopathies, encephalopathies, and multi-organ involvement. Among these, mutations in the *BCS1L* gene, which encodes an assembly factor for ETC complex III (CIII), are the most common cause of CIII deficiency. The most severe phenotype caused by mutations compromising the function of the BCS1L (BCS1-like AAA-ATPase) is called GRACILE syndrome (**G**rowth **R**estriction, **A**minoaciduria, **C**holestasis, liver **I**ron overload, **L**actic acidosis, and **E**arly death), and it occurs in Finland. An experimental knock-in (KI) mouse model carrying the Finnish mutation (*Bcs1l*$^{p.S78G}$, homozygous Ser78Gly missense mutation) recapitulates most of the clinical manifestations and exhibits early mortality.

This thesis project identified surprising parallels between the phenotype of the CIII-deficient *Bcs1l*$^{p.S78G}$ KI mice and mouse models of typical laminopathic and DNA repair-deficient progeroid syndromes, or premature aging disorders. Progeroid features in these mice include growth impairment, kyphosis, reduced bone mineral density and fat mass, lymphoid atrophy, and thin skin. Consistent with progeroid pathology, *Bcs1l*$^{p.S78G}$ mice exhibit an early and widespread DNA damage response, cell cycle arrest, and mitotic defects in regenerating organs such as the liver, kidney, and exocrine pancreas, leading to extensive cellular senescence in the affected tissues, characterized by irreversible cell cycle arrest. This study was the first one to demonstrate that primary oxidative phosphorylation (OXPHOS) deficiency can cause segmental progeria and suggested that the detrimental cell cycle re-entry against depletion of energy and biosynthetic precursors is driving most of the progeroid pathology. While exploring pyrimidine nucleotide biosynthesis as a potential defective mechanism in CIII-deficient mice linked to premature aging, we encountered the challenge of lacking an enzymatic deoxyribonucleotide (dNTP) quantification method sensitive enough for the mouse tissues of our interest. To address this, we designed a novel assay utilizing a long synthetic template, the EvaGreen DNA dye, and robust high-fidelity DNA polymerase. This assay revealed increased purine dNTP concentrations in the *Bcs1l*$^{p.S78G}$ liver, indicating a potential disruption in nucleotide balance or liver proliferative activity.

Mitochondrial diseases lack effective treatments, and their clinical management depends solely on supportive care. Theoretically, the most effective approach to treat monogenic genetic diseases caused by nuclear gene mutations is gene replacement therapy, for example, using viruses as delivery vectors. The liver shows early CIII deficiency and histopathological change in the *Bcs1l*$^{p.S78G}$ mouse model. However, it is not known how much of the systemic and metabolic phenotypes, such as growth restriction, loss of adipose tissue, and hypoglycemia, are dependent on the liver disease. To investigate this, we performed a gene therapy trial with recombinant adeno-associated viruses (rAAVs) expressing wild-type mouse BCS1L. The gene was expressed under either a broad or a hepatocyte-specific promoter. As outcomes, we assessed growth and survival, liver disease progression, blood glucose, body temperature, and molecular disease markers in tissues. Hepatocyte-specific rescue of CIII activity was sufficient to fully prevent liver disease, significantly improve systemic metabolism, and double the survival of the treated mice. This improvement highlights the capacity of the liver to mediate broad metabolic benefits despite CIII deficiency in many other tissues. A striking novel discovery in this study was that basal liver thermogenesis played an essential role in sustaining normal body temperature (euthermia) in the juvenile CIII-deficient mice, independently of brown adipose tissue (BAT)-driven thermogenesis. This finding underscores the significant but poorly studied role of the liver in body temperature regulation. Additionally, raising the ambient temperature to restore normothermia in the hypothermic mice showed therapeutic benefits, further suggesting that maintaining body temperature may alleviate some consequences of OXPHOS deficiency.

In conclusion, these studies elucidate novel disease mechanisms in CIII deficiency at the molecular, cellular, and metabolic levels. The outcomes emphasize the need to understand the underlying tissue-specific pathology in mitochondrial diseases and the metabolic adaptations due to systemic organ crosstalk. Such understanding paves the way for designing treatments for mitochondrial diseases. In this regard, our findings already reveal significant beneficial effects of liver-targeted gene therapy in a preclinical model of a multiorgan mitochondrial disease.

TIIVISTELMÄ

Mitokondriotaudit ovat monimuotoinen monogeenisten eli yhden geenin mutaatioiden aiheuttamien aineenvaihduntatautien ryhmä, joissa usein mitokondrioiden elektroninsiirtoketjun eli hengitysketjun toiminta on heikentynyt. Ne ilmenevät potilailla hyvin monilla tavoin, esimerkiksi lihassairauksina, aivosairauksina ja monielinsairauksina. BCS1L-geenin mutaatiot ovat yleisin hengitysketjun kompleksi III (KIII) puutossairauksien aiheuttaja, ja ne johtavat vajavaiseen KIII:n kokoamiseen. Vakavin näistä on GRACILE-oireyhtymä, jolle tyypillisiä oireita ovat kasvuhäiriö, munuaistauti (aminohappojen erittyminen virtsaan), maksatauti (sapen erityshäiriö), raudan kertyminen maksaan, maitohapon kertyminen elimistöön ja kuolema imeväisiässä. Lastenlääkäri Vineta Fellmanin vuonna 1998 kuvaama GRACILE-oireyhtymä kuuluu suomalaiseen tautiperimään. Taudin tutkimiseksi on kehitetty suomalaista pistemutaatiota (Ser78Gly) kantava hiirimalli (*Bcs1l*$^{p.S78G}$), joka saa monilta osin samankaltaisen sairauden kuin GRACILE-potilaat mukaan lukien lyhyt elinikä.

Tässä väitöskirjatyössä osoitan yllättäviä yhteneväisyyksiä KIII-puutoksen *Bcs1l*$^{p.S78G}$ -hiirimallin ja tyypillisten progerian eli ennenaikaisen vanhenemisen hiirimallien välillä. Tyypillisiä vanhenemismuutoksia *Bcs1l*$^{p.S78G}$ -hiirissä ovat esimerkiksi selkäkyttyrä (kyfoosi), alentunut luuntiheys (osteoporoosi) ja kehon rasvamäärä, imukudosten (perna ja kateenkorva) surkastuminen ja ohut iho. Progerioille tunnusomaisesti hiirillä nähdään voimakas DNA-vauriovaste, solysyklin pysähtyminen ja solunjakautumisvaiheen ongelmia kudoksissa, joissa tapahtuu uusiutumista (regeneraatio), esimerkiksi maksassa, munuaistiehyissä ja haimassa. Tämä johtaa sairaissa kudoksissa solujen vanhenemiseen (senesenssi), jossa solunjakautuminen on pysähtynyt pysyvästi. Tutkimus osoitti ensimmäisen kerran, että suora hengitysketjupuutos voi aiheuttaa ennenaikaista vanhenemista ja että solunjakautuminen tilanteessa, jossa energiasta ja raaka-aineista on pulaa, johtaa vanhenemismuutoksiin ja progeriaan. Tutkiessamme heikentynyttä pyrimidiininukleotidien biosynteesiä mahdollisena tautimekanismina hiirissä törmäsimme ongelmaan, että julkaistut entsymaattiset deoksiribonukleotidien (dNTP) mittausmenetelmät eivät olleet riittävän herkkiä mittaamaan hiiren kudosten alhaisia pitoisuuksia. Ongelman ratkaisemiseksi kehitimme uuden mittausmenetelmän, joka perustuu pidempään DNA:n mallijuosteeseen, EvaGreen-väriin ja oikolukevaan DNA-polymeraasiin. Tällä mittausmenetelmällä osoitimme, että puriini-dNTP:t ovat koholla mutanttihiiren maksassa, mikä viittaa joko nukleotidien epätasapainoon tai lisääntyneeseen käyttöön solunjakautumisen vuoksi.

Mitokondriotauteihin ei ole olemassa tehokkaita hoitoja, ja potilaiden hoito on vain oireenmukaista tukihoitoa. Teoriassa monogeenisten sairauksien, mukaan luettuna tumageenien mutaatioiden aiheuttamat mitokondriosairaudet, tehokkain hoito on geenihoito tai geenikorjaushoito, esimerkiksi käyttäen viruksia DNA:n kantajina. *Bcs1l*$^{p.S78G}$ -hiirillä hengitysketjupuutos ja kudosvaurio ilmenevät maksassa jo varhaisessa vaiheessa. Ei kuitenkaan tiedetä, kuinka paljon koko elimistön energia-aineenvaihduntaan liittyvät muutokset kuten kasvuhäiriö, rasvakudoksen häviäminen ja alhainen verensokeri riippuvat maksasairaudesta. Tämän selvittämiseksi teimme geeniterapiakokeen, jossa veimme adenoassosioituvilla virusvektoreilla (AAV9) villityyppisen *Bcs1l*-geenin nuoriin hiiriin. Geenin ilmentymistä ajoi joko vain maksasoluissa tai kaikissa soluissa aktiivinen säätelyalue. Vastemuuttujina tutkimme hiirten kasvua ja elinikää, maksataudin etenemistä, verensokeria, ruumiinlämpöä ja tunnettuja molekyylitason muutoksia kudoksissa. Pelkkä maksasoluihin kohdistettu geenihoito riitti estämään maksataudin kehittymisen, parantamaan energia-aineenvaihduntaa ja kaksinkertaistamaan sairaiden hiirten eliniän. Tämä vahva parantuminen kertoo maksan kyvystä pitää yllä elimistön energia-aineenvaihduntaa huolimatta hengitysketjupuutoksesta useissa muissa kudoksissa. Tutkimuksen hämmästyttävin havainto oli, että maksan mitokondriotoiminnan ja sitä myötä lämpöä tuottavan aineenvaihdunnan korjaaminen riittää pitämään yllä nuorten hiirten normaalia ruumiinlämpöä riippumatta ruskean rasvakudoksen (BAT) lämmöntuotosta. Tulos vahvistaa käsitystä maksan tärkeästä mutta vähän tutkitusta roolista ruumiinlämmön säätelyssä. Lisäksi alilämpöisten hiirten pitäminen niin korkeassa lämpötilassa lämpökaapissa, että ne saavuttivat normaalin ruumiinlämmön, vähensi maksatautimuutoksia ja esti maksasolujen vanhenemista.

Yhteenvetona nämä tutkimukset paljastivat aivan uusia solu-, molekyyli- ja aineenvaihduntatason tautimekanismeja hengitysketjupuutoksen hiirimallissa. Tulokset vahvistavat sitä ajatusta, että on tärkeää ymmärtää eri kudosten tautimekanismeja ja aineenvaihdunnan vuoropuhelua eri elinten välillä mitokondriotaudeissa. Sellainen tieto auttaa kehittämään hoitoja, mistä esimerkkinä osoittamamme maksaan kohdistetun geeniterapian merkittävä hoitovaikutus prekliinisessä mitokondriotaudin hiirimallissa.

সারাংশ

মাইটোকন্ড্রিয়ার ক্রিয়াগত ব্যর্থতা জনিত ব্যাধিগুলি, একধরনের একক-জিনগত বিপাকীয় অবস্থার একটি সমষ্টি, যা প্রায়ই ইলেকট্রন পরিবহন তন্ত্রের (ইটিসি), যা শ্বসন নামেও পরিচিত, কার্যকারিতা হ্রাস দ্বারা চিহ্নিত হয়। এই ব্যাধিগুলি বিভিন্ন ধরণের ক্লিনিক্যাল উপসর্গ প্রদর্শন করে, যা বহু-অঙ্গকে প্রভাবিত করে, যার মধ্যে রয়েছে মায়োপ্যাথি, এনসেফালোপ্যাথি ইত্যাদি । এর মধ্যে, ইটিসি কমপ্লেক্স থ্রী (সিথ্রী)-এর অ্যাসেম্বলি ফ্যাক্টর *BCS1L* জিনের মিউটেশনগুলি সিথ্রী-এর ঘাটতির সবচেয়ে প্রধান কারণ । BCS1L মিউটেশনগুলির সাথে যুক্ত সবচেয়ে গুরুতর অবস্থা হল GRACILE (গ্রেসাইল) সিনড্রোম (গ্রোথ রিস্ট্রিকশন, অ্যামিনোঅ্যাসিডুরিয়া, কোলেস্টাসিস, লিভার আয়রন ওভারলোড, ল্যাকটিক অ্যাসিডোসিস, এবং আরলি ডেথ), যা প্রথম ফিনল্যান্ডে বর্ণিত হয়েছিল । একটি নক-ইন মাউস মডেল (*Bcs1l*$^{p.S78G}$, হোমোজাইগাস Ser78Gly মিসসেন্স মিউটেশন *Bcs1l*-এ) গ্রেসাইল সিনড্রোমের ক্লিনিক্যাল উপসর্গের অধিকাংশ পুঙ্খানুপুঙ্খ ভাবে প্রদর্শন করে এবং বিশেষ মাইটোকন্ড্রিয়াল ডিএনএ (mtDNA) ব্যাকগ্রাউন্ডের উপস্থিতিতে তাদের তুলনামূলক শীঘ্র-মৃত্যু ঘটে ।

এই গবেষণামূলক প্রবন্ধটি সিথ্রী-ঘাটতিযুক্ত *Bcs1l*$^{p.S78G}$ নক-ইন মাউস এবং ক্লাসিক্যাল ল্যামিনোপ্যাথি এবং ডিএনএ রিপেয়ার-ডেফিসিয়েন্ট প্রোজেরয়েড সিনড্রোমের বা অকাল বার্ধক্যজনিত ব্যাধির মাউস মডেলের ফেনোটাইপের মধ্যে সদৃশতা চিহ্নিত করে । এই মাউসগুলিতে প্রোজেরয়েড বৈশিষ্ট্যগুলির মধ্যে রয়েছে বৃদ্ধি প্রতিবন্ধকতা, কাইফোসিস, হাড়ের ঘনত্ব এবং চর্বি হ্রাস, লিম্ফয়েড অ্যাট্রোফি, এবং শীর্ণ ত্বক। প্রোজেরয়েড মাউস মডেলের অনুরূপ, *Bcs1l*$^{p.S78G}$ মাউস মডেলে বিস্তৃত ডিএনএ ড্যামেজ রেস্পন্স, কোশ চক্রে বাধা, এবং যকৃৎ, বৃক্ক, এবং অগ্ন্যাশয়ের মতো পুনরুৎপাদনশীল অঙ্গগুলিতে কোষ বিভাজনগত ত্রুটি দেখা যায় । এটি ক্ষতিগ্রস্ত কলাগুলিকে সেলুলার স্নেসেন্সের দিকে পরিচালিত করে। এই প্রবন্ধটিই প্রথম চিহ্নিত করে যে শ্বসনপ্রক্রিয়া বা অক্সিডেটিভ ফসফরাইলেশন (অকসফস)-এর ঘাটতি সেগমেন্টাল প্রোজেরিয়া ঘটাতে পারে এবং শক্তি এবং বায়োসিন্থেটিক প্রিকর্সর হ্রাস সত্ত্বেও কোশ চক্রের পুনঃপ্রবেশ অধিকাংশ ক্ষেত্রে প্রোজেরয়েড প্যাথলজির চালক হিসেবে কাজ করে। সিথ্রী-ঘাটতিযুক্ত মাউস মডেলে অকাল বার্ধক্যজনিত সম্ভাব্য ত্রুটিপূর্ণ প্রক্রিয়া হিসাবে পাইরিমিডিন নিউক্লিওটাইড বায়োসিন্থেসিস-এর বিশ্লেষন করার সময়, আমরা এনজাইম্যাটিক ডিঅক্সিরাইবোনিউক্লিওটাইডের (ডিএনটিপি) পরিমাণ নির্ধারণ করার একটি সূক্ষ্ম পদ্ধতির অভাবের সম্মুখীন হয়েছিলাম, যা যকৃত এবং অন্যান্য কলার জন্য উপযুক্ত। এই সমস্যা সমাধানের জন্য, আমরা একটি নতুন প্রক্রিয়া উদ্ভাবন করি, যা একটি দীর্ঘ কৃত্রিম টেমপ্লেট, এভাগ্রীন ডিএনএ ডাই, এবং একটি স্থায়ী, ইনহিবিটর-প্রতিরোধী উচ্চ গুনগত মানসম্পন্ন ডিএনএ পলিমারেজ ব্যবহার করে। এই প্রক্রিয়াতে প্রকাশ হয়েছিলো যে *Bcs1l*$^{p.S78G}$ যকৃতে পিউরিন ডিএনটিপি-এর পরিমান বেশী, যা নিউক্লিওটাইড ভারসাম্যজনিত বা যকৃতের কোষ বিভাজনগত সম্ভাব্য সমস্যার ইঙ্গিত দেয়।

মাইটোকন্ড্রিয়ার ক্রিয়াগত ব্যর্থতা জনিত রোগের কার্যকর চিকিৎসার অভাব রয়েছে, এবং এর ক্লিনিক্যাল ব্যবস্থাপনা কেবলমাত্র সহায়ক চিকিৎসার উপর নির্ভরশীল। তত্ত্বগতভাবে, নিউক্লিয়ার জিন মিউটেশন দ্বারা সৃষ্ট একক-জিনগত রোগের জন্য সবচেয়ে কার্যকর পদ্ধতি হল জিন প্রতিস্থাপন । রোগের প্রাথমিক হিস্টোপ্যাথলজিকাল লক্ষণ এবং সিথ্রী-এর ঘাটতি মূলত যকৃত এবং বৃক্কে দেখা যায়। বৃদ্ধি প্রতিবন্ধকতা, মেদ কলার ক্ষতি এবং রক্তশর্করাস্বল্পতা সহ বিপাকীয় উপসর্গগুলির কতটা যকৃতের উপর নির্ভরশীল তা জানা যায়নি।

