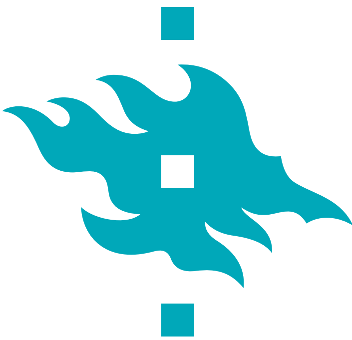

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM
INVESTIGANDAM UNIVERSITATIS HELSINKIENSIS

JUAN CRUZ LANDONI

MITOCHONDRIAL DNA REPLICATION DEFECTS

CONSEQUENCES FOR NEONATAL HEART DISEASE & PREMATURE AGEING

STEM CELLS AND METABOLISM RESEARCH PROGRAM
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

Stem Cells and Metabolism Research Program, Faculty of Medicine &
Doctoral Programme in Integrative Life Sciences
University of Helsinki
Finland

MITOCHONDRIAL DNA REPLICATION DEFECTS

CONSEQUENCES FOR NEONATAL HEART DISEASE & PREMATURE AGEING

Juan Cruz Landoni Martín

ACADEMIC DISSERTATION

To be presented for public examination, with permission from the Faculty of Medicine of the University of Helsinki, in Biomedicum Helsinki Lecture Hall 1, on the 20th of May 2022 at eleven o'clock.

Helsinki | Finland | 2022

SUPERVISOR
Academy Professor **Anu Suomalainen Wartiovaara**, MD, PhD
Stem Cells and Metabolism Research Program, Faculty of Medicine, Biomedicum Helsinki; Neuroscience Centre, Helsinki Institute of Life Science & HUSlab, Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland.

OPPONENT
Associate Professor **Nika Danial**, PhD
Department of Cancer Biology, Dana–Farber Cancer Institute & Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, United States of America.

EXTERNAL REVIEWERS
Adjunct Professor **Jaakko Pohjoismäki**, PhD
Department of Environmental and Biological Sciences, University of Eastern Finland, Joensuu, Finland.

Professor **Carlos Moraes**, MD, PhD
Department of Neurology, University of Miami Miller School of Medicine, Miami, Florida, United States of America.

THESIS COMMITTEE
Associate Professor **Pekka Katajisto**, PhD
Institute of Biotechnology, Helsinki Institute of Life Science & Molecular and Integrative Bioscience Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland; Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden.

Docent **Cory Dunn**, PhD
Institute of Biotechnology, Helsinki Institute of Life Science, University of Helsinki, Helsinki, Finland

FACULTY REPRESENTATIVE
Professor **Eero Mervaala**, MD, PhD
Department of Pharmacology, Faculty of Medicine, University of Helsinki, Helsinki, Finland

COVER GRAPHICS
Front: Artificial intelligence -powered paintings generated using the *WOMBO Dream* algorithm, prompted by keywords of the thesis: Mitochondrial DNA, mouse, ageing, heart.

Back: Immunofluorescent image of the mitochondrial network of a human cell in culture, labelling the transporter of the outer membrane protein TOM20 in black.

The Faculty of Medicine uses the Ouriginal system (plagiarism recognition) to examine all doctoral dissertations.

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

Print: ISBN 978-951-51-8096-4 ISSN 2342-3161

Online: ISBN 978-951-51-8097-1 ISSN 2342-317X

Painosalama Oy, Turku 2022

"Go! Confront the problem! Fight! Win!"
— Edna Mode.

"Life is too important to be taken seriously"
— Oscar Wilde

"N'oublie jamais, celui qui croit savoir n'apprend plus."
"Never forget, those who believe they know, learn no more"
— Pierre Bottero

"Juguemos a que el mundo no es como nos dicen; que el sol se mueve, que los colores primarios no existen, y que los gases son líquidos"
"*Let's play that the world is not how we've been told; that the sun moves, that primary colours don't exist, and that gases are liquids"*
— Fenomenoide, Expedición Ciencia

ABSTRACT

Mitochondria are organelles crucially involved in energy metabolism (hence the "powerhouses of the cell") and most other cellular processes. They accommodate the second essential genome of the human cell: mitochondrial DNA (mtDNA). The integrity and quantity of mitochondrial genomes, as well as the systems in charge of its maintenance, have been associated with a wide variety of rare and common human diseases, as well as ageing. The causes behind such striking diversity in presentation remain largely unknown and an outstanding question for many fields of biology and medicine.