অকসফস ঘাটতির কারণে সৃষ্ট সামগ্রিক বিপাকীয় কার্যকারিতা-গত সমস্যায় যকৃতের ভূমিকা তদন্ত করার জন্য, ওয়াইল্ড-টাইপ *Bcs1l* প্রকাশকারী রিকম্বিনেন্ট অ্যাডেনো-অ্যাসোসিয়েটেড ভাইরাস ইনজেকশন দেওয়া হয়। এই জিনটি একটি বিস্তৃত অথবা একটি যকৃত-কোষ-নির্দিষ্ট প্রোমোটারের অধীনে প্রকাশ করা হয়েছিল। অতঃপর, আমরা বিভিন্ন প্যারামিটার মূল্যায়ন করি, যার মধ্যে রয়েছে বৃদ্ধি, যকৃতের রোগের অগ্রগতি, শক্তি বিপাক, শরীরের তাপমাত্রা, এবং জীবনকাল। আমাদের ফলাফলগুলিতে দেখা গিয়েছিলো যে যকৃত-কোষ-নির্দিষ্ট সিথ্রী ঘাটতির পুনরুদ্ধার যকৃতের রোগ সম্পূর্ণরূপে প্রতিরোধ করতে, সামগ্রিক বিপাকীয় উপসর্গগুলির উল্লেখযোগ্যভাবে উন্নতি ঘটাতে এবং মাউসের আয়ুষ্কাল দ্বিগুণ করতে সক্ষম। এই উন্নতি বিভিন্ন টিস্যুতে সিথ্রী-এর ঘাটতি থাকা সত্ত্বেও বিপাকীয় সুবিধা প্রদানে যকৃতের সক্ষমতাকে তুলে ধরে। এই গবেষণার একটি আকর্ষণীয় প্রাপ্তি হল যে, বেসাল লিভার থার্মোজেনেসিস সিথ্রী-ঘাটতিযুক্ত মাউসে স্বাভাবিক শরীরের তাপমাত্রা (ইউথার্মিয়া) বজায় রাখতে একটি অপরিহার্য ভূমিকা পালন করে, যা বাদামী মেদ কলা (ব্যাট)-চালিত থার্মোজেনেসিস নিরপেক্ষ। এই পর্যবেক্ষণ তাপমাত্রা নিয়ন্ত্রণে যকৃতের গুরুত্বপূর্ণ ভূমিকা তুলে ধরেছে। উপরন্তু, পারিপার্শ্বিক তাপমাত্রা বাড়ানোয় হাইপোথার্মিক মাউস স্বাভাবিক শরীরের তাপমাত্রা বজায় রাখতে সাহায্য পায়, যা অকসফস ঘাটতির কিছু ক্ষতিকর পরিণতি কমাতে সাহায্য করে ।

পরিশেষে, এই গবেষণা সিথ্রী- এর ঘাটতির অণু, কোষীয় এবং বিপাকীয় স্তরে নতুন প্যাথোমেকানিজমগুলিকে চিত্রিত করে। তদ্ব্যতীত, এই ফলাফলগুলি মাইটোকন্ড্রিয়াল ব্যাধির কলা-নির্দিষ্ট প্যাথোলজি এবং বিভিন্ন অঙ্গের পারস্পরিক যোগাযোগের ফলে সৃষ্ট বিপাকীয় অভিযোজন সম্পর্কে বোঝার প্রয়োজনীয়তাকে জোর দেয়, যা মাইটোকন্ড্রিয়াল রোগেগুলির সম্ভাব্য চিকিৎসার সন্ধানের জন্য গুরুত্বপূর্ণ। এই গবেষণার ফলাফলগুলি বহু-অঙ্গ সংশ্লিষ্ট মাইটোকন্ড্রিয়াল রোগের একটি প্রাক-ক্লিনিক্যাল মডেলে, যকৃত-নির্দেশিত জিন থেরাপির থেরাপিউটিক প্রভাবকে বিশেষভাবে তুলে ধরে।

ABBREVIATIONS

AAV – adeno-associated virus
ADP – adenosine diphosphate
AMP – adenosine monophosphate
AOX – alternative oxidase
ATP – adenosine triphosphate
BCS1L – BCS1-like AAA-ATPase
BAT – brown adipose tissue
CDKN1a – cyclin-dependent kinase inhibitor 1a
CIII – complex III (ubiquinol-cytochrome c reductase)
CI – complex I
CIV – complex IV
CHDH – choline dehydrogenase
CoA – coenzyme A
CoQ – coenzyme Q
CV – complex V
CYB – cytochrome b
CYC1 – cytochrome c1
DCA – dichloroacetate
DEXA – dual-energy X-ray absorptiometry
DHODH – dihydroorotate dehydrogenase
dNTP – deoxyribonucleoside triphosphate
DNA – deoxyribonucleic acid
DOA – dominant optic atrophy
ETF – electron transfer flavoprotein
EGFP – enhanced green fluorescent protein
ETC – electron transport chain
ETHE1 – ethylmalonic encephalopathy 1
FAD – flavin adenine dinucleotide
FAO – fatty acid oxidation
FFA – free fatty acids
FRDA – Friedreich's ataxia
GTP – guanosine triphosphate
GRACILE – growth restriction, aminoaciduria, cholestasis, liver iron overload, lactic acidosis, and early death
GI – gastrointestinal
HE – heterozygous
HO – homozygous
HPLC-MS – high-performance liquid chromatography-mass spectrometry
IMM – inner mitochondrial membrane
IMS – inter-membrane space
iBAT – interscapular brown adipose tissue
KO – knock-out
LHON – Leber's hereditary optic neuropathy
LYRM7 – LYR motif-containing protein 7
MELAS – mitochondrial encephalomyopathy, lactic Acidosis, and stroke-like episodes
MT-CYB – mitochondrial cytochrome b
mtDNA – mitochondrial DNA
NAD – nicotinamide adenine dinucleotide

NAD(P)H – nicotinamide adenine dinucleotide phosphate (reduced form)
nDNA – nuclear DNA
NDUFS – NADH-ubiquinone oxidoreductase core subunit
OXPHOS – oxidative phosphorylation
OMM – outer mitochondrial membrane
PDK – pyruvate dehydrogenase kinase
POLG – DNA polymerase gamma
qPCR – quantitative polymerase chain reaction
rAAV – recombinant adeno-associated virus
RNA – ribonucleic acid
rNTP – ribonucleotide triphosphate
RISP – rieske iron-sulfur protein
ROS – reactive oxygen species
SASP – senescence-associated secretory phenotype
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SQOR – sulfide quinone oxidoreductase
TCA – tricarboxylic acid
TAZ – tafazzin protein
Tk2 – thymidine kinase 2
TTC19 – tetratricopeptide repeat domain 19
TYMP – thymidine phosphorylase
TTP – thymidine triphosphate
UCP1 – uncoupling protein 1
UMP – uridine monophosphate
UQCC – ubiquinol-cytochrome c reductase complex assembly factor
UQCRC – ubiquinol-cytochrome c reductase core protein
UQCRB – ubiquinol-cytochrome c reductase binding protein
UQCRFS1 – ubiquinol-cytochrome c reductase rieske iron-sulfur polypeptide 1
UQCRQ – ubiquinol-cytochrome c reductase complex III subunit q
WT – wild-type

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

Publications included in the thesis

I. Janne Purhonen, **Rishi Banerjee**, Allison E McDonald, Vineta Fellman and Jukka Kallijärvi. A sensitive assay for dNTPs based on long synthetic oligonucleotides, EvaGreen dye and inhibitor-resistant high-fidelity DNA polymerase. Nucleic Acids Research, gkaa516, (2020).

II. Janne Purhonen, **Rishi Banerjee**, Vilma Wanne, Nina Sipari, Matthias Mörgelin, Vineta Fellman and Jukka Kallijärvi. Mitochondrial complex III deficiency drives c-MYC overexpression and illicit cell cycle entry leading to senescence and segmental progeria. Nature Communications 14, 2356 (2023).

III. **Rishi Banerjee**, Divya Upadhyay, Tomáš Zarybnický, Christa Kietz, Satu Kuure, Vineta Fellman, Janne Purhonen and Jukka Kallijärvi. Hepatic mitochondrial respiration is crucial for euthermia in complex III-deficient mice with impaired brown adipose tissue thermogenesis. bioRxiv 2024.09.23.612616. (*submitted*)

In the text, the publications are referred to by their Roman numbers. The published articles are reprinted, following the license of the copyright owners.

Author's contribution

I. I took part in the design of the experiments. I set up all the cell culture experiments and generated the Pacific oyster AOX-expressing mammalian cell line. I was also involved in the collection of mouse tissues and the extraction of dNTPs from both tissue and cell samples. I conducted most of the optimization steps needed for the assay development and was involved in measuring dNTPs from different sample panels, respirometry analysis, and immunofluorescence staining of Pacific oyster AOX-expressing mammalian cell line. I was involved in writing the manuscript draft and the revision.

II. I contributed to the design of the experiments. I took part in many of the key methods for this study, including body composition analysis, DEXA scan, SDS PAGE, western blot, qPCR, measurement of dNTPs, and image quantification. I was involved in animal maintenance, dietary interventions, rAAV administrations, and tissue collection. I helped revise the manuscript.

III. I participated in the design of all experiments, conducted all virus injections, and performed most of the mouse monitoring and most of the laboratory analyses of mouse samples (quantification of tissue histology, qPCR, ATP measurements, isolation of mitochondria, measurement of respiratory enzyme activities, blue-native PAGE, western blot, differential gene expression analysis and pathway analyses for transcriptomics data, statistics). I prepared all the manuscript figure panels and their revised versions, and most of the individual images and tables, wrote the first manuscript draft, and significantly contributed to revising the manuscript text.

1 INTRODUCTION

Mitochondrial disorders represent the most common group of inborn errors of metabolism. Unfortunately, the therapeutic options for mitochondrial diseases are currently limited to symptomatic treatment, such as supportive care that may improve the quality of life and potentially increase the life expectancy of some patients (Russell et al. 2020). Meanwhile, modern genetic tools have led to an increased number of identified causative mutations underlying mitochondrial diseases. In this thesis project, my primary aim was to achieve a better understanding of the disease mechanisms and evaluate potential therapies for childhood mitochondrial diseases, using GRACILE syndrome, a neonatal mitochondrial complex III deficiency, as a model disease (Fellman et al. 1998). GRACILE syndrome is caused by a homozygous (HO) missense mutation in a mitochondrial complex III (CIII) assembly factor gene, *BCS1L* (*BCS1L*$^{p.S78G}$, Serine at 78 replaced by Glycine), reported in the Finnish population. (Visapää et al. 2002). The resulting phenotype of this CIII deficiency disorder consists of severe fetal **G**rowth **R**estriction, **A**minoaciduria, **C**holestasis, **I**ron overload in the liver, **L**actic acidosis, and **E**arly death during infancy (Fellman et al. 1998). To study the disease mechanism and search for potential therapies for GRACILE syndrome, Prof. Fellman's research group generated a KI mouse model carrying the same patient mutation (Levéen et al. 2011). Homozygous *Bcs1l*$^{p.S78G}$ mice successfully recapitulate most of the clinical manifestations of GRACILE syndrome, including early-onset liver and kidney disease with growth restriction (Levéen et al. 2011). In a mtDNA background that exacerbates the CIII deficiency, the homozygotes with double mutations *(Bcs1l*$^{p.S78G}$*; mt-Cyb*$^{p.D254N}$) succumb to a metabolic crisis by 4-5 weeks of age (Purhonen et al., 2020b). This was the first viable and physiologically relevant mouse model for CIII deficiency, quite closely mimicking the human disorder. For this thesis study, we have used both mouse strains to explore the molecular and metabolic mechanisms and to investigate a therapeutic possibility.

2 REVIEWS OF LITERATURE

2.1 Structure and key functions of mitochondria

The mitochondrion, a double membrane-enclosed organelle performing cellular respiration, ATP production, and heat generation, is a hallmark component of the eukaryotic cell (Lane and Martin 2010; Kühlbrandt 2015). The matrix is enclosed by two phospholipid bilayers, namely the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM), which are separated by an inter-membrane space (IMS). The OMM maintains the integrity of the mitochondrial structure and creates a barrier between inner contents and the cytosol. This membrane is permeable to most small molecules and contains various membrane transporter proteins (Endo and Kohda 2002). IMM forms an impermeable barrier between the mitochondrial matrix and IMS (Bartolák-Suki et al. 2017). Moreover, the folding of IMM forms the cristae that are packed with the respiratory complexes (Fig. 1).

Based on tissue and cell type and metabolic states, the size, shape, and abundance of mitochondria vary widely (McCarron et al., 2013). Because of their origins as symbiotic free-living bacteria, mitochondria are the only organelles within the cytoplasm of animal cells that contain DNA, which exists as small, multi-copy circular genomes (Lane and Martin 2010). Thus, unlike other organelles, mitochondrial function is dependent on proteins encoded both by the nucleus and mitochondrial DNA (mtDNA). Human mtDNA is double-stranded, circular, and has a size of 16,569 base pairs and encodes 13 proteins that are essential structural components and catalytic subunits of mitochondrial respiratory complexes, as well as the RNA required for mitochondrial gene expression: 2 ribosomal RNAs and 22 transfer RNAs (Anderson et al., 1981). Similarly, mouse mtDNA consists of 16,295 base pairs (Bibb et al., 1981). More than 99% of the mitochondrial proteome is encoded by nuclear DNA, followed by translation in cytoplasmic ribosomes and subsequent transport into mitochondria (Hendrickson et al., 2010; Taanman, 1999). mtDNA is located in the mitochondrial matrix, the innermost compartment, rich in enzymes and proteins that are involved in metabolic processes like pyruvate and fatty acid oxidation, as well as the TCA cycle.

The discovery of the TCA cycle (Krebs cycle) as a central hub for energy production and biosynthesis, together with the identification of mitochondrial respiratory enzymes that generate an electrochemical gradient to drive ATP synthesis, firmly established

mitochondria as the powerhouse of the cell. Over the past several decades, research has increasingly recognized the involvement of mitochondria in numerous aspects of cell signaling and cellular metabolic pathways other than ATP synthesis (Spinelli and Haigis 2018) (refer to Table 1). Even though the identification of the complete human mitochondrial proteome has also been largely achieved, many of the proteins still lack functional annotation (Rath et al., 2021). Additionally, mitochondria play a crucial role in signaling pathways related to the cell cycle, differentiation, and apoptosis (Chen et al., 2023).

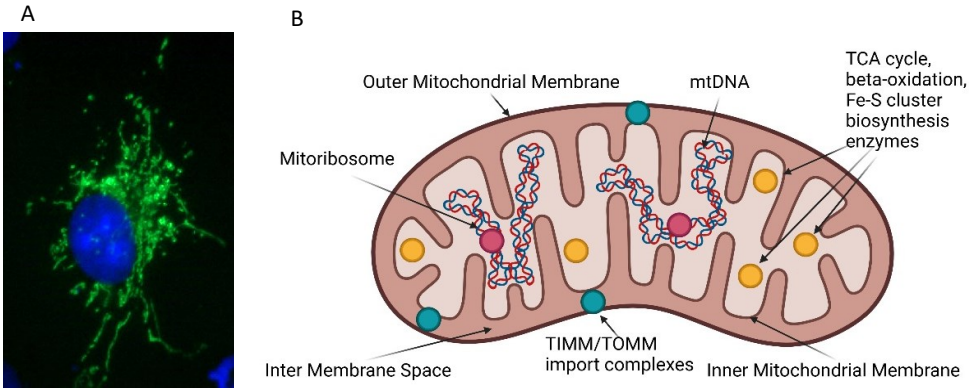

Figure 1: A) Immunofluorescence staining of mitochondria (MT-CO1) in cultured COS-1 cells. B) Structural components of mitochondria. Image B was prepared using Biorender.com.

The cell takes up various nutrient molecules from the circulation, including carbohydrates, amino acids, fatty acids, and ketone bodies as fuel. Amino acids (such as glutamate and aspartate) undergo deamination in the mitochondria, and fatty acids are transported via the carnitine shuttle system to enter mitochondrial β-oxidation. These nutrients ultimately produce acetyl-coenzyme A (acetyl-CoA). Acetyl-CoA serves as the primary fuel for the TCA cycle, a crucial metabolic pathway that provides carbon backbones for the biosynthesis of amino acids, nucleotides, and other structural and signaling molecules.

Mitochondria engage in dynamic interactions with other cellular components in many different ways (Martínez-Reyes and Chandel 2020). Adenosine monophosphate (AMP)-activated protein kinase (AMPK) detects OXPHOS deficiency by assessing adenylate phosphorylation status in the cell, thereby initiating an appropriate adaptive mitochondrial response (Herzig and Shaw 2018). Furthermore, one of the most significant signaling pathways that is initiated from mitochondria is through the release of cytochrome c, which

triggers the apoptosis of the cell (Kluck et al. 1997). The detailed physiological roles of mitochondria are listed in Table 1.

Table 1: Some key mitochondrial functions linked to the electron transport chain (ETC).

Pathway/Process	Key enzymes/proteins	Function/Role
Respiratory electron transfer	Complexes I-IV, coenzyme Q (CoQ), Cytochrome c	Transfers electrons, pumps protons, and generates proton motive force for ATP synthesis.
Oxidative phosphorylation	ATP synthase	Phosphorylation of ADP to ATP using membrane potential.
Reactive oxygen species (ROS) production	Complex III (Qo site), other ETC sites	Produces ROS (e.g., superoxide) during electron leak; implicated in signaling and oxidative stress.
Fatty acid oxidation (FAO)	Electron transfer flavoprotein (ETF), ETFDH, Acyl-CoA dehydrogenases	Oxidizes fatty acids to acetyl-CoA, linking to the TCA cycle and ketogenesis.
Choline and betaine metabolism	Choline dehydrogenase (CHDH), Betaine-homocysteine S-methyltransferase (BHMT)	Involved in methylation cycles, lipid metabolism, and methionine synthesis.
Pyrimidine nucleotide biosynthesis	Dihydroorotate dehydrogenase (DHODH), Carbamoyl-phosphate synthetase 2 (CAD), Uridine monophosphate synthase (UMPS)	Synthesizes RNA/DNA precursors (e.g., Uridine monophosphate, UMP); links to transcription, translation, and glycosylation.
Mitochondrial fatty acid synthesis (mtFAS)	Acetyl-CoA, malonyl-CoA, mtFAS enzymes	Synthesizes fatty acids within mitochondria; produces octanoic acid, a precursor for lipoic acid synthesis, critical for mitochondrial enzyme complexes.
Iron-sulfur cluster biosynthesis	Cysteine desulfurase (NFS1), ISCU2, Frataxin, Ferredoxin 2 (FDX2)	Assembles [2Fe–2S] and [4Fe–4S] clusters are essential for electron transport, metabolic reactions, and DNA-related enzyme functions.
Apoptosis	Cytochrome c, BAX/BAK	Programmed cell death
Uncoupling	Uncoupling protein 1 (UCP1)	Adaptive heat production in brown adipose tissue

(Banerjee et al., 2022; Miinalainen et al., 2003; Nowinski et al., 2020; Peña-Blanco and García-Sáez, 2018; Shi et al., 2021; Trayhurn, 2017; Venkatesan et al., 2014)

2.1.1 Respiratory electron transfer and oxidative phosphorylation

Mitochondria are primarily known for their role in the generation of ATP, which directly or indirectly powers almost all cellular functions (Spinelli and Haigis 2018). Mitochondria achieve the conversion of the chemical energy of nutrients into ATP by a series of chemical reactions that carry electrons coming from nutrients through the ETC to molecular oxygen. Hundreds of protein subunits, assisted by assembly factors and cofactors, assemble to form the four canonical respiratory complexes embedded in IMM, named complexes I, II, III, and IV. The electron transfer is initiated by NADH-ubiquinone oxidoreductase of complex I (CI) and succinate dehydrogenase of complex II (CII) which subsequently oxidizes NADH to NAD^+ and reduces flavin adenine dinucleotide (FAD) to $FADH_2$, respectively, consequently transferring electrons to coenzyme Q (CoQ), see Fig. 2A. Additionally, at least seven other mitochondrial dehydrogenases use CoQ as electron acceptor. These include DHODH, SQOR (sulfide quinone oxidoreductase; hydrogen sulfide detoxification), PRODH (proline oxidation, protein turnover), CHDH, and glycerol 3-phosphate dehydrogenase (GPDH: glycerol phosphate shuttle, lipid metabolism, nicotinamide adenine dinucleotide; NAD^+/NADH balance) (Banerjee, Purhonen, and Kallijärvi 2022). The next enzyme in the electron transport chain is complex III (CIII, cytochrome bc_1 complex, cytochrome c reductase), which accepts electrons from reduced CoQ and passes them on to the soluble IMS electron carrier, cytochrome c. The last block of the electron transport chain is the terminal oxidase, cytochrome c oxidase (complex IV, CIV). CIV or cytochrome c oxidase transfers the electrons from cytochrome c to molecular oxygen, which is reduced to water. The electron transfer at CI, CIII, and CIV is coupled to the translocation of protons (H^+) from the matrix to the IMS, generating an electrochemical potential (Schoepp-Cothenet et al. 2013). ATP synthase (Complex V; CV) acts as an ion channel, allowing those protons to flow back into the matrix, which drives a rotating motor-like engine that phosphorylates ADP into ATP (Klusch et al., 2017). This whole process of respiratory electron transfer and the phosphorylation of ADP is called oxidative phosphorylation (OXPHOS) (Mitchell and Moyle 1965). ATP production through oxidative phosphorylation is highly efficient, generating an average of 36 ATP molecules per glucose molecule, compared to glycolysis, which yields only 2 net ATP molecules per glucose.

2.1.2 Alternative oxidases (AOXs)

AOXs found in plants, as well as in some fungi and protists (Jiye Li et al. 2024), are membrane-associated terminal oxidases located on the matrix side of IMM. AOXs have also been identified in various lower animal species across nine different phyla (McDonald and Vanlerberghe, 2006, 2004). AOXs are mostly not constitutively active but take over respiration when cytochrome-linked RC complexes (CIII and CIV) are inactive and reduced CoQ accumulates (McDonald and Gospodaryov 2019; Wagner, Wagner, and Moore 1998; Vanlerberghe 2013). AOX transfers electrons from reduced CoQ to molecular oxygen, which is an exothermic process, without coupling to proton translocation (Fig. 2B). Some plants, like lotus, magnolia, and philodendron, use the AOX pathway for floral thermogenesis, which helps to regulate flower temperature, emit scents and improve pollination (McDonald, 2022). Therefore, AOXs do not play a role in contributing to the proton gradient and ATP production. Instead, AOX helps in maintaining metabolic flexibility and sustains cellular function while dissipating excess reducing potential under stressful conditions as well as oxidative damage caused to cells. AOX genes have been lost during the evolution of vertebrates. Transgenic AOXs from the tunicate *Ciona intestinalis*, the fungi *Emericella nidulans* and the pacific oyster *Crassostrea gigas* have been ectopically expressed and studied in different mammalian cell lines (Guarás et al., 2016; Hakkaart et al., 2006; Perales-Clemente et al., 2008; Purhonen et al., 2020a) and also in mice (Rajendran et al. 2019; El-Khoury et al. 2014; Szibor et al. 2017). Xenogeneic AOXs localize to the mammalian mitochondrial inner membrane and restore respiration when the CIII-CIV segment is blocked. When CIII and CIV are functioning properly, AOXs remain mostly inactive (El-Khoury et al. 2014; Szibor et al. 2017). AOXs have proven to be valuable mechanistic tools for studying the roles of various segments of the respiratory electron transfer chain (Banerjee, Purhonen, and Kallijärvi 2022).

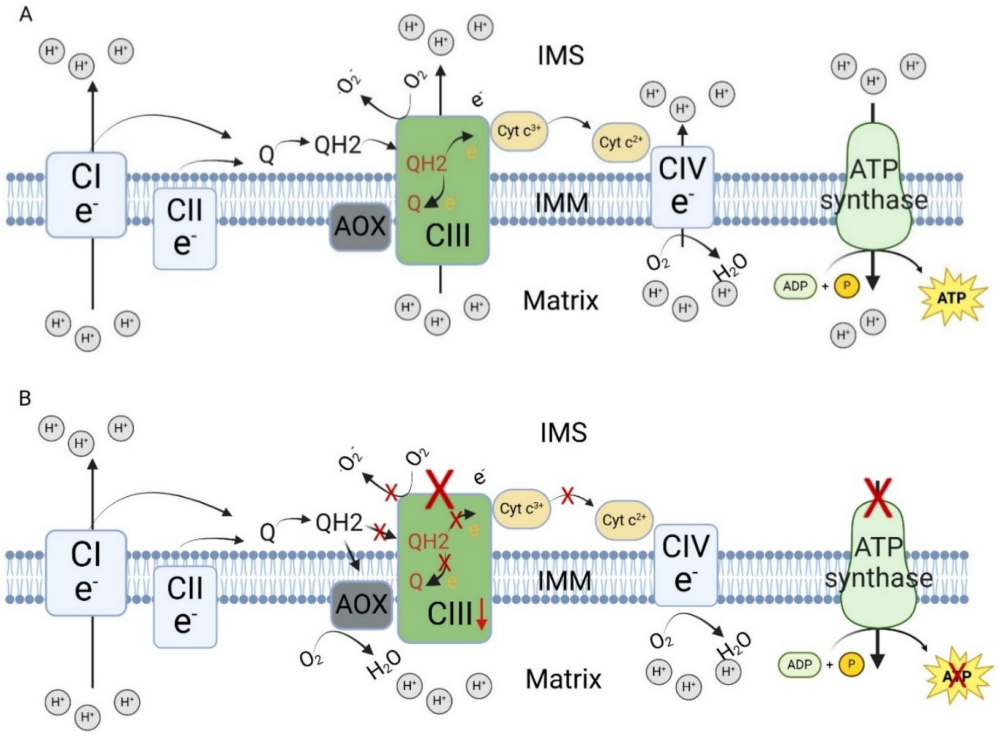

Figure 2: Respiratory electron transfer and oxidative phosphorylation in standard condition (AOX is inactive) and in CIII deficiency (AOX is active). Images were prepared using Biorender.com.

2.1.3 Mitochondria in nucleotide metabolism

Nucleotides serve as the building blocks of DNA and RNA, as the primary energy carriers in cells, and as essential signaling molecules. They consist of three components: a nitrogenous base, a pentose sugar (ribose or deoxyribose), and one to three phosphate groups. The nucleotides are divided into two classes based on the structure of their nitrogenous bases: purines and pyrimidines. Purine nucleotides consist of adenine (A) or guanine (G) bases, which have a double-ring structure, and pyrimidine nucleotides contain cytosine (C), thymine (T), or uracil (U) bases, which have a single-ring structure. Ribonucleotide triphosphates (rNTPs) are the building blocks of RNA. They have a higher concentration in cells. Deoxyribonucleotide triphosphates (dNTPs) are the nucleotides used in DNA synthesis, and they act both as the carbon structure and supply the chemical energy necessary for polymerization in the form of high-energy phosphate bonds (Mullen and Singh, 2023). In animal cells, ribonucleotides can either be synthesized *de novo* from amino acids and sugars, subsequently reduced to dNTPs by ribonucleotide reductase (Mullen and

Singh, 2023), or recycled from various intermediates and degradation products through salvage pathways (Wang 2010). NAD (nicotinamide adenine dinucleotide)/NADH and NADP (Nicotinamide Adenine Dinucleotide Phosphate) /NADPH are dinucleotides that play crucial roles in cellular metabolism. NAD and NADP act as electron carriers in many oxidation reactions. They can exist in oxidized (NAD^+ and $NADP^+$) or reduced (NADH and NADPH) forms, respectively.

Mitochondria play a crucial role in the biosynthesis and recycling of nucleotides. *De novo* synthesis of purine nucleotides produces adenine and guanine nucleotides. Mitochondria provide metabolic precursors carbamoyl phosphate and aspartate during pyrimidine biosynthesis (Nakhle, Rodriguez, and Vignais 2020). Carbamoyl phosphate is synthesized from bicarbonate, ammonia (derived from amino acids), and ATP in the cytosol, catalyzed by carbamoyl phosphate synthetase II (CPS II). As carbamoyl phosphate serves as a nitrogen donor in the subsequent steps of pyrimidine synthesis (Fig. 3), its availability is critical for the biosynthetic pathway (Moffatt and Ashihara, 2002). Aspartate is another crucial precursor that enters the pyrimidine biosynthetic pathway and combines with carbamoyl phosphate to form carbamoyl aspartate, catalyzed by the enzyme aspartate transcarbamoylase (ATCase) (Moffatt and Ashihara, 2002). This is an initial step of pyrimidine biosynthesis that leads to the formation of dihydroorotate. Dihydroorotate is then oxidized to orotate by DHODH, which is located in the IMM (Spinelli and Haigis 2018). Subsequently, orotate is converted to UMP by several successive reactions involving orotate phosphoribosyltransferase and UMP synthase; the latter substance is then phosphorylated to give uridine-5′-diphosphate (UDP) and, subsequently, to uridine-5′-triphosphate (UTP), which then may further be a substrate for conversion into cytidine triphosphate (CTP) and thymidine triphosphate (TTP) (Moffatt and Ashihara, 2002). Disruption of the respiratory electron transfer makes mammalian cells dependent on an external supply of uridine, as the rate-limiting enzyme of the uridine biosynthesis pathway, DHODH, uses CoQ as an electron acceptor (Perales-Clemente et al. 2008; Löffler et al. 1997), and this relies on CIII.

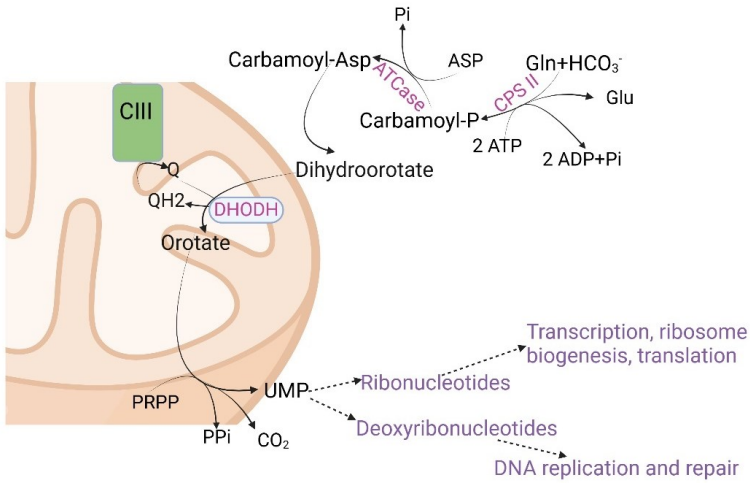

Figure 3: Mitochondria as the hub for pyrimidine nucleotide biosynthesis. The image was prepared using Biorender.com.

The concentration and the relative balance of nucleotides are crucial for maintaining cellular and metabolic homeostasis, as they directly affect nuclear DNA and mtDNA replication and fidelity, impacting DNA repair, genome integrity, cell cycle regulation, oncogenesis, and many other essential cellular processes (Reichard, 1988; Chabes et al., 2003; Mathews, 2014; Wheeler et al., 2005). Mitochondrial metabolism has an important role in the overall balance and availability of nucleotides within the cell. Mitochondria regulate cellular nucleotide pools via their biosynthesis and phosphorylation, which depends on ATP (Chakrabarty and Chandel 2022). Moreover, mitochondrial respiration is vital for the maintenance of the redox status of NAD+/ NADH (Covarrubias et al. 2021).

Mitochondrial diseases can cause disturbances in nucleotide metabolism, leading to imbalanced dNTP and rNTP pools. Considering the multiple functions of nucleotides in the cell, defects in nucleotide metabolism enzymes result in many different developmental, metabolic, neurological, and immunological diseases (Nyhan 2005). Pyrimidine and purine nucleotide biosynthesis connect to the respiratory electron transfer directly and indirectly (Spinelli and Haigis 2018; Nakhle, Rodriguez, and Vignais 2020). Therefore, dysfunctional OXPHOS can cause defects in nucleotide biosynthesis, leading to nucleotide depletion and imbalance. The depletion of dNTPs inhibits global DNA replication and causes fork stalling, leading to a temporary halt in the progression of the replication machinery. Stalled replication forks seem to be quite stable, and replication can resume once dNTP levels are replenished. Nucleotide depletion also causes transcription stress and cell cycle arrest,

which can lead to genome instability. If the G1 checkpoint is bypassed and the cell cycle progresses to the S-phase, this results in replication stress and DNA damage (Pai and Kearsey 2017; Linke et al. 1996; Pelletier et al. 2020).

2.2 Respiratory complex III (CIII, cytochrome bc_1 complex)

2.2.1 CIII structure and assembly

Mitochondrial CIII, also known as the cytochrome bc_1 complex, consists of 11 subunits in mammals, including three catalytic subunits - cytochrome b (CYT B/CYB), Rieske iron-sulfur protein (RISP, UQCRFS1), and cytochrome c_1 (CYT C1/CYC1) is highly conserved across bacteria and eukaryotes (Esser and Xia, 2024). Early studies in *Saccharomyces cerevisiae* were critical in understanding the assembly and function of CIII and its associated factors, such as BCS1, which plays a role in RISP translocation (Cruciat et al., 1999; Wagener et al., 2011). Cryo-EM analysis (Guo et al. 2018) of the mammalian CIII revealed that it is a tightly packed homodimer in the IMM, although high-resolution X-ray diffraction structures of CIII were already available prior to these studies (Iwata et al., 1998). The dimer is comprised of 21 subunits (Zong et al. 2018). The assembly of CIII begins with the synthesis of the only mtDNA-encoded subunit cytochrome b (MT-CYB), followed by the incorporation of nuclear DNA (nDNA)-encoded subunits. Three assembly factors, UQCC1, UQCC2, and UQCC3, take part in the initial steps of the assembly and stabilize the precomplex. After that, the core proteins (UQCRC1 and UQCRC2) are incorporated into the precomplex, followed by the dimerization of CIII (Stephan and Ott 2020). The final stages of CIII assembly involve the insertion of the crucial catalytic subunit RISP (Wagener and Neupert 2012). The RISP precursor is synthesized in the cytosol and imported into mitochondria via the common TOM-TIM import system. In the matrix, a LYR-motif-containing chaperone (MZM1L/LYRM7) binds to it, serving as a scaffold, which further recruits the Fe-S transfer complex to form a 2Fe-2S cluster (T.-Z. Cui et al., 2012; Sánchez et al., 2013). Thereafter, BCS1L, an IMM translocase, translocases RISP and incorporates it into the pre-CIII complex to form the fully assembled and catalytically active CIII (Cruciat et al., 1999; Fernandez-Vizarra et al., 2007; Hinson et al., 2007; Wagener et al., 2011).