One of the most prolific models for mitochondrial dysfunction is the prematurely ageing mtDNA Mutator mouse, carrying a defective mtDNA replicase that causes the accumulation of point mutations in mtDNA. Prompted by the observation that the Mutator mouse phenotype closely mimics that of other premature ageing models (typically related to nuclear DNA damage), we investigated the cell cycle and nuclear integrity in a stem cell model and its relationship with mtDNA replication. We discovered that, in addition to mtDNA mutations, Mutator mice also present accelerated mtDNA replication and rewiring of nucleotide metabolism. We developed the state-of-the-art methodology for deoxynucleotide quantification to confirm this observation and found that deoxynucleotides are being prioritised to mitochondria in stem cells, depriving nuclear replication of building blocks and causing DNA damage in the nucleus. The data reframe the mtDNA Mutator model as a secondary nuclear DNA instability model, unifying mouse premature ageing models under the same proposed mechanism.

To further explore the aforementioned hypothesis and test recent reports on the beneficial effects of boosting mtDNA, we crossed the mtDNA Mutator mice with mice overexpressing the mtDNA helicase Twinkle, which further increased mtDNA replication and amount. This resulted in an unexpected and fatal neonatal heart failure phenotype. Characterising the mechanisms behind this pathology revealed intriguing effects in mtDNA integrity and maintenance, as well as large-scale metabolic stress responses which appear to disrupt normal heart development and maturation.

Altogether, the thesis provides new paradigms on the roles of mtDNA replication in physiology. On one hand, its ability to influence cellular metabolism and threaten nuclear DNA stability in progenitor/stem cells, causing premature ageing. On the other, its role in early life maturation, opening the door to novel discoveries on its relationship to heart function, adaptation to oxygen, cell death signalling, and related human diseases.

TABLE OF CONTENTS

ABSTRACT 4
TABLE OF CONTENTS 5
LIST OF ORIGINAL PUBLICATIONS 7
ABBREVIATIONS 8
1 INTRODUCTION 9
2 REVIEW OF THE LITERATURE 10
2.1 Mitochondria: Nature & Rise to Power 10
Invading Asgard *10*
Threads and granules *11*
Mitochondrial networking *11*
2.2 The powerhouse and beyond 13
The Metabolic Hub *13*
The Respiratory Chain *14*
From invader to overlord *15*
The Iron Maiden *15*
2.3 Sneaky Little Genomes 16
Mitochondrial DNA *16*
MtDNA replication and maintenance *17*
DNA polymerase gamma *18*
Twinkle *18*
TFAM *18*
mtDNA replication models *19*
Mitochondrial Gene expression *19*
2.4 Mitochondrial dysfunction 20
Mitochondrial diseases *20*
MtDNA maintenance disorders *20*
2.5 Building blocks and Currency 21
Nucleotide Metabolism *21*
Nucleotides in mitochondrial disease *22*
Measuring dNTPs *22*
2.6 Ageing & progeroid syndromes 23
The Mitochondrial Theory of Ageing *24*
Questions and concerns *24*
2.7 Treatments for mitochondrial dysfunction 25
Genetic interventions & transplants *25*