2.2.2 The BCS1L translocase

BCS1L is a member of the AAA superfamily of ATPases (Hanson and Whiteheart, 2005). The BCS1L monomer, which consists of 419 amino acids, has three domains: a transmembrane domain in the N-terminus containing the sequence for protein sorting and mitochondrial targeting, followed by a unique BCS1L-specific domain, and an ATPase domain in the C-terminus, which plays a role in the substrate binding and translocation (Cruciat et al., 1999; Hikmat et al., 2021; Nobrega et al., 1992; Wagener and Neupert, 2012). Recently, the structure of yeast Bcs1 (Kater et al. 2020) and mouse (Kwan Tang et al. 2020) BCS1L was solved. Unlike typical hexameric AAA ATPases, BCS1L is a heptamer that carries out a specialized airlock-type mechanism to translocate the folded RISP substrate across the inner membrane (Fig. 4C) without risking the membrane potential (Kwan Tang et al. 2020), powered by ATP hydrolysis (Wagener and Neupert 2012).

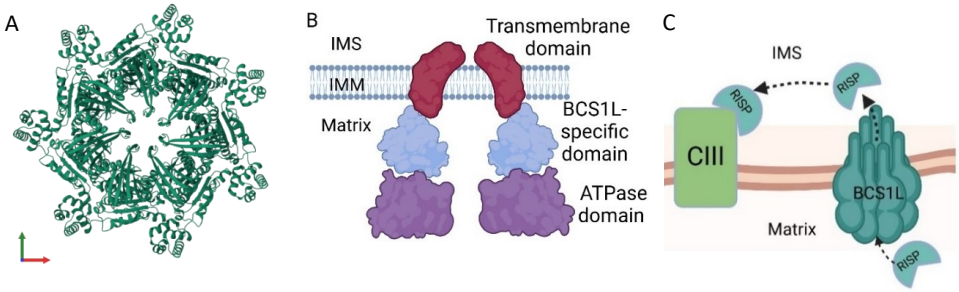

Figure 4: The structure of BCS1L from *Mus musculus*. A) The heptameric structure, obtained from the alphafold protein structure database (Uniprot: Q9CZP5), B) different domains of BCS1L, C) BCS1L translocates RISP, and forms fully assembled and catalytically active CIII. Images B and C were prepared using Biorender.com.

2.3 Mitochondrial diseases

Mitochondrial diseases are a heterogeneous group of monogenic metabolic disorders characterized by the malfunction of mitochondria, typically affecting mitochondrial OXPHOS directly or indirectly, with a collective lifetime risk of 1 in 3,200 (Tan et al., 2020). These inborn errors of metabolism are caused by mutations in nDNA and mtDNA (Russell et al. 2020). Mitochondrial diseases resulting from mutations in nDNA follow Mendelian inheritance, whereas mtDNA is maternally inherited. Because mitochondria are crucial for almost all cell types in the human body, their dysfunction can result in a variety of clinical manifestations ranging from myopathy, hepatopathy, cardiomyopathy, kidney disease, endocrine and hematological disturbance, and neurological disorder. The symptoms occur

in isolation or various combinations along with a wide range of age onset and severity. The reason behind this diversity remains largely unknown (Russell et al., 2020).

2.3.1 CIII deficiencies

Mutations in genes encoding CIII subunits or its assembly factors are collectively known as CIII deficiencies. Like other mitochondrial disorders, symptoms of CIII deficiencies vary widely, ranging from exercise intolerance to early-onset lethal metabolic crises and severe neurological manifestations (Hikmat et al., 2021). Different mutations in the same gene and impacting the same protein region can still result in vastly different phenotypes (Hinson et al., 2007). The first mutations identified as causing CIII deficiency were in the mitochondrial cytochrome b (MT-CYB) subunit (Fisher and Meunier 2001; Kaphan et al. 2018) and since, over 40 mutations have been identified in MT-CYB. Most of these mutations cause exercise intolerance and progressive myopathy (Andreu et al. 1999; Meunier et al. 2013), and sometimes cardiomyopathy (Valnot et al. 1999) with a delayed disease onset, typically during childhood or adulthood. The sole confirmed case of homoplasmic *MT-CYB* mutation leading to CIII deficiency has been reported to cause a neonatal-lethal multisystemic disease (Fragaki et al. 2009). The MT-CYB protein contains regions that are highly conserved across bacteria, plants, and animals. However, it is highly polymorphic (Meunier et al. 2013), with over 450 such changes having been discovered (Ruiz-Pesini et al. 2007). The majority of these changes are sporadic and heteroplasmic, with high occurrence in skeletal muscle (Sprason et al., 2024) with no clinical consequences.

Additionally, very rare mutations in the UQCRB, UQCRQ, UQCRC2, UQCRFS1 (RISP), and CYC1 subunit genes have been identified in patients, mostly with juvenile-onset mitochondrial diseases (Banerjee, Purhonen, and Kallijärvi 2022). The most commonly mutated gene is *BCS1L* (Hikmat et al. 2021; Čunátová and Fernández-Vizarra 2024). See Table 2 for a list of known mutations causing CIII deficiency in patients.

Table 2. Genes mutated in human CIII deficiency

Category	Gene	Phenotype (no. of patients in the bracket)	OMIM
CIII Subunit Genes	*MT-CYB*	Cardiomyopathy, exercise intolerance, multisystem disorder (<20) (Andreu et al., 1999; Meunier et al., 2013; Valnot et al., 1999)	516020
	UQCRB	Hypoglycemia, lactic acidosis, transient liver dysfunction (1) (Haut et al., 2003)	191330
	UQCRQ	Severe psychomotor retardation, mildly elevated blood lactate (1) (Barel et al., 2008)	612080
	UQCRC2	Liver failure, lactic acidosis, hypoglycemia (4) (Gaignard et al., 2017; Miyake et al., 2013)	191329
	UQCRFS1	Lactic acidosis, cardiomyopathy, alopecia totalis (2) (Gusic et al., 2020)	191327
	CYC1	Ketoacidosis, recurrent metabolic crises, hyperglycemia (2) (Gaignard et al., 2013)	123980
	UQCRH	Severe lactic acidosis, hyperammonaemia, hypoglycaemia, and encephalopathy (Vidali et al., 2021)	620137
Assembly Factor Genes	*BCS1L*	From severe visceral (GRACILE syndrome) to mild sensorineural (80) (Hikmat et al., 2021)	603358 252000
	TTC19	Leigh syndrome, progressive neurodegeneration, cerebellar ataxia (13) (Ardissone et al., 2015; Conboy et al., 2018; Fernández-Vizarra and Zeviani, 2015; Ghezzi et al., 2011; Habibzadeh et al., 2019; Koch et al., 2015)	613814
	LYRM7	Leukoencephalopathy, lactic acidosis (12) (Cherian et al., 2021; Hempel et al., 2017; Invernizzi et al., 2013; Natarajan et al., 2021; Zhang et al., 2019)	615831
	UQCC2	Intrauterine growth retardation, lactic acidosis, delayed development (2) (Feichtinger et al., 2017; Tucker et al., 2013)	614461
	UQCC3	Lactic acidosis, hypoglycemia, hypotonia, developmental delay (1) (Wanschers et al., 2014)	616097

2.3.2 Diseases caused by *BCS1L* mutations

Mutations in *BCS1L* are the most commonly diagnosed cause of CIII deficiencies globally (Hikmat et al. 2021; Čunátová and Fernández-Vizarra 2024). The mutated translocase cannot sufficiently incorporate RISP into CIII and thereby leads to loss of CIII activity. All of these identified mutations are recessive. Even though recent cryoelectron microscopy (Kater et al. 2020; Kwan Tang et al. 2020) has shed some light on the phenotypic variability

among these mutations, the genotype-phenotype correlations are largely unclear and spread across the three domains of the protein with no clear correlation between genotype and phenotype. One thing all of them have in common is their early onset, typically during the fetal period or soon after birth. GRACILE syndrome, of Finnish disease heritage (Uusimaa et al., 2022), is the most severe disease caused by *BCS1L* mutations (Fellman et al., 1998). The major clinical manifestations are severe fetal growth restriction (-4 SD), early-onset lactic acidosis, hepatopathy with cholestasis and iron accumulation, and proximal tubulopathy accompanied by characteristic aminoaciduria (Fellman et al. 1998). The patients also lack subcutaneous adipose tissue, and few have exhibited unusual reactions to sound stimuli, indicating sensorineural hearing loss similar to Björnstad syndrome. So far, 41 infants of Finnish origin with classical GRACILE syndrome have been reported (Čunátová and Fernández-Vizarra, 2024; Fellman, 2012; Fellman et al., 2008, 1998; Guo et al., 2022; Hikmat et al., 2021; Kahraman et al., 2025; Kotarsky et al., 2010; Visapää et al., 2002). GRACILE-like disorders due to a homozygous *BCS1L* mutation have also been published, being most common in Turkey, but similar phenotypes are also caused by compound heterozygous (HE) mutations (Akduman et al., 2016; de Lonlay et al., 2001; Kasapkara et al., 2014; Serdaroğlu et al., 2016). Likewise, Björnstad syndrome with neurosensory hearing loss and pili torti is caused by several *BCS1L* mutations (Hinson et al., 2007). Interestingly, the most common and severe mutations seem to be *c.232A>G*, *c.296C>T* and *c.166C>T*, which cause neonatal disorders both as homozygous and compound heterozygous mutations (Čunátová and Fernández-Vizarra, 2024; de Lonlay et al., 2001; Hikmat et al., 2021; Visapää et al., 2002). Some mutations have also been shown to cause muscle weakness and optic atrophy. Published mutations up to 2024 are documented in a recent study by (Čunátová and Fernández-Vizarra, 2024). With the increasing awareness of *BCS1L* mutations and the availability of whole exome sequencing, the numbers of new mutations are continuously increasing. This published study highlights that comprehensive genotype-phenotype characterization of Finnish disease heritage disorders, monogenic conditions caused by founder mutations more prevalent in Finland than elsewhere, can facilitate the identification of additional mutations in the same genes globally. Thereby, in the case of GRACILE syndrome, it is obvious that the research on the present knock-in mouse model has importance for understanding pathomechanisms and may pave the way for new therapeutic strategies for mitochondrial disorders in general.

2.4 Mouse models of CIII deficiency

Only a few viable mouse models of CIII deficiency exist, as fully knocking out any CIII subunit is likely embryonically lethal. RISP and UQCRQ knock-out (KO) cells and tissues-specific KO mice have been utilized to investigate the role of CIII in respiratory electron transfer and other cellular processes and molecular signaling, like cell differentiation and angiogenesis (Tormos et al. 2011; Diebold et al. 2019). Mice carrying an artificial heterozygous *Uqcrfs1P*$^{p.P224S}$ mutation showed only minor sex-specific phenotypes of aging (Hughes and Hekimi 2011). Mice lacking TTC19 (Bottani et al. 2017) and PARL (Spinazzi et al. 2019), which is essential for TTC19 proteolytic processing, are viable and exhibit mild CIII deficiency with central nervous system symptoms similar to patients with TTC19 mutations (Fernández-Vizarra and Zeviani, 2015). The only patient mutation KI animal model of CIII deficiency is the *Bcs1l*$^{p.S78G}$ mouse carrying the GRACILE mutation (Levéen et al. 2011). CIII-deficient mouse models of different severities are documented in Table 3.

The *Bcs1l*$^{p.S78G}$ mice, carrying the same Finnish missense mutation as classical GRACILE syndrome patients, are born healthy but fail to grow shortly after weaning, exhibiting symptoms including lactic acidosis, hypoglycemia, hepatopathy (with glycogen depletion, lipid accumulation), and kidney tubulopathy (Levéen et al. 2011). At birth and until weaning age (3 weeks), the CIII activity and the amount of fully assembled CIII are normal and then decrease linearly. At the end stage, visceral organs like the liver and kidney (Levéen et al., 2011) show very low CIII activity (20 and 40% respectively) and reduced mitochondrial respiration (Levéen et al., 2011; Purhonen et al., 2020b). The survival of *Bcs1l*$^{p.S78G}$ mice is 4-5 weeks on a genetic background that carries an mtDNA variant *mt-Cyb*$^{p.D254N}$. In a wild-type mtDNA background, the mean survival is between P150 and P210 (Levéen et al., 2011; Purhonen et al., 2020b; Rajendran et al., 2016). In the mutant mice, liver histology reveals periportal hepatocyte degeneration, glycogen depletion, and advancing fibrosis. Hepatic iron accumulation is present but less pronounced than what is observed in human patients. Kidney histology shows a reduction in tubular mass and luminal casts, which are indicative of proteinuria.

Table 3. Mouse models of complex III deficiency; Abbreviations: not determined (n.d.), not applicable (n.a.), conditional knock-out (CKO).

Mutation(s)	CIII Activity (% of WT)	Survival	Phenotype	References
Parl KO and CKO (Δexon2-3)	~70	2 months	Leigh-like severe encephalomyelopathy	(Cipolat et al., 2006; Spinazzi et al., 2019)
Risp CKO (**Δ**exon2) in neurons (CaMKIIα-Cre)	25 (cortex), 14 (hippocampus)	3–3.5 months	Neuronal cell death, mild astrogliosis at end stage	(Diaz et al., 2012; Garcia et al., 2008)
Bcs1l$^{p.S78G}$ KI (Ser78Gly)	25–35 (liver), 50–65 (kidney), 50 (heart), 35 (muscle)	6 months	Growth restriction, hepatopathy, tubulopathy, loss of white adipose tissue (WAT), hypoglycemia, cardiomyopathy, localized cerebral astrogliosis	(Levéen et al., 2011; Purhonen et al., 2017; Rajendran et al., 2019)
Mt-Cyb$^{p.D254N}$ (spontaneous)	75–100	n.d.	Decreased energy expenditure, whole-body respiratory exchange ratio (P30)	(Purhonen et al., 2020b)
Bcs1l$^{p.S78G}$; *Mt-Cyb*$^{p.D254N}$	10–25 (liver), 30–40 (kidney), 30–35 (heart), 25 (muscle)	35 days	Growth restriction, hepatopathy, tubulopathy, loss of WAT, hypoglycemia, lactic acidosis	(Levéen et al., 2011; Purhonen et al., 2020b)
Risp$^{p.P224S}$ KI (Pro224Ser)	HO: n.a., HE: 80–85 (liver)	HO: lethal, HE males: 10% decrease in lifespan	HO: embryonic lethal, HE males: slight decrease in lifespan	(Hughes and Hekimi, 2011)
Ttc19 KO (Δexon7)	40–50 (brain, liver, skeletal muscle)	>18 months	Motor dysfunction, astrogliosis, decreased energy expenditure (females)	(Bottani et al., 2017)
Uqcrq CKO (Δexon 1) in endothelium (Cdh5CreERT2)	n.d.	15–30 days	Severely compromised angiogenesis, lethal by P30	(Diebold et al., 2019)
Uqcrh-KO (Two-exon deletion in *Uqcrh*)	<20%	~12 weeks	Failure to thrive post-weaning, Hyperglycemia, Cardiac contractile dysfunction	(Spielmann et al., 2023; Vidali et al., 2021)
BRAWNIN (*C12orf73*)-KO	<10%	n.d.	Unspecified	(Liang et al., 2022; Zhang et al., 2020)

2.5 Liver, the metabolic hub of the organism

2.5.1 Liver anatomy and physiology

The liver, the largest visceral organ, is located in the right upper quadrant of the abdominal cavity, resting just below the diaphragm, lying at the right of the stomach and surrounding the gall bladder. The liver consists of four lobes, which are further divided into lobules, the functional units of the liver. Each lobule houses millions of hepatocytes. Most of the blood enters the liver via the portal vein under low-pressure conditions, the rest comes through the hepatic artery. Since the portal vein originates from the gastrointestinal tract (GI), the capillary bed of the GI tract already extracts most O_2, resulting in low O_2 content in portal venous blood. In contrast, the blood from the hepatic artery originates directly from the aorta, so it is rich in oxygen. The portal vein, hepatic artery, hepatic bile duct along with lymphatics, and nerves connect with the liver at the location known as the hilus.

The liver lobule consists of a central vein branch encircled by liver parenchyma and several portal areas, each of which contains three elements: a branch of the portal vein, arteriole(s), and bile duct(s) (Trefts et al., 2017). The direction of bile flow through the bile ductulus is opposite to the direction of the blood flow through sinusoidal capillaries. Even though the liver appears quite homogenous histologically, as ~80% of the liver mass is hepatocytes, the organization of the liver lobule leads to metabolite and oxygen gradients across the liver (Stanger 2015). These gradients create a partitioning of functions within the liver, which is called metabolic zonation, with areas having higher oxygen content engaging in increased oxidative metabolism. For example, after feeding, the periportal hepatocytes conduct gluconeogenesis and beta-oxidation, while the perivenous hepatocytes perform triglyceride synthesis and glycolysis (Stanger 2015). Other cells in the liver include bile duct epithelial cells, Kupffer cells, stellate cells, and sinusoidal endothelial cells.