Control of oxidative damage *26*
Enhancement of mitochondrial biogenesis *26*
Improvement of mitochondrial DNA homeostasis *27*
Modulation of mtDNA copy number *27*
Challenges and outlooks *28*
3 AIMS OF THE STUDY **29**
4 MATERIALS & METHODS **29**
4.1 List of Methodologies 29
4.2 Ethical statements and licenses 30
4.3 Mouse models 30
MtDNA Mutator mice *30*
Twinkle overexpressor mice *30*
Mutator Twinkle-overexpressor mice *30*
4.4 Quantitation of dNTP pools 30
4.5 Statistical analyses 31
5 RESULTS **32**
5.1 Disrupted cell cycle and replication stalling in Mutator mouse stem cells 32
5.2 Nuclear DNA damage in Mutator stem cells *in vitro* and *in vivo* 33
5.3 mtDNA over-replication and nucleotide metabolic shift 33
5.4 TFAM overexpression can rescue the Mutator stem cell DNA damage 35
5.5 A unifying mechanism for mouse premature ageing 35
5.6 Overexpressing Twinkle helicase increases mtDNA copy number by replication 36
5.7 Enhanced replication in the mutator background causes fatal neonatal heart failure. 37
5.8 The cardiac failure is dose-responsive to $Polg^{D257A}$ alleles and affects other organs ... 38
5.9 Increasing mtDNA replication intensifies the likelihood of mtDNA damaging events 39
5.10 Multi-omic analysis reveals perinatal signalling and metabolic remodelling 40
5.11 Ferroptotic cell death induced by mitochondrial dysfunction in the heart 43
5.12 Development of a new dNTP quantitation method 45
6 DISCUSSION **47**
7 CONCLUSIONS **51**
8 ACKNOWLEDGEMENTS **53**
9 REFERENCES **56**
10 ORIGINAL PUBLICATIONS **72**

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which can be found appended. In the text, the original publications are referred to by their Roman numerals.

I. Riikka H. Hämäläinen, **Juan C. Landoni**, Kati J. Ahlqvist, Steffi Goffart, Sanna Ryytty, M. Obaidur Rahman, Virginia Brilhante, Katherine Icay, Sampsa Hautaniemi, Liya Wang, Marikki Laiho & Anu Suomalainen (2019). "Defects in mtDNA replication challenge nuclear genome stability through nucleotide depletion and provide a unifying mechanism for mouse progerias". *Nature Metabolism* 1 (10), 958-965. (Hämäläinen *et al.*, 2019).

II. **Juan C. Landoni**, Tuomas Laalo, Steffi Goffart, Riikka Kivelä, Karlo Skube, Eija Jokitalo, Anni Nieminen, Sara Wickström, James Stewart, Anu Suomalainen. (2022). "Overactive mitochondrial DNA replisome causes neonatal heart failure via ferroptosis". *Manuscript | BioRxiv Preprint.* (Landoni *et al.*, 2022).

III. **Juan C. Landoni**, Liya Wang, & Anu Suomalainen (2018) "Quantitative solid-phase assay to measure deoxynucleoside triphosphate pools". *Biology Methods and Protocols* 3 (1), bpy011. (Landoni *et al.*, 2018).

ABBREVIATIONS

DNA	Deoxyribonucleic acid
mtDNA	Mitochondrial DNA
RNA	Ribonucleic acid
POLG	Polymerase gamma (holocomplex)
TWNK	Twinkle helicase
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
dNTP	deoxynucleoside triphosphate
d[ ]TP	deoxy[Adenosine/Thymidine/Cytidine/Guanosine] triphosphate
ROS	Reactive oxygen species
CoA	Coenzyme A
TCA	Tricarboxylic acid cycle
NADH	Reduced nicotinamide adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
OXPHOS	Oxidative phosphorylation system
Fe-S	Iron-sulphur (clusters)
SSBP1	Mitochondrial single-stranded DNA binding protein
TFAM	Transcription factor A mitochondrial
ER	Endoplasmic reticulum
m/t/rRNA	Messenger/transfer/ribosomal RNA
mtRNA	Mitochondrial RNA
MRG	Mitochondrial RNA granule
ISR^{mt}	Mitochondrial integrated stress response
PEO	Progressive external ophthalmoplegia
dNTP	Deoxynucleoside 5´ triphosphate
MIRAS	Mitochondrial recessive ataxia syndrome
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
TK2	Thymidine kinase 2
DGUOK	Deoxyguanosine kinase
HPLC	High Performance Liquid Chromatography
NAC	N-acetyl cysteine
WT	Wildtype
TwOE	Twinkle-overexpressor
$Polg^{Het}$/Het	$Polg^{D257A}$ heterozygote
$Polg^{Mut}$/Mut	$Polg^{D257A}$ homozygote; Mutator

1 INTRODUCTION

Due to their origins as free-living bacteria, mitochondria are the only cytosolic organelles in the animal cell to contain DNA, in the form of small multi-copy circular genomes. The recent decades have seen a rapid accumulation of knowledge concerning mitochondria, mitochondrial DNA (mtDNA), and the effects of their malfunction, originating from fundamental research on patients and laboratory models of mitochondrial dysfunction. Nonetheless, a myriad of critical questions remains unresolved, particularly regarding the remarkable diversity of consequences of mitochondrial malfunction, and the exact mechanisms that cause it. Answering these questions is crucial for our understanding of physiology, and for the development of therapies to treat the constantly expanding collection of mitochondria-related human diseases.

In this thesis, I discuss the current knowledge of mitochondria and mtDNA: their origins, characteristics, replication and expression systems, and the consequences of their dysfunction. Furthermore, I review some of the exciting novel therapeutic approaches for mitochondrial dysfunction, their suitability, and their limitations. I will focus on aspects of particular relevance for the present work but will refer to fantastic work and reviews on each topic if you wish to read further.

Further on, I describe the projects and results obtained during my doctoral work and published in peer-reviewed journals, as well as some unpublished data. While most of the presented results are my personal contribution, science is a collaborative endeavour, and as such the projects also include the critical work and expertise of many talented colleagues, collaborators, and mentors. To recognise and acknowledge this, I will use the personal pronoun "we" throughout the thesis.

We have explored the roles that mtDNA replication can play in the organism by manipulating its homeostasis, beginning with the rewiring of metabolism leading to premature ageing, and ending with the devastating consequences for neonatal hearts. The discoveries provide novel insight and challenge some established paradigms in several fields, with relevance that goes beyond mitochondrial research and into fundamental biology and clinical medicine.

I hope that my writing can do justice to the fascinating and constantly surprising world that is mitochondrial biology, and that you enjoy this voyage as much as I enjoyed charting it.

2 REVIEW OF THE LITERATURE

2.1 Mitochondria: Nature & Rise to Power

Invading Asgard

Just like any good story, the tale of mitochondria begins with a meal. In this case, a primitive cell engulfing a free-living bacterium, some two thousand million years ago. Against all odds, the larger cell did not digest the bacterium. Instead, the two organisms entered a symbiotic relationship – an arrangement that likely gave rise to most complex life as we know it. This widely accepted endosymbiotic theory (Sagan (neé Margulis), 1967; Margulis, 1970) was inspired by the unexpected discovery that mitochondria, just like bacteria, contain their own circular genome named mitochondrial DNA (mtDNA) (Nass *et al.*, 1965), alongside many other bacteria-like qualities such as their expression systems and behaviour (Gray *et al.*, 1999).

Fifty years after this hypothesis, numerous aspects of this event have been uncovered at the ends of the earth. The endosymbiont that gave rise to mitochondria has been meticulously described to be an ancient α-proteobacterium, although its exact phylogenetic positioning is still under debate (Gray *et al.*, 1999; Martijn *et al.*, 2018). In contrast, the identity of the host cell remained enigmatic until recently, when new species of archaea were discovered near the hydrothermal vent named Loki's Castle, deep in the Atlantic Ocean (Spang *et al.*, 2015). Lokiarchaeota and other Norse-god-named relatives compose the proposed Asgard superphylum, (Zaremba-Niedzwiedzka *et al.*, 2017), and present a striking genomic, structural, and molecular similarity to eukaryotes. This makes Asgardarcheota our closest known prokaryotic relatives.