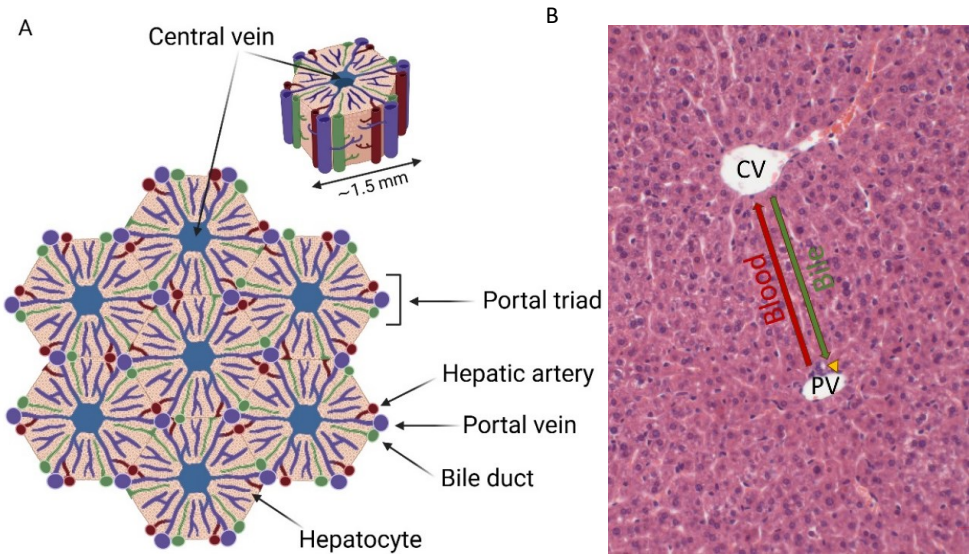

Figure 5: A) Hepatic lobule structure B) A cross-section of liver lobule stained with hematoxylin and eosin. CV, central vein; PV, portal venule. The direction of blood and bile are shown. Yellow arrowheads point to bile ducts and hepatic arterioles (small duct-like structures) next to the PV. Image A was prepared using Biorender.com.

At weeks 34–37 in humans, corresponding to days 18–21 in mice, the liver undergoes a metabolic switch from a glucose-consuming organ to an organ that produces and stores glucose. This switch is supported by an increase in gluconeogenic and glycogenic enzyme expression and a decrease in glycolytic enzyme expression (Trefts et al., 2017). In the fed state, high insulin and low glucagon levels promote glucose uptake and anabolic processes in the liver, inhibiting gluconeogenesis and promoting glycogen and lipid synthesis. During fasting, glucagon levels rise, and insulin levels fall, driving glycogenolysis, gluconeogenesis, fat oxidation, and macronutrient metabolism to maintain blood glucose. This metabolic shift is driven by increased cyclic AMP (cAMP) and regulated by glucocorticoid signaling, which are very important nutrient-sensing mechanisms (Trefts et al., 2017). The liver plays an important role in storing iron, copper, and vitamin A. Moreover, it harbors many vital detoxification processes, including the urea cycle and glutathione metabolism. Most of the water-soluble nutrients, xenobiotics, and drugs undergo hepatic detoxification before reaching other tissues. Moreover, the liver is an endocrine organ (Watt et al. 2019) that secretes insulin-like growth factors and numerous hepatokines, including both chemokines and mitokines. In essence, the liver is undeniably the center of organismal metabolism and helps maintain homeostasis in every tissue of the body.

2.5.2 Hepatic manifestations in OXPHOS disorders

Hepatic manifestations due to OXPHOS deficiency occur in 20% of neonates but are rare in adults (Kotarsky et al., 2010). The two primary types of mitochondrial liver diseases are mtDNA depletion syndromes (El-Hattab and Scaglia, 2013) and CIII deficiencies caused by mutations in nuclear DNA-encoded subunits and assembly factors (Fellman and Kotarsky 2011). These disorders have a wide range of liver disease phenotypes. In neonates, within the first weeks to months of life, acute liver failure can occur, with typical manifestations like steatosis, cholestasis, glycogen depletion, and hypoglycemia with lactic acidosis. Iron overload in the liver is a typical manifestation of GRACILE syndrome but is also present in some mtDNA depletion disorders (Fellman and Kotarsky 2011; Mandel et al. 2001; Pronicka et al. 2011). Other phenotypes of these mtDNA depletion syndromes include hepatomegaly, progressive liver failure, steatosis, cholestasis, fibrosis (Lee and Sokol, 2007a, 2007b).

Recent findings further highlight the strong link between mitochondrial dysfunction and pediatric acute liver failure. A report by (Lenz et al., 2024) demonstrated that the majority of pediatric acute liver failure cases are caused by mutations in genes encoding mitochondrial proteins. Animal models indicate that insufficient mitochondrial ATP production or fatty acid oxidation are not the sole factors for these hepatic manifestations (Cho et al., 2017; Lee et al., 2016). Therefore, the reason why some of the OXPHOS defects cause hepatopathy remains unclear.

2.6. Progeroid manifestations in mitochondrial diseases

Progeria syndromes are rare genetic disorders that accelerate physiological aging, causing individuals to exhibit premature aging characteristics and appear significantly older than their actual age. Most premature aging or progeroid syndromes are caused by dysfunctional nuclear lamina proteins (e.g, in Hutchinson-Gilford progeria syndrome, HGPS) or DNA repair enzymes (e.g., in Werner syndrome), consequently leading to defective genome maintenance and repair (Milosic et al., 2024). Even though mitochondrial diseases often reduce the lifespan, premature aging itself is not characteristic of these disorders. Historically, a conceptual role for mitochondria in aging was suggested to be that mtDNA mutations accumulate over time, which leads to progressive defects in mitochondrial respiratory chain function and subsequently increasing ROS production (Somasundaram et

al., 2024). ROS would then cause oxidative damage to tissues and further mtDNA damage, creating a vicious cycle that contributes to aging-related degeneration. To test this concept, mice with a mutation that impairs the proofreading ability of the mitochondrial DNA (mtDNA) replicative polymerase γ (POLG) were generated. This forced mtDNA mutagenesis results in relatively late-onset premature aging-like manifestations (Kujoth et al. 2005; Trifunovic et al. 2004). These mice, known as mutator mice, accumulate mtDNA mutations, which inevitably lead to the gradual decline of various mitochondrial functions, including the respiratory complexes and mitochondrial translation. Until our findings in the *Bcs1l*$^{p.S78G}$ mice, the mutator mice were the only mitochondrial premature aging model (Trifunovic et al., 2004). Characteristics that resemble segmental progerias, such as short stature, progressive emaciation, hearing loss, anemia, alopecia, and skin lesions, are found in some OXPHOS disorders, including some CIII deficiencies (Gusic et al. 2020; Hikmat et al. 2021; Hock, Robinson, and Stroud 2020; Wolny et al. 2009). Recently, mutations in the mitochondrial outer membrane proteins MTX2 and TOMM7 have been reported to underlie progeroid patient phenotypes (Elouej et al. 2020; Garg et al. 2022). In summary, mitochondrial dysfunction can lead to progeria in both humans and mice, although the mechanisms leading to defective nuclear genome maintenance are largely unknown. Also, it has remained uncertain whether primary OXPHOS deficiency can cause progeria.

2.7 Therapeutic strategies for mitochondrial disorders

2.7.1 Dietary and pharmacological interventions

Previous research has demonstrated that specific dietary modifications can improve the health of patients with mitochondrial disorders. Therefore, it is critical to assess the nutritional needs and deficiencies of the patients individually (Zong et al., 2024). High-carbohydrate diets have been reported to increase oxidative stress, posing metabolic challenges for individuals with impaired oxidative phosphorylation (Jiang et al., 2021). In line with that, similar dietary interventions adversely affected the health and survival of complex III deficient mice (Rajendran et al., 2016). We have also reported the beneficial effect of ketogenic diet on the hepatopathy in *Bcs1l*$^{p.S78G}$ mice (Purhonen et al., 2017). Various pharmacological strategies have been tested as novel therapies in experimental models of various mitochondrial diseases. Even though these interventions are quite limited,

a list of examples of some of the different pharmacological interventions in patients and animal models is listed in Table 4.

Table 4: Pharmacological interventions in mouse models of mitochondrial disease

Intervention	Suggested Target/Mechanism	Disease Model/Disorder	Effects/Findings
Nicotinamide riboside	Supports NAD^+ levels, prevents disease-related mitochondrial ultrastructural changes, enhances mitochondrial DNA stability, and further activates the mitochondrial unfolded protein response	MELAS patient, Deletor mice, with adult-onset mitochondrial myopathy, *Bcs1l*$^{p.S78G}$ mice	Improved blood lactate and pyruvate levels and decreased lesion volume in MELAS patients (Majamaa et al., 1996) and delays the progression of mitochondrial myopathy in Deletor mice and patients (Khan et al., 2014; Pirinen et al., 2020). No therapeutic effect on CIII deficient mice (Purhonen et al., 2018)
Riboflavin	Improves the efficiency of ETC	CI (NADH dehydrogenase) deficient patient	Improved myopathy and encephalomyopathy (Bernsen et al., 1993).
Succinate and coenzyme Q10	Succinate donates electrons directly to FAD, bypassing CI, and CoQ10 stabilizes the CIV	Kearns-Sayre/chronic external ophthalmoplegia plus syndrome	Prevented death due to respiratory failure (Shoffner et al., 1989).
Vitamins C and K	Electron transport capabilities and the ATP synthesis rate were improved	CIII deficient patient	Improved function of skeletal muscle (Eleff et al., 1984).
Dimethylglycine (DMG)	DMG-mediated respiratory chain function improvement is not obvious	Saguenay-Lac-Saint-Jean cytochrome-c oxidase (SLSJ-COX) deficiency patient	Oxygen consumption (VO_2) levels were not improved (Liet et al., 2003).
Pyrimidine Supplementation	Enhancement of the substrate for purine nucleoside salvage pathway	Deoxyguanosine kinase (*dguok*) KO zebrafish	Significant increase in liver mtDNA copy number (Munro et al., 2019).
Bezafibrate	Peroxisome prolife rator-activated receptor (PPAR) agonist; enhances	Multiple respiratory chain deficiencies	Prevented disease progression in muscle-specific knock-out mouse models of COX10 (Yatsuga and Suomalainen,

	mitochondrial biogenesis and fatty acid oxidation		2012). No therapeutic effect in *Surf1* and *Cox15* mice KO mice (Viscomi et al., 2011).
Rapamycin	Inhibits Mechanistic Target of Rapamycin (mTOR) signaling, promoting mitophagy and reducing mitochondrial stress	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial *(Ndufs4)* KO mice, *Twinkle* myopathy, *Tk2-H126N* knock-in encephalopathy	Delayed onset of neurological symptoms, reduced neuroinflammation, and improved histopathology and motor performance in multiple preclinical trials (Barriocanal-Casado et al., 2019; Kallijärvi and Fellman, 2019).
AICAR	Activates AMPK, promoting mitochondrial biogenesis and energy homeostasis	CIV deficiency-induced myopathy and *Bcs1l*$^{p.S78G}$ mice	Improved endurance, muscle function, and biogenesis in multiple models (Peralta et al., 2016; Viscomi et al., 2011). No therapeutic effect on CIII deficient mice (unpublished)
EPI-743 (Vincerinone)	Antioxidant; targets NAD(P)H quinone oxidoreductase to reduce oxidative stress	Leigh syndrome, MELAS, Leber's Hereditary Optic Neuropathy (LHON), FRDA	Improved neurologic function (Enns et al., 2012), reversed vision loss and quality of life (Sadun et al., 2012).
Idebenone	Antioxidants; bypasses CI to facilitate electron transfer in the ETC	LHON, FRDA, MELAS	Improved neurological function (Suárez-Rivero et al., 2021) and recovery in visual acuity (Klopstock et al., 2011)
Dichloroacetate (DCA)	Activates pyruvate dehydrogenase complex by inhibiting Pyruvate dehydrogenase kinase (PDK), enhancing mitochondrial metabolism	Lactic acidosis in mitochondrial disorders	Reduced lactate levels and improved metabolic balance in clinical studies (Tinker et al., 2021).
Elamipretide (SS-31)	Improves cristae networks ETC efficiency	Barth syndrome	Alleviated cardiomyopathy (Sabbah, 2022).

2.7.2. Gene therapy

Gene therapy means transferring genetic material to affected cells to correct the genetic defect and thus prevent or treat the disease caused by the defect. Although delivering and expressing ectopic genes at a physiologically appropriate level throughout the body remains difficult, gene therapy strategies can target specific cells or tissues to achieve a beneficial effect. Preclinical studies have demonstrated that organ-specific gene therapy can improve both cell-autonomous and systemic phenotypes in various mitochondrial disorders. A list of some preclinical gene therapy trials on mitochondrial disease models is documented in Table 5.

Table 5: Preclinical gene therapy trials in mouse models of mitochondrial disease

Mitochondrial Disorder	Gene Therapy Approach	Mouse Model	Outcome	Reference
CI deficiency (NADH:Ubiquinone Oxidoreductase Core Subunit S3 /NDUFS3)	AAV-mediated delivery of *Ndufs3*	Skeletal muscle conditional *Ndufs3*-KO mice	Improvement of the mitochondrial myopathy and revert muscle function after disease onset	(Pereira et al., 2020)
Leigh syndrome	Gene therapy using AAV vectors to restore CI and CIV deficiencies	Multiple models (e.g., *Ndufs4*, Surfeit locus protein 1 /*Surf1* KO)	Extended lifespan and provided behavioral and biochemical improvement	(Corrà et al., 2022; Ling et al., 2021; Reynaud-Dulaurier et al., 2020; Silva-Pinheiro et al., 2020)
CI deficiency (NDUFS3)	AAV-PHP.eB-mediated *Ndufs3* delivery	CNS-specific *Ndufs3* CKO mice	Survival extended from 5–6 to >15 months, prevented motor/cognitive deficits, and reversed neuroinflammation and mitochondrial dysfunction	(Walker et al., 2024)
CIV deficiency (COX10)	AAV-PHP.eB-mediated *Cox10* delivery	CNS-specific *Cox10* CKO mice	Survival extended from 5–6 to >15 months, rescued neuronal loss and gliosis	(Walker et al., 2024)

Mitochondrial Disorder	Gene Therapy Approach	Mouse Model	Outcome	Reference
Dominant optic atrophy (DOA)	AAV-mediated delivery of OPA1 Mitochondrial Dynamin Like GTPase (*OPA1)*	Mouse model of DOA	Rescued retinal ganglion cell survival and optic nerve degeneration.	(Sarzi et al., 2018)
Barth syndrome	AAV delivery of *TAZ* (tafazzin) gene	Tafazzin knockdown (Barth syndrome model)	Improves cardiac protein expression profiles	(Suzuki-Hatano et al., 2019)
Friedreich ataxia (FRDA)	AAV-mediated delivery of frataxin gene	Conditional frataxin deletion mouse model (*Mck-Cre-Fxn*$^{L3/L-}$ mice)	Reversal of cardiomyopathy	(Perdomini et al., 2014)
MPV17-related disease	AAV-mediated delivery of *MPV17*	*Mpv17* KO model	Restores liver mtDNA copy number and OXPHOS	(Bottani et al., 2014)
Mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome	AAV-mediated delivery of thymidine phosphorylase (*TYMP*)	*Tymp/Upp* KO mice	Reduced accumulation of toxic metabolites	(Torres-Torronteras et al., 2018)
Ethylmalonic encephalopathy (EE)	AAV delivery of *ETHE1* gene (ethylmalonic encephalopathy 1)	*Ethe1* KO mice	Improved survival and reduced sulfur metabolite buildup	(Di Meo et al., 2012)
Autosomal recessive human thymidine kinase 2 (TK2) deficiency	AAV-mediated delivery of functional *TK2*	*Tk2* KI mice	Delayed disease onset and increased survival	(Lopez-Gomez et al., 2021)

Leber's hereditary optic neuropathy (LHON) is currently the sole primary mitochondrial disease being investigated in clinical trials for gene replacement therapy (Newman et al. 2022). This disorder is caused by mutations in the mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4 (*MT-ND4*) gene. Phase III trials of Lenadogene nolparvovec have shown both efficacy and good tolerability in LHON patients.

Despite the potential of gene therapy for mitochondrial diseases, significant challenges remain, such as vector design, targeted delivery, transgene expression, and immunotoxicity. Advancements in viral vectors, CRISPR, and prenatal gene therapy make the future increasingly promising for treating these disorders (Soldatov et al. 2022; Di Donfrancesco et al. 2022; Slone and Huang 2020).

A commonly used approach to deliver therapeutic genes to specific tissues or organs involves engineered viral vectors capable of cellular uptake and transferring their therapeutic DNA into the nucleus. One of the commonly used viral vectors is adeno-associated viral vectors (AAVs). AAVs belong to the *Parvoviridae* family and were initially discovered in the 1960s as contaminants in adenovirus preparations (Atchison, Casto, and Hammon 1965). Recombinant adeno-associated viruses (rAAVs) consist of small (4.7 kb), single-stranded DNA in which the genes necessary for viral replication have been replaced with the therapeutic DNA for delivery. AAVs are not linked to any disease in humans or animals and stay episomic in cells for a long time after a single administration, lowering the risk of insertional mutagenesis and their ability to transduce terminally differentiated cells along with their low immunogenicity, making them popular for gene delivery strategies (Lundstrom 2018). Furthermore, the liver in mice is highly amenable to viral transduction by rAAVs, with efficiencies reaching up to 90% (Cunningham et al. 2015). These factors make *Bcs1l*$^{p.S78G}$ mice an especially promising model for rAAV intervention.