Following the endosymbiotic event, both the host cell and mitochondria co-evolved greatly into tightly regulated and efficient systems across all lineages of the Eukarya domain. Most of the mitochondrial genetic material was transferred to the nucleus, keeping only a handful of genes in mtDNA, and delegating the transcription and translation responsibilities to the host cell. Nonetheless, mtDNA and its gene expression remain essential for most cells, and mitochondria cannot be made *de novo*: every mitochondrion originates from a previous mitochondrion (Lane & Martin, 2010).

When observing the genetics of mitochondrial proteins, yet another intruder in this story was identified: viruses left their genomic mark too. Many of the key proteins involved in mtDNA replication and maintenance, such as the mitochondrial DNA polymerase gamma, the replicative helicase of mtDNA Twinkle, and the mitochondrial RNA polymerase appear to share ancestry with T-odd phage proteins (Shutt & Gray, 2006). This relatedness shaped their function and can also make them sensitive to anti-viral drugs, as e.g. DNA polymerase gamma is known to be inhibited by antiretroviral drugs targeting HIV (Arnaudo *et al.*, 1991; Lim & Copeland, 2001). The infection providing these genes are presumed to have occurred around the time of the endosymbiotic event, early in eukaryotic evolution (Shutt & Gray, 2006).

Supported by phylogenetic observations and bioenergetic calculations, it is likely that the mitochondrial endosymbiotic event was the sole catalyst for the massive expansion in structural and genomic complexity of eukaryotes. It allowed for the association between two

genomes with independent evolutionary history and the development of bioenergetically efficient membranes. Consequently, eukaryotes can now energetically afford the 200 000-fold expansion of the expressed genome and develop the complex multicellular processes that characterise macroscopic life as we know it (Lane & Martin, 2010). In addition, the bacterial and viral nature of mitochondrial proteins has been critical to their entire evolution, interaction with the host cell, and relevant to the fight against human disease.

Threads and granules

Mitochondrion (plural *mitochondria*) is a compound word originating from the Greek words μίτος (*mitos*), meaning "thread"; and χονδρίον (*khondrion)*, meaning "small granule". The organelle was named at the end of the 19th century (Benda, 1898) with impressive accuracy, as accumulating modern data display the highly dynamic shaping of these organelles in response to cellular signalling and energetic demands (Collins *et al.*, 2002; Wai & Langer, 2016). Mitochondria can be found as the independent bean-shaped granules we know from textbooks, as a large network of interconnected threads, or anything in between (Figure 1B) (Lewis & Lewis, 1915).

Heritage of their endocytotic origin, mitochondria are composed of a double membrane, each with distinct structures and functions. The outer mitochondrial membrane was originally the barrier between the host cell and the endosymbiont. It functions as a semi-permeable envelope for the movement of molecules and proteins and has evolved into a communication platform for cell signalling (Chandel, 2015). The inner mitochondrial membrane has undergone colossal changes since its time as a bacterial membrane, developing the structurally intricate and dynamic system of invaginations known as cristae (Figure 1A). The cristae host the machinery for mitochondrial respiration and ATP synthesis and can modulate their density and surface according to cellular energetic demands (Hackenbrock, 1966).

The two membranes also create two additional compartments isolated from the cell's cytosol. The mitochondrial matrix, enclosed by the inner membrane, is the compartment where most metabolic reactions within mitochondria happen, as well as where the mitochondrial genome is hosted. Between the two membranes is the inter-membrane space, critical for the electrochemical and bioenergetic proprieties of mitochondria, as described later.

Mitochondrial networking

The mitochondrial network undergoes active and dynamic reshaping, and the multiple functions of its fission and fusion events have been the spotlight of many recent discoveries (Figure 1C). Like many findings in molecular biology, the machinery behind mitochondrial dynamics was unveiled by fundamental research in yeast and human diseases where those processes were affected. The proteins involved in mitochondrial fission and fusion are often also involved in metabolic adaptations, response to stress, and regulation of mitochondrial proliferation, distribution, and recycling, further emphasising the importance of mitochondrial behaviour in cellular signalling (Labbé *et al.*, 2014; Pernas & Scorrano, 2016; Wai & Langer, 2016; Kraus *et al.*, 2021).