3 THE AIM OF THE STUDY

This thesis project aimed to elucidate disease mechanisms in CIII deficiency and investigate the potential of a pre-clinical gene therapy intervention to ameliorate their disease progression.

The specific objectives were:

1) To develop a sensitive enzymatic assay for the quantification of dNTPs, particularly in postmitotic mouse tissues with low dNTP levels

2) To unravel the molecular and cellular mechanism leading to senescence and juvenile-onset segmental progeria in *Bcs1l*$^{p.S78G}$ mice

3) To perform a hepatocyte-targeted gene therapy trial, aiming to dissect the liver-dependent and independent systemic disease mechanisms in *Bcs1l*$^{p.S78G}$ mice

4 METHODS

The original publications describe the methods in detail, the summary of which is presented in Table 6. Here, the experimental design is summarized, and the most crucial methods for this thesis are presented.

Table 6. List of methods

Method	Study
Blood glucose	II, III
Body composition analysis	II
Body temperature and nociception	III
Histochemistry	
- Paraffin-embedded sections	II, III
- Cryosections	III
- Hematoxylin and eosin	II, III
- Periodic acid-Schiff	II, III
- Oil-Red-O	III
- Immunostaining	I, II, III
Protein analyses	
- SDS-PAGE and Western Blot	II, III
- Blue-Native PAGE and Western blot	III
Mitochondrial enzyme activity measurements	III
Gene expression analyses	
- qPCR	II, III
- Transcriptomics	III
ATP measurements	III
Mitochondrial respirometry	I
dNTP quantification	I, II
Statistics	I, II, III

4.1 Mouse strains, health monitoring, and ethics

The *Bcs1l*$^{p.S78G}$ KI mice in the congenic C57B/6JCrl background (Rajendran et al. 2016) were used in studies I-III. We crossbred *Ciona intestinalis* AOX transgenic mice (Szibor et al. 2017) with *Bcs1l*$^{p.S78G}$ heterozygotes to generate breeding pairs capable of producing *Bcs1l*$^{p.S78G}$ homozygous offspring, both with and without the hemizygous AOX transgene. Wild-type and heterozygous *Bcs1l*$^{p.S78G}$ littermates served as healthy controls. The AOX

transgene, integrated into the *Rosa26* locus, is expressed ubiquitously under the regulation of a robust synthetic cytomegalovirus enhancer fused to the chicken beta-actin (CAG) promoter (Szibor et al. 2017).

The behavioral changes and weight loss were monitored to minimize the suffering of the mice, to prevent spontaneous deaths, and to estimate survival. More than 15% weight loss was set as the criteria for euthanasia. For survival, analyses were done blindly to minimize bias. Female and male mice were analyzed separately, and if no difference was found, the groups were combined.

The animal studies were approved by the animal ethics committee of the State Provincial Office of Southern Finland (ESAVI/6365/04.10.07/2017, ESAVI/16278/2020 and ESAVI/31141/2023) and were performed according to FELASA (Federation of Laboratory Animal Science Associations) and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. I have completed the Laboratory Animal Science examination and was trained to do mouse experiments.

4.2 Novel enzymatic assay to quantify dNTPs (I)

The quantitation of dNTP pools was performed using a novel sensitive assay for dNTPs based on long synthetic oligonucleotides, EvaGreen dye, and inhibitor-resistant high-fidelity DNA polymerase.

The polar metabolites were isolated from cultured cells and mouse tissue using physical homogenization and cold 60% methanol extraction, followed by incubation at 95°C for 3 min to quench residual enzymatic activity. Then, the supernatants were run through 3-kDa cut-off centrifugal filters (Amicon Ultra-0.5 ml, Merck), and hydrophobic metabolites were removed by washing the extracts twice with diethyl ether. Residual diethyl ether was evaporated using Speed-Vac Plus SC110A centrifugal vacuum evaporator (Savant Instruments, Farmingdale, NY, USA), and the extract was diluted according to the initial biological sample used.

The measurement was carried out in four separate reactions, one per canonical dNTP (dATP, dCTP, dGTP, dTTP). Each reaction mixture included long synthetic oligonucleotides, EvaGreen dye, and inhibitor-resistant high-fidelity DNA polymerase, with three non-limiting dNTPs. Then, an equal volume of sample was added, which then supplied the dNTP to be measured, and the assay was run in the thermal cycler (CFX384, BioRad). To eliminate

the interfering signal from ribonucleotides, thermostable RNAse HII (IDT) was included in the reaction mixture. Finally, a sigmoidal standard curve was generated from the baseline-corrected end-point fluorescence values of the standard samples, and the exact concentration of each dNTP of the samples was interpolated.

4.4 Assessment of cellular senescence and progeria-like manifestations (II)

We used the *Bcs1l*$^{p.S78G}$ mice to determine whether an isolated deficiency in mitochondrial complex III (CIII) is enough to cause cellular senescence and progeria-like disease. The included radiographic imaging and body composition analysis, along with histological studies of the skin, liver, kidney, thymus, and spleen. Additionally, molecular characterization involved gene expression analysis using quantitative PCR (qPCR) and RNA-sequencing (RNA-Seq), protein level analysis through western blotting and immunohistochemistry, and examining markers of cellular senescence (p21, γH2AX, TP53). To investigate the mechanism behind senescence and segmental progeria, we used ubiquitously expressing AOX mice and liver-specific AAV expressing Omomyc.

4.5. Hepatocyte-targeted gene replacement therapy (III)

Presymptomatic (postnatal day 19-23, P19-23) mutant mice were injected intraperitoneally with 100 µl saline containing 5×10^{10} rAAV, serotype 9. Hepatocyte-specific ApoE enhancer and α1-antitrypsin (AAT) promoter were used to drive mouse BCS1L expression. As a control, the same rAAV backbone expressing EGFP was injected. The groups of mutant mice injected with rAAV carrying *Bcs1l* or *EGFP* transgene will be referred to as AAT-*Bcs1l* and AAT-*EGFP*, respectively. Along with the expression cassette, the hepatocyte-specific vector (AAT-rAAV) also has flanking PiggyBac transposon recognition sequences, which allow genomic integration upon parallel expression of PiggyBac transposase (Cunningham et al. 2015). As a third group, to ensure persistent expression in the growing liver, we co-injected the PiggyBac transposase-encoding rAAV. As a control for hepatocyte-specific effects, we used a construct with a broadly active CAG promoter. Phenotypic assessment involved monitoring survival rates, body temperature, growth, and blood glucose levels. Histological studies were performed on liver, kidney, skeletal muscle, and BAT, with molecular characterization through qPCR, western blotting, and immunohistochemistry to evaluate mitochondrial function and markers of cellular stress. Body temperature was

assessed by measuring core and skin temperatures and analyzing cold-inducible gene expression (*Rbm3*, *Cirbpv4*) using qPCR. Thermogenesis response was studied through the analysis of UCP1 and ADRB3 expression in brown adipose tissue and skeletal muscle by qPCR and western blotting. Sensory neuropathy was examined using immunostaining of footpad nerve tissues to assess sensory neuron integrity.

4.6 Statistics

For data that followed a normal distribution, we performed a one-way ANOVA followed by selected comparisons using t-tests with Welch's correction. For the rest, the Mann-Whitney U test was performed. Survival curves were examined utilizing the log-rank Mantel-Cox test. These statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software Inc.). Details regarding the specific statistical tests used and the *p*-values are indicated in the figure legends and shown within the graphs.

5 RESULTS

5.1 CIII-deficient mice show juvenile-onset segmental progeria

We examined *Bcs1l*$^{p.S78G}$ knock-in mice on two mtDNA backgrounds: homozygotes with severe CIII deficiency (*mt-Cyb*$^{p.D254N}$) that succumb by P30-35 to cachexia and hypoglycemia (Purhonen et al., 2020b), and homozygotes with WT mtDNA that show juvenile disease onset but survive until P200 and develop late-onset dilating cardiomyopathy (Rajendran et al. 2019).

Radiographic analysis of the thoracic-lumbar curvature in juvenile 1-month-old *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice revealed severe kyphosis, consistent with previous reports (Rajendran et al. 2019; Purhonen et al. 2018; 2017). (**II,** Fig. 1a). Body composition analysis further showed reduced body fat and bone mineral density in both the mutant mice (**II,** Fig. 1b, c and Supplementary Fig. 1a–c). Patients with GRACILE syndrome exhibit thin, wrinkled skin (Fellman et al. 1998). In line with that, histological examination of *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice skin revealed a lack of the dermal fat layer (**II,** Fig. 1d). *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice exhibited albuminuria and significantly reduced urinary MUP levels (**II,** Fig. 1e), similar to naturally aged and *Zmpste24-/-* mice (a model for HGPS) (Garratt et al. 2011; Varela et al. 2005). Furthermore, like many progeroid mouse models that frequently exhibit thymus and spleen involution, the thymic remnants of *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice typically weighed less than 10% of WT thymus (**II,** Fig. 1f, h), and their spleens were also atrophic (**I,** Fig. 1g, h).

We then investigated cellular and molecular indicators of premature (non-replicative) senescence, a characteristic of premature aging, in the tissues of the mutant mice. In earlier studies, we observed an atypical hepatic progenitor cell response in *Bcs1l*$^{p.S78G}$ mice (Purhonen et al. 2017). Typically, the liver regenerates through hepatocyte hypertrophy and proliferation, with minimal involvement of progenitor cells unless hepatocyte proliferation is impaired (Español–Suñer et al. 2012; Lu et al. 2015). Juvenile *Bcs1l*$^{p.S78G}$ and *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice, expected to exhibit normal growth- and regeneration-related hepatocyte proliferation, demonstrated notable increases in the senescence marker cyclin-dependent kinase inhibitor 1A; CDKN1A (p21) (**II,** Fig. 1i) and its protein-level expression (**II,** Fig. 1j). Moreover, the mutant mice liver exhibited transcriptional upregulation of Cathepsin L (**II,** Fig. 1n), a cysteine protease found in lysosomes and the nucleus, and is linked to nuclear lamina damage (Gonzalez-Suarez et al. 2011; Islam et al. 2022) and

implicated in cleavage events during cellular senescence (Duarte et al. 2014). Further gene expression analysis of the senescence-associated secretory phenotype (SASP) revealed a 50- to 600-fold increase in amphiregulin (Areg) and elevated expression of several associated chemokines and cytokines in the mutant liver (**II,** Supplementary Fig. 1i). In the transcriptomics data of liver and kidney of adult *Bcs1l*$^{p.S78G}$ mice, the most highly upregulated gene was *Gpnmb* (Rajendran et al. 2019; Purhonen et al. 2017), a transmembrane glycoprotein associated with aging and recently used to target and remove senescent cells (Suda et al. 2021). We observed a 100-fold increase in *Gpnmb* mRNA expression in the mutant mice as early as one month of age (**II,** Fig. 1o).

Irreparable DNA damage is a frequent cause of cellular senescence (Hernandez-Segura, Nehme, and Demaria 2018). Therefore, we measured the DNA double-strand break marker γH2AX (S139-phosphorylated histone H2AX), which showed a several-fold increase in liver lysates from the mutant mice (Fig. 2c). Abnormal nuclear architecture can both result from and contribute to DNA damage, and it is also a characteristic feature of cellular senescence and progeroid syndromes (Hernandez-Segura, Nehme, and Demaria 2018; Carrero, Soria-Valles, and López-Otín 2016). The livers of *Bcs1l*$^{p.S78G}$ mice exhibited hepatocyte anisokaryosis and karyomegaly (**II,** Fig. 3a–d), indicative of G2 phase cell cycle arrest and an abnormal increase in polyploidy (Barnhoorn et al. 2014; Núñez et al. 2000; Selfridge et al. 2001). Unlike the uniformly round nuclei of WT hepatocytes, mutant hepatocytes frequently showed laminopathy-like nuclear envelope invaginations and blebs (**II,** Fig. 3a–c, e). By approximately P100, some *Bcs1l*$^{p.S78G}$ mutant hepatocyte nuclei contained cytoplasmic inclusion-like structures similar to those seen in hepatocytes of DNA repair-deficient *Xpg*$^{-/-}$ and *Ercc*$^{-/\Delta}$ mice, as well as in normally aged livers (Barnhoorn et al. 2014; Andrew 1962; Dollé et al. 2011). Furthermore, western blot analysis of liver lysates from juvenile *Bcs1l*$^{p.S78G}$ mutants revealed a changed ratio of lamin C to lamin A (**II,** Supplementary Fig. 2f), indicating a significant impact on nuclear structural integrity.

5.2 Development of a sensitive enzymatic assay for dNTP quantification

This project in the thesis arose from the technical requirements in project II, specifically, the need to quantify minute dNTP concentrations in mouse tissue biopsies. After understanding the importance of dNTP balance, it became necessary to measure them, which has historically been a challenging task. This difficulty arises because the cellular concentration of dNTPs is very low, and the available biological material from mice is often limited. Additionally, dNTP concentrations differ significantly between cell types, stages of the cell cycle, and subcellular compartments (Gandhi and Samuels 2011; Wheeler and Mathews 2011; Pancsa et al. 2022). Traditional enzymatic methods for measuring dNTPs have depended on incorporating radioactive dNTPs into a complementary DNA strand by a DNA polymerase (Ferraro et al., 2010; Sherman and Fyfe, 1989). These methods have primarily been used to measure dNTPs in actively proliferating cultured cells with high dNTP concentrations and minimal complexity of interfering biological matrix (Ferraro et al., 2010; Hunting and Henderson, 1981; Landoni et al., 2018; Sherman and Fyfe, 1989). Although enzymatic methods have measured dNTP concentrations in the liver (Söderhäll, Larsson, and Skoog 1973) and skeletal muscle samples (Ylikallio et al. 2010; Nikkanen et al. 2016; Forsström et al. 2019), no method validation data for tissue samples have been reported. Recently, a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS)-based method was developed to quantify tissue dNTP levels from a small tissue sample (15–30 mg) (Olafsson et al. 2017). While the HPLC method is robust, not all laboratories have the access to necessary HPLC-MS equipment and expertise.

In the last few decades, due to significant improvements in molecular biology techniques, the possibility of designing synthetic DNA sequences drastically improved (Sherman and Fyfe, 1989) and also, the development of high-fidelity thermostable polymerases further reduced NTP misincorporation (Ferraro et al. 2010). Lately, a simple probe hydrolysis-based enzymatic fluorometric assay was developed to measure dNTPs from cultured cells (Wilson et al. 2011). This utilizes synthetic DNA templates and a Taq DNA polymerase. Due to its limited sensitivity and robustness, especially with complex tissue extracts containing low dNTP concentrations, this method couldn't measure tissue dNTP levels reliably in our model.

Our novel enzymatic assay for dNTP quantification can be performed in a single microplate well, requiring only the addition of a reagent mix and the sample. A standard qPCR instrument is used for temperature control and fluorescence reading. The method employs

the highly sensitive EvaGreen detection chemistry for double-stranded DNA (dsDNA) and leverages advances in chemical oligonucleotide synthesis to produce ultralong (~200 nt) ssDNA templates at a reasonable cost. EvaGreen, a well-characterized intercalating DNA fluorochrome, offers higher specificity for dsDNA over single-stranded DNA (ssDNA) compared to SYBR Green (Mao, Leung, and Xin 2007).

We primarily used Q5 DNA polymerase in this work because it retains specificity despite the typical rNTP-to-dNTP ratios in cultured cells. Due to the template design and the excess of non-limiting dNTPs, rNTP interference is minimal, limited to a single misincorporation at the detection site. RNAse HII then cleaves the ribonucleotide bond, allowing specific signal detection by reading the fluorescence at a temperature where specific full-length products remain dsDNA while unspecific products denature to ssDNA. Additionally, the chosen DNA polymerase tolerates inhibitory impurities often present in concentrated tissue extracts.

After optimization, we achieved reliable quantification down to 30 fmol using 197-nucleotides long (nt) templates and 90 fmol using 50-nt templates. The actual lowest limit of quantification (LLOQ) is likely lower, especially when using the product melting temperature profile for end-point readout. The cost of our assay, excluding sample preparation, is approximately 34 EUR (37 USD) per 100 detection reactions with 197-nt templates.

In summary, we developed a simple, inexpensive, and robust enzymatic fluorometric assay capable of measuring dNTPs from non-proliferating cells or tissue extracts. The assay requires less than 4 mg of tissue or 0.1×10^6 cultured cells without technical replicates. Additionally, it supports 96 and 384-well formats, enabling the simultaneous measurement of numerous samples.

Given the role of mitochondria in nucleotide biosynthesis and maintaining their redox and phosphorylation statuses, we quantified rNTPs (Purhonen, Hofer, and Kallijärvi 2023), pyridine dinucleotides, and dNTPs in the liver of P30 mice. Despite rNTP depletion, dNTP levels were not depleted in the mutant liver tissue (**II,** Fig. 6e), likely due to tight allosteric feedback regulation (Nordlund and Reichard, 2006) Interestingly, purine dNTPs (dATP and dGTP) concentrations were increased, suggesting a skewed nucleotide balance or an altered proliferative status of the liver.

5.3 Mechanisms of progeroid disease in CIII-deficient mice

To investigate the mechanisms linking CIII deficiency to aberrant cell cycle progression, we administered a 16-hour *in vivo* bromodeoxyuridine or 5-bromo-2'-deoxyuridine (BrdU) labeling of replicating DNA to the mice. *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice exhibited a significant increase in hepatic and renal DNA synthesis (**II,** Fig. 9d, f and Supplementary Fig 9b, e). In published transcriptomics analysis of mutant liver at P45, *c-myc* emerged as the top-predicted transcriptional regulator driving the observed gene expression alterations (Purhonen et al. 2017). At protein level c-Myc expression was hugely upregulated in the P30 liver of *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice (**II,** Fig. 7f). A hallmark of c-MYC-driven cell cycle progression, as seen in cancer, is the bypass of metabolic checkpoints, a phenomenon also observed in normal cells (Felsher et al. 2000). We evaluated several cell cycle markers (**II,** Fig. 9a and Supplementary Fig. 9a), and despite significant nucleotide depletion, which typically inhibits cell cycle entry or progression to the S-phase (Linke et al. 1996), *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice exhibited increased hepatic expression of cyclin D1 and proliferating cell nuclear antigen (PCNA). Cyclin D1 and PCNA are markers that peak during the G1- and S-phase of the cell cycle, respectively, indicating insufficient metabolic checkpoints at G0 and G1, consistent with elevated c-MYC expression. In contrast, levels of cyclin A2 and Ki-67, markers that peak during G2 to early mitosis, remained unchanged. These data suggest that many hepatocytes and renal tubular epithelial cells in *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice failed to complete the cell cycle and became arrested in the G2 phase, leading to polyploidy, aneuploidy, senescence, or cell death.

To establish causal evidence for a role for c-MYC's role in driving DNA damage in CIII deficiency, we injected recombinant adeno-associated viral vectors (rAAV) expressing Omomyc specifically in hepatocytes of post-weaned *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice (**II,** Fig. 10a). Omomyc, a dominant-negative mutant fragment of c-MYC (Massó-Vallés and Soucek 2020), significantly reduced DNA damage as indicated by decreased H2AX phosphorylation across the entire liver (**II,** Fig. 10b, g). While Omomyc primarily functions by inhibiting c-MYC binding to its target DNA sequences (Beaulieu et al. 2019), it also lowered endogenous c-MYC levels (**II,** Fig. 10c), consistent with previous reports (Demma et al. 2019).

5.4 Effect of AOX in juvenile CIII-deficient mice

We utilized transgenic expression of *Ciona intestinalis* AOX (Rajendran et al. 2019) to examine the link between respiration, nuclear DNA damage, and senescence. This approach aimed to restore coenzyme Q oxidation and electron flow via CI-CII, upstream of CIII. AOX expression extended the median survival of *Bcs1l*$^{p.S78G}$;*mt-Cyb*$^{p.D254N}$ mice threefold to 111 days, with some individuals surviving beyond 200 days (**II,** Fig. 4a). AOX intervention prevented weight loss and growth failure (**II,** Fig. 4b), as well as mitigated typical liver pathology (**II,** Fig. 4c and Supplementary Fig. 3), while maintaining kidney function and morphology (**II,** Fig. 4d, e and Supplementary Fig. 4). At the molecular level, AOX nearly completely suppressed the c-Myc induction (**II,** Fig. 7f, g), abolished the DNA damage response and cellular senescence in the liver, evidenced by the absence of γH2AX, TP53 (p53), and CDKN1A induction (**II,** Fig. 4f–h). Moreover, AOX prevented the increase in hepatic and renal DNA synthesis (**II,** Fig. 9d, f and Supplementary Fig 9b, e) and the accumulation of large hepatocyte nuclei, which correspond to cells in the S-G2 phase of the cell cycle and those with increased polyploidy (**II,** Fig. 9b, c). Interestingly, in the kidney, AOX partially reduced the induction of γH2AX (**II,** Fig. 4j), suggesting attenuated active DNA damage. Similar protective effects were observed in the liver of juvenile *Bcs1l*$^{p.S78G}$;*mt-Cyb*$^{p.D254N}$ mice, where AOX prevented the upregulation of γH2AX and CDKN1A and mitigated liver disease (Supplementary Fig. 5a, c–e). However, these benefits diminished by P150 (**II,** Supplementary Fig. 5b and ref. (Rajendran et al. 2019)).

5.5 Hepatocyte-targeted gene therapy in CIII-deficient mice

To explore the potential of gene therapy and investigate the roles of the liver, we performed a liver-targeted rAAV transduction in the CIII-deficient mice (genotype: *Bcs1l*$^{p.S78G}$;*mt-Cyb*$^{p.D254N}$). rAAV vectors encoding either *Bcs1l* or *EGFP* genes driven by a hepatocyte-specific ApoE enhancer, and α1-antitrypsin (AAT) promoter (Cunningham et al. 2015) (**III,** Fig. 1A, B). The AAT-*Bcs1l* achieved high transduction efficiency where a hepatic *Bcs1l* mRNA expression was increased about 20 times (**III,** Fig. 1C). Furthermore, blue native gel electrophoresis and immunoblotting of UQCRFS1 (RISP) verified that there was a restoration of complex III assembly with an improvement in CIII activity, leading to increased levels of liver ATP (**III,** Fig. 2A-C). This restoration of CIII activity normalized the compensatory mitochondrial biogenesis, shown by the mitochondrial mass markers

VDAC1 and HSP60 (**III,** Fig. 2E). Moreover, AAT-*Bcs1l* prevented all the manifestations of the hepatopathy, such as expansion of portal areas, increase in ductular reactions and cell death (**III,** Fig. 2F-I). AAT-*Bcs1l* also prevented the upregulation of the disease marker gene (Purhonen et al. 2017) glutathione S-transferase 1 (*Gsta1*) and mitochondrial dysfunction-associated mitokine, growth-differentiation factor 15 (*Gdf15*) (**III,** Fig. 2J, K).

Quite impressively, a single injection of AAT-*Bcs1l* prolonged the median survival by 100%. (Fig. 6A). The prolonged survival in the treated mice was likely due to the prevention of a lethal metabolic crisis, specifically severe hypoglycemia (Fig. 6B). Growth during fetal development and throughout adolescence demands substantial metabolic resources, and in *Bcs1l*$^{p.S78G}$ mice, growth

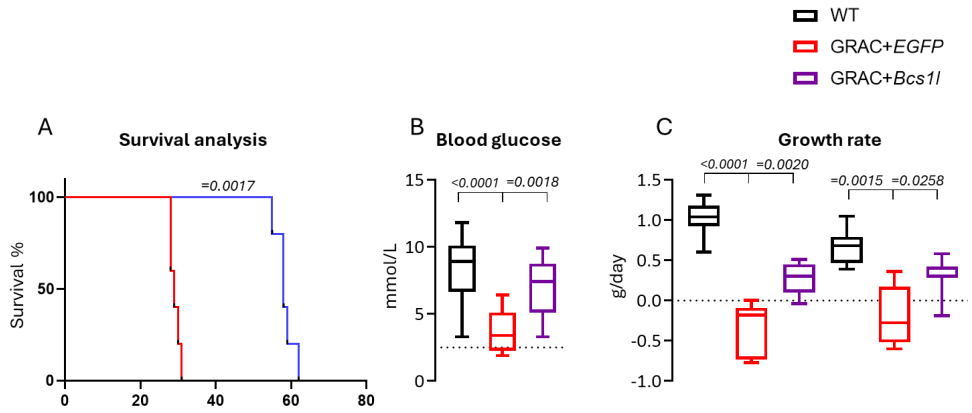

Figure 6: rAAV-mediated gene therapy restores growth and extends the lifespan of CIII-deficient mice two-fold: A) Survival analysis of mutant mice injected with rAAV-*EGFP* compared to those treated with three distinct rAAVs expressing *Bcs1l*. B) Blood glucose levels at P28, with the dotted line marking the critical threshold (<2.5 mmol/L) associated with spontaneous mortality. C) Sex-segregated growth rate of mice between P25 and P28

significantly slows after 3 weeks of age, along with the gradual decline in CIII activity (Levéen et al. 2011). Hepatocyte-targeted gene therapy partially restored the expression of key growth regulators, *Igf-1* and *Ghr*, in the liver and improved overall growth (Fig. 6C & **III,** Fig. 1G & H). This suggests that the restoration of hepatic mitochondrial function helped to normalize growth signaling, enhancing the availability of metabolic resources necessary for proper growth and development.

5.6 Thermogenesis in the CIII-deficient mice

Adaptive, or facultative, thermogenesis is driven by skeletal muscle as well as brown and beige adipose tissues, functioning under neuronal regulation (Tran et al. 2022). In newborn humans and small homeothermic animals like mice, brown adipose tissue (BAT) serves as a primary site for heat generation. In healthy individuals, thermoreceptors in the skin and visceral organs detect temperature changes and relay signals through the hypothalamus to sympathetic nerve endings in the BAT (Tran et al. 2022). Mutant mice exhibited hypothermia (32–36°C) at P28–P30 (Fig. 7A). Hepatocyte-specific restoration of CIII function via AAT-*Bcs1l* normalized body temperature (Fig. 7A). Mutant mice displayed sensory neuropathy with fragmented peripheral nerve markers in foot pads and decreased sensory innervation, which AAT-*Bcs1l* partially improved (**III,** Fig. 3D-G). Dysfunction in interscapular BAT (iBAT) thermogenesis was evident in mutants. iBAT showed low mitochondrial CIII activity (Fig. 7B), inflammation (**III,** Fig. 4E, F), suppressed browning and thermogenic gene expression (**III,** Fig. 4G), and unresponsiveness to cold-induced activation (Fig. 7C & **III,** Fig. 4M & N). AAT-*Bcs1l* corrected some liver-related inflammatory markers but did not rescue BAT function (Fig. 7B, C, & **III,** Fig. 4C-G, M & N). Given that skeletal muscle thermogenesis is likely regulated by neuronal adrenergic signaling similar to brown adipose tissue (BAT) in rodents, it was anticipated that this process would also be impaired in the mutant mice. On the contrary, skeletal muscle thermogenesis showed adaptive responses, including increased sarcolipin (*Sln)*, *Pdk4*, and fatty acid metabolism genes (Fig. 7D), to compensate for BAT dysfunction but could not fully counteract the systemic thermogenic deficits. AAT-*Bcs1l* restored skeletal muscle metabolism to normal by addressing liver-driven metabolic shifts, highlighting systemic crosstalk.

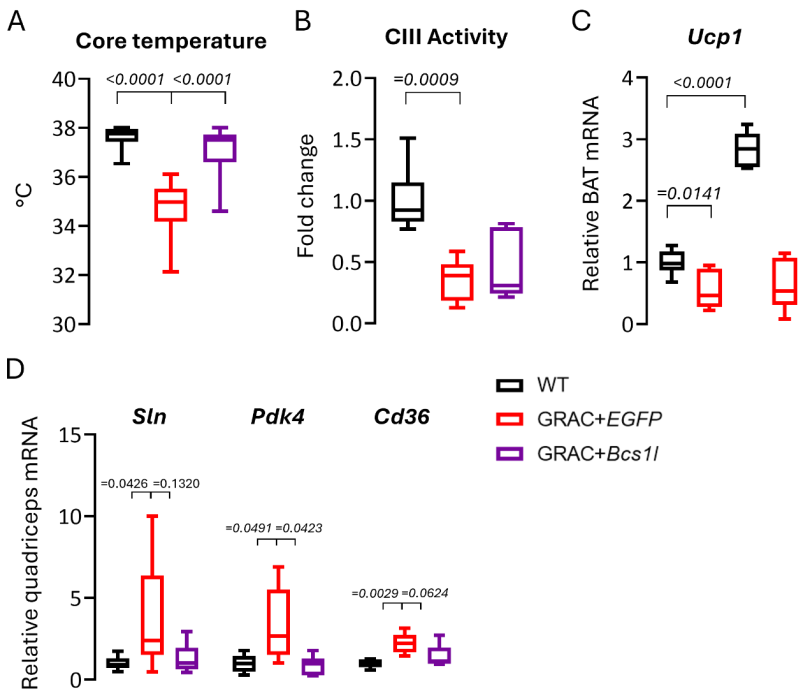

Figure 7: Hepatocyte-targeted gene therapy reverses hypothermia in CIII-deficient mice without BAT activation: A) Estimated core temperature of P28 mice, B) CIII activity in P28 BAT, adjusted relative to CIV activity C) Impact of cold exposure on Ucp1 mRNA expression in P28 BAT D) mRNA levels of *Sln*, *Pdk4*, and *Cd36* in P28 quadriceps

5.7 Hepatic mitochondrial respiration is necessary to maintain euthermia in juvenile CIII-deficient mice

The restoration of CIII function in hepatocytes normalized liver energy metabolism, preventing key pathological changes such as glycogen depletion (**III,** Fig. 6b), micro-vesicular fat accumulation (**III,** Fig. 6A), and the associated transcriptional alterations that reflected impaired fatty acid oxidation (Fig 8A & **III,** Fig. 6F & G). The mutant liver showed a shift towards increased fatty acid oxidation to compensate for low blood glucose and depleted glycogen stores (Fig. 8A). However, probable dysfunction in mitochondrial β-oxidation, which is directly tied to the loss of CIII function and the mitochondrial coenzyme Q pool, limited this adaptive response. AAT-*Bcs1l* gene therapy largely corrected these

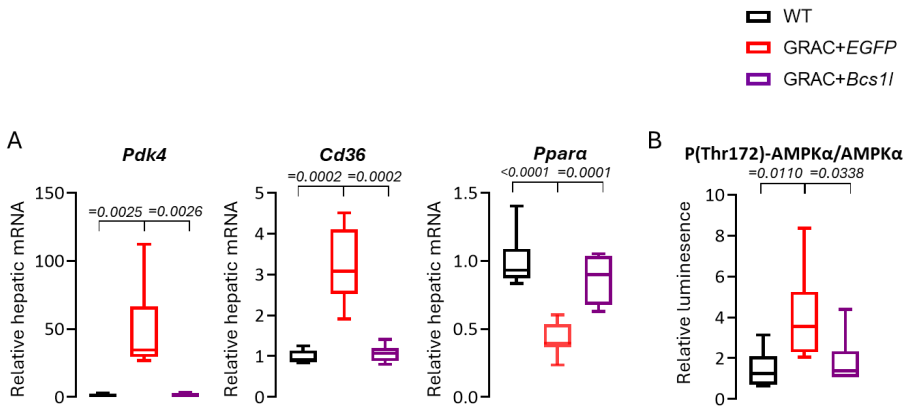

Figure 8: Restoring basal liver metabolism maintains normal body temperature in CIII-deficient mice: A) *Pdk4*, *Cd36*, and *Pparα* mRNA expression from P28 liver, B) Western blot quantification of the phosphorylation status of AMPKα from P28 liver lysates

abnormalities by re-establishing mitochondrial respiration (Fig. 8A, B & **III,** Fig. 6A, B, F-H).

The critical role of the liver in systemic thermogenesis underscores its important contribution to whole-body energy homeostasis. Unlike BAT and skeletal muscle, which rely on adaptive mechanisms triggered by external stimuli, hepatic basal metabolism operates continuously to support energy and heat production (Tran et al. 2022; Rui 2014). Despite the persistent dysfunction in BAT and skeletal muscle, the basal metabolic heat generation through restored mitochondrial respiration was sufficient to maintain the core body temperature in CIII-deficient mice. Moreover, housing the mutant mice at 35ºC relieved the metabolic stress and hepatocyte senescence (**III,** Fig. 7F-J).

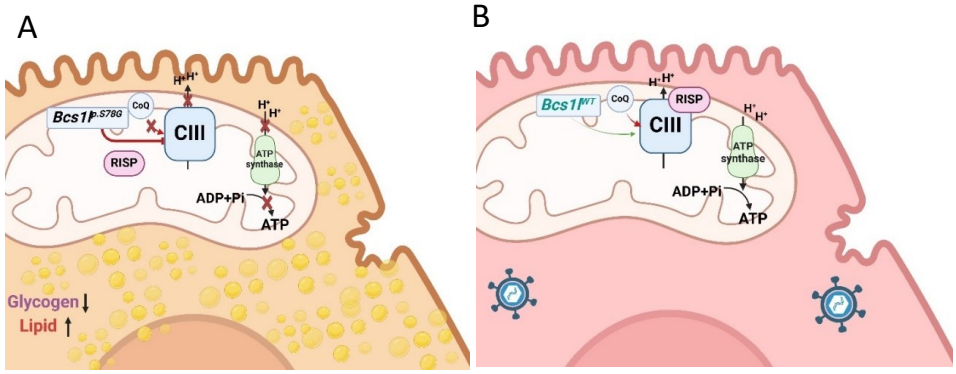

Figure 9: CIII deficient hepatocyte in *Bcs1l*$^{p.S78G}$ mice before and after rAAV gene replacement therapy. The image was prepared using Biorender.com.

6 DISCUSSION AND FUTURE PROSPECTS

In the first study, we successfully developed and validated a novel enzymatic fluorometric assay for quantifying dNTP concentrations in tissue samples and non-proliferating cells. The first enzymatic assay for dNTPs was published in 1969 (Solter and Handschumacher, 1969) with subsequent studies making minor improvements in sensitivity and specificity (Ferraro et al., 2010; Forsström et al., 2019; Hunting and Henderson, 1981; Landoni et al., 2018; Nikkanen et al., 2016; Sherman and Fyfe, 1989; Söderhäll et al., 1973; Ylikallio et al., 2010). Previous studies quantified dNTPs in cultured cells (Ferraro et al., 2010; Hunting and Henderson, 1981; Landoni et al., 2018; Sherman and Fyfe, 1989) and some tissues, but validation of the methods for tissue samples is largely lacking. Our enzymatic assay provides a simple, cost-effective, high-throughput method for dNTP quantification with a detection limit of 30 fmol. Using this tool, we identified elevated purine dNTP levels in *Bcs1l*$^{p.S78G}$ liver, indicating disrupted nucleotide balance and/or altered proliferative states.
Few studies have previously explored tissue dNTP levels in the context of health and disease (Olafsson et al. 2017; Ylikallio et al. 2010; Nikkanen et al. 2016; Forsström et al. 2019). With growing interest in nucleotide metabolism in cancer biology and metabolic disorders like mitochondrial diseases (Nikkanen et al. 2016; Buj and Aird 2018), the development of a user-friendly dNTP assay for routine laboratory was important.
In the second study, the *Bcs1l*$^{p.S78G}$ KI mice on two distinct mtDNA backgrounds provided valuable insights into the molecular and cellular mechanisms leading to premature aging in OXPHOS deficiency. Studies of aging in model organisms showed a decline in mitochondrial function, which might in turn result in the observed age-dependent decline in organ function (Chistiakov et al., 2014; Sun et al., 2016). A similar trend has been observed in humans as well, along with a predisposition to certain age-related diseases (Petersen et al. 2003). The relationship between mitochondrial function and aging has been most extensively researched in skeletal muscle. Most studies have found that aging typically leads to a decline in mitochondrial enzyme activity (e.g., citrate synthase), a reduction in respiratory capacity per mitochondrion (e.g., substrate-dependent oxygen consumption), and an increase in ROS production (H. Cui et al., 2012; Joseph et al., 2012; Short et al., 2005). The CIII deficient *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mice exhibited hallmark features of segmental progerias including kyphosis, reduced body fat and bone mineral density, dermal fat loss, thymic and splenic atrophy, and signs of systemic senescence. Premature aging has previously been associated with *Polg*$^{p.D257A}$ mtDNA mutator mice among OXPHOS

deficiency models (Trifunovic et al., 2004). However, traits resembling segmental progerias, such as growth failure, emaciation, and skin lesions, are common in OXPHOS disorders, including some CIII deficiencies (Gusic et al. 2020; Hikmat et al. 2021; Hock, Robinson, and Stroud 2020; Wolny et al. 2009). Even though the mechanism behind these premature aging manifestations is not well understood, one hypothesis suggests that aging phenotypes in mutator mice arise from excessive mtDNA repair depleting dNTPs (Hämäläinen et al. 2019). However, findings from *Bcs1l*$^{p.S78G}$ mice show that OXPHOS deficiency can directly lead to nuclear DNA damage and premature aging without mtDNA involvement.

Bcs1l$^{p.S78G}$ mice showed markers of premature senescence (e.g., CDKN1A, γH2AX, and SASP genes), nuclear abnormalities, and DNA damage. Hepatocyte-specific c-MYC inhibition using Omomyc reduced DNA damage, highlighting its role in OXPHOS deficiency. Further exploration of this potential retrograde signal between mitochondria and nucleus is needed. Genetic experiments, such as crossing *Bcs1l*$^{p.S78G}$ mice with *c-Myc* KO or hypomorphic allele mice, could provide valuable insights. The triggers of c-MYC induction in response to OXPHOS deficiency remain unclear. However, it could also be a potential downstream event after stimulation with the EGFR ligand AREG, which is a novel mitokine and part of the senescence-associated secretory phenotype (Hino et al. 2022).

AOX expression tripled median survival and alleviated disease phenotypes, including weight loss, organ dysfunction, and senescence markers (γH2AX, TP53, CDKN1A) in the mice. It corrected aberrant cell cycle progression, preventing polyploidy, aneuploidy, and pathological DNA synthesis. The positive impact of AOX may be linked to an unknown redox regulation mechanism of the CoQ pool, a reduction in mitochondrial membrane potential, or further suppression of already low mitochondrial ROS production. The concentration of cellular H_2O_2 is known to influence cell proliferation, either promoting or inhibiting it, depending on its level (Burdon 1995). Further research is needed to explore these effects. Overall, this study underscores a novel pathomechanism of mitochondrial dysfunction and c-MYC dysregulation in driving DNA damage, senescence, and cell cycle abnormalities in CIII deficiency.

In the third study, we highlight the potential of liver-targeted gene therapy using AAT-*Bcs1l* as an effective therapeutic approach for addressing CIII deficiency and its systemic effects in *Bcs1l*$^{p.S78G}$*;mt-Cyb*$^{p.D254N}$ mutant mice. Gene therapy offers a promising treatment for monogenic recessive diseases. The strategy involves reintroducing the wild-type version of

a mutant gene or other therapeutic genes using suitable delivery strategies to treat the genetic condition. Even though delivering and expressing an ectopic gene across the entire body remains challenging, current technologies can target specific cells or tissues to achieve therapeutic effects. Liver-targeted gene therapy showed significant rescue of lethal phenotype in a mouse model of mitochondrial neurogastrointestinal encephalomyopathy caused by *TYMP* gene mutations and also in a zebrafish model of Leigh syndrome French Canadian type (LSFC) caused by *LRPPRC* mutation (Soldatov et al. 2022; Sabharwal et al. 2022). In GRACILE syndrome patients and *Bcs1l*$^{p.S78G}$ CIII deficient mice, the liver is one of the main affected organs. Thus, hepatocyte-targeted gene replacement therapy holds promise for treating liver damage caused by severe CIII deficiency. Interorgan communication between the affected and unaffected organs in mitochondrial diseases is still poorly understood (Li, Cui, and Tian 2022; Boardman et al. 2023). For instance, in *Bcs1l*$^{p.S78G}$ mice, the extent to which systemic phenotypes like growth restriction, lipodystrophy, and hypoglycemia are liver-dependent remains unknown (Tomašić et al. 2020). Thus, rescuing the liver in such a multi-organ disorder can shed light on the systemic effect of CIII deficiency in the liver.

In this project, we showed that a single intraperitoneal injection of AAT-*Bcs1l* restores CIII activity and liver ATP levels, fully preventing hepatopathy and doubling median survival by averting metabolic crises (e.g., hypoglycemia). It normalized growth regulators (*Igf-1*, *Ghr*) and improved systemic thermoregulation by stabilizing hepatic respiration and energy metabolism. AAT-*Bcs1l* corrected liver-driven metabolic shifts, restoring skeletal muscle metabolism and highlighting the liver's role in systemic energy homeostasis and basal heat production. Thermoregulation in mitochondrial disease models remains largely unexplored, except few studies on adipose tissue-specific KOs (Vernochet et al. 2014; Pereira et al. 2021).

Cold-induced thermogenesis is an energy-intensive process that helps endotherms maintain their body temperature when the ambient temperature drops. Metabolic networks evolved to handle starvation and cold exposure, two energy-demanding processes that require dynamic communication between tissues to maintain survival (Hue and Taegtmeyer 2009). During starvation, WAT mobilizes lipid storages as free fatty acids (FFAs), which are then used by the liver and muscles to preserve glucose for the brain. In cold exposure, the triglyceride is broken down in WAT, which activates thermogenesis in brown adipocytes (Lafontan and Berlan 1993). Despite early suggestive evidence of a significant role of tissue

communication in energy metabolism, the specific role and physiological effects of the liver in adapting to cold conditions have been overlooked. Consequently, recent literature on this topic remains scarce (Abumrad 2017). Since the 1970s, the focus has largely shifted to BAT thermogenesis, especially in relation to obesity (Trayhurn 2017). As the body's primary metabolic center, the liver generates heat through two main sources: basal mitochondrial heat, resulting from proton leak, and metabolic heat, primarily from ATP hydrolysis, where 60% of the energy is released as heat. Recently, one study has proposed that FFAs activate hepatic fatty acid oxidation, resulting in the release of acylcarnitines in the circulation, which works as a fuel for BAT thermogenesis (Simcox et al. 2017). Furthermore, the liver plays a key role in the metabolism of thyroid hormones (Zavacki et al., 2005). These hormones are essential regulators of body temperature, energy expenditure, and overall metabolism. They impact several processes, including glucose absorption, fatty acid oxidation and synthesis, and the transformation of white fat into brown or beige fat (Zekri, Flamant, and Gauthier 2021). Our findings underscore the liver's largely forgotten critical role in systemic thermogenesis and the potential of hepatocyte-targeted gene therapy to address the complex metabolic and systemic manifestations of mitochondrial diseases.

In summary, this thesis significantly expands the characterization of the disease mechanisms in the *Bcs1l*$^{p.S78G}$ mouse model of CIII deficiency and evaluates the potential of rAAV-based gene therapy in treating the disease. With the help of modern prenatal genetic technology, detecting rare genetic disorders is possible by the 12th week of gestation. In cases of inherited genetic diseases, in utero gene therapy (IUGT) holds the promise of preventing early, irreversible, and fatal pathological changes (Rashnonejad et al. 2019). Fetal therapeutic interventions may be particularly beneficial for early-onset mitochondrial disorders, such as GRACILE syndrome, where mitochondrial dysfunction starts before birth (Fellman et al. 1998).

ACKNOWLEDGEMENTS

The doctoral journey has been a transformative experience marked by growth, challenges, and invaluable learning. As this journey reaches its final stage, I reflect on how transformative it has been, both academically and personally. Throughout this journey, I have been fortunate to receive unwavering support and guidance from mentors, colleagues, collaborators, funding agencies, administrative staff, friends, and family. Without their contributions, this work would not have been possible.

I am deeply grateful to my supervisor, Dr. Jukka Kallijärvi, for introducing me to the field of mitochondrial disorders and providing steadfast support throughout my research. His expertise and guidance have been indispensable in shaping this work and my intellectual development. His encouragement, patience, and confidence in my abilities have been crucial for my growth. I am truly fortunate to have had him as my mentor. I would also like to express my heartfelt appreciation to my co-supervisor, Prof. Vineta Fellman, whose feedback and encouragement were key in developing and refining my research. Her extensive expertise, enthusiasm for our work, and dedication to securing funding for the GRACILE research have been invaluable. I could not have asked for a more supportive and enriching laboratory environment to face inspiring challenges, experiment, and develop essential skills.

This work was conducted at the Folkhälsan Research Center in Biomedicum Helsinki, and I would like to acknowledge the center and Samfundet Folkhälsan for providing outstanding facilities, infrastructure, and administrative support throughout my research. My gratitude also extends to the Finnish Doctoral Programme in Oral Sciences (FINDOS) for offering the salaried position that enabled me to pursue this work. I am especially thankful for the financial support from the Jane and Aatos Erkko Foundation, Samfundet Folkhälsan, the Sigrid Juselius Foundation, Finska Läkaresällskapet, the Finnish Foundation for Pediatric Research, Medicinska Understödsföreningen Liv och Hälsa rf, the Magnus Ehrnrooth Foundation, and the Orion Research Foundation. Additionally, I would like to acknowledge the Medicine Fund, Jubilee Fund, Samfundet Folkhälsan, ASGCT, and ESGCT for their travel grants, which facilitated my active participation in national and international conferences. The graphics in this thesis were created with BioRender, and ChatGPT is acknowledged for language editing assistance and the thesis cover image. I am grateful to

the Stem Cells and Metabolism (STEMM) Research Program for fostering a collaborative environment.

I express my sincere gratitude to Prof. Luis Carlos Lopez Garcia for serving as my opponent during the public defense and providing valuable insights, as well as to Prof. Anna-Elina Lehesjoki for taking on the role of the custos. My appreciation also extends to my thesis pre-examiners, Asst. Prof. Erika Fernandez-Vizarra and Docent Riikka Martikainen for their thorough evaluation and constructive feedback. I am deeply thankful to my thesis committee members, Prof. Henna Tyynismaa and Dr. Christopher Carroll, for their continuous support throughout my doctoral research, particularly during our annual meetings. I would also like to acknowledge all my co-authors and collaborators for their dedication and hard work in advancing our projects.

I extend my heartfelt gratitude to all current and former members of my research group for creating a collaborative and intellectually stimulating environment. I would like to express my special thanks to Janne Purhonen for generously sharing his extensive practical experience, offering valuable insights during experimental troubleshooting, and being a great conference companion. I am deeply appreciative of Christa Kietz for her insightful discussions and collaborative spirit, as her constructive feedback greatly refined my research. My sincere appreciation also goes to our technical assistant, Vilma Wanne, for her outstanding support in laboratory work, ensuring smooth genotyping and various routine tasks. Her dedication to managing numerous qPCRs and Western blots was invaluable. I would like to thank my fellow PhD student, Divya Upadhyay, for being both a dedicated colleague and a close friend. Her collaborative spirit, thoughtful insights, and the many coffee break conversations we shared helped foster a supportive and enjoyable work environment, making this journey all the more meaningful. I extend my deepest gratitude to Nasrin for being a good friend for many years and for assisting with the Bengali translation of the thesis abstract. I also want to thank Oliver Ros for bringing energy and fresh perspectives to the team and Elisa Alppila for her contributions during the early stages of my research. I would like to acknowledge all the technical and administrative staff, particularly Ann-Liz, for their invaluable support in lab management and all the behind-the-scenes work, which has been essential to the success of this research. My appreciation extends to the organizing team for coordinating my seminar session at the Folkhälsan Research Center and STEMM Research Program. Lastly, I appreciate the management at

Folkhälsan and the University of Helsinki for handling all documentation related to my work contracts and financial arrangements.

I would not have made it through this journey without the incredible friends who stood by me. A thirteen-year-long friendship with Toiba has transformed our relationship into one akin to family, providing unwavering support and encouragement, even from a distance. The Blah gang! Abhishek, Sachin, Suravi, Maxime, Aastha, and Susheel have been my pillars of support, with special thanks to Abhishek and Sachin for the unforgettable trips we shared. I would like to extend my sincere gratitude to Adrita for our enriching coffee shop discussions, to Madhuparna for being an exceptional dance partner, and to Kinchit for her unwavering warmth and kindness. Additionally, I wish to acknowledge Vaishali, Sharath, Aishwarya, and others, whose friendship and support have played a crucial role in my personal and professional development. I am also deeply thankful to my friends in the Bengali and Indian communities in Finland for their care and encouragement throughout this journey. To my extended network of colleagues, mentors, and friends from school, college, and beyond, I truly appreciate each individual's contribution, whether through words of encouragement, sharing in both joyful and challenging moments, or simply offering their presence, their support has made this experience more meaningful.

A special thanks to Animesh, whose understanding, encouragement, and patience have been instrumental throughout this process. His steadfast support has not only helped me through the challenges of this thesis but has also given me a new family to lean on. I am deeply grateful to my family, whose unwavering support has been the foundation of this journey, and to my extended family, including my uncles, aunts, cousins, nieces, nephews, and late grandparents, for their invaluable support and blessings.

Finally, I dedicate this thesis to my pillars of strength, my loving parents, whose values, prayers, and encouragement have shaped who I am and continue to guide me. My Maa has been a guiding force in my life, from being my first tutor to always encouraging me to pursue my dreams. Her unwavering support has been invaluable in shaping my vision and giving me the wings to achieve my goals. Similarly, my Baba has been a steady and silent cheerleader, offering his unwavering support and wisdom from behind the scenes. His thoughtful guidance and constant belief in me have been a source of strength throughout this journey. I am here today because of the foundation they both have laid for me, and I carry their blessings and lessons with me every day.

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ORIGINAL PUBLICATIONS

Mitochondrial Disease and the Importance of the Liver

Mitochondrial disorders are rare inherited diseases that affect the body's ability to produce energy, often leading to complex symptoms across multiple organs. One such disease, GRACILE syndrome, is caused by mutations in the BCS1L gene, which disrupts mitochondrial complex III function. In a mouse model carrying the Finnish GRACILE mutation, researchers discovered features resembling premature aging—such as growth delay, bone loss, and skin thinning—linked to DNA damage and impaired cell division. Surprisingly, targeting the liver with gene therapy significantly improved overall health, doubled survival, and even restored normal body temperature despite ongoing defects in other tissues. These findings not only highlight the liver's central role in maintaining whole-body metabolism but also suggest new ways to treat mitochondrial diseases using organ-specific approaches.

Rishi Banerjee began his academic training in biotechnology in India before moving to Finland, where he completed his master's degree at the University of Helsinki. His doctoral research investigates complex III deficiency in rare mitochondrial disorders, linking it to premature aging and metabolic dysfunction through liver-targeted gene therapy and metabolic phenotyping in mouse models.

Dissertationes Universitatis Helsingiensis is a publication series for university dissertations founded and published by the University of Helsinki Doctoral School. The series was founded in 2023.

ISBN 978-952-84-0946-5 (Paperback)
ISBN 978-952-84-0945-8 (PDF)
ISSN 2954-2898 (Paperback)
ISSN 2954-2952 (PDF)
Unigrafia, Helsinki
Printed in PunaMusta, Joensuu 2025