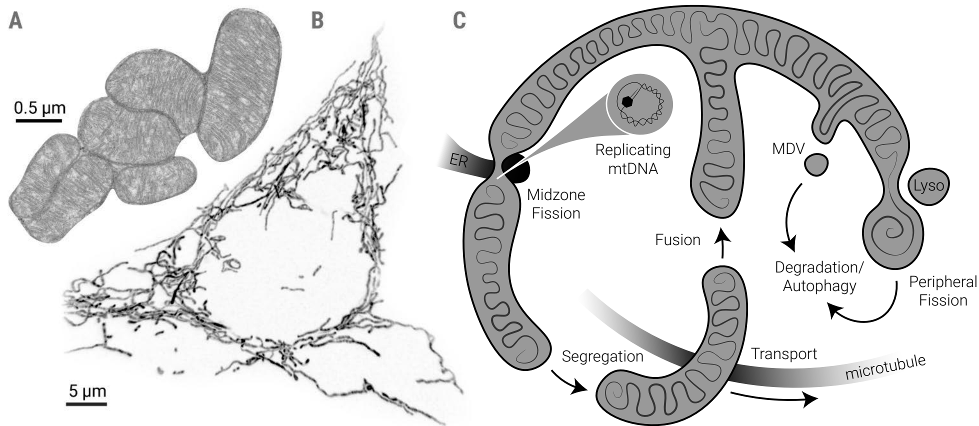

Figure 1: Dynamic threads and granules. **(A)** Electron-micrograph of mouse cardiac mitochondria, showing the invaginations of the inner membrane. **(B)** Immunofluorescent image of the mitochondrial network of an osteosarcoma cell in culture, labelling the transporter of the outer membrane protein TOM20. **(C)** Graphical summary of the key processes involved in mitochondrial network dynamics. Abbreviations: ER, endoplasmic reticulum; MDV, mitochondria-derived vesicle; Lyso, lysosome.

Like most cellular structures, mitochondria are transported and positioned by the cytoskeleton (De Vos *et al.*, 2005; Hollenbeck & Saxton, 2005), and when mitochondria approximate, they can fuse into a single entity (Meeusen *et al.*, 2004). The outer mitochondrial membrane contains dynamin-related proteins mitofusin 1 and 2, which can complex with the opposite mitochondrion's mitofusins and mediate the fusion of the membranes (Santel & Fuller, 2001; Ishihara *et al.*, 2004; Koshiba *et al.*, 2004; Song *et al.*, 2009). The inner mitochondrial membrane is fused by the action of another dynamin-related protein named OPA1 (Shepard & Yaffe, 1999; Wong *et al.*, 2000). OPA1 and its proteolytic interactors form a complex regulatory system for inner membrane and cristae dynamics, able to respond to many cellular and metabolic cues (Frezza *et al.*, 2006; Ehses *et al.*, 2009; Head *et al.*, 2009; Wai & Langer, 2016).

The process opposing fusion is mitochondrial fission. The main executioner of fission is dynamin-related protein 1 (DRP1), a GTPase that oligomerizes around the outer membrane and induces constriction and severing (Labrousse *et al.*, 1999; Pitts *et al.*, 1999; Smirnova *et al.*, 2001; Kamerkar *et al.*, 2018). DRP1 requires membrane-anchored adaptors, including MFF (Gandre-Babbe & Van Der Bliek, 2008), FIS1 (James *et al.*, 2003), MiD49 and MiD51 (Palmer *et al.*, 2011). The adaptors can independently and cooperatively recruit DRP1 to the mitochondrial membrane and different adaptors seem to be implicated in different types of fission: e.g. FIS1 is involved in degradation-related fission, while MFF associates with proliferative fission events (Shen *et al.*, 2014; Helle *et al.*, 2017; Kleele *et al.*, 2021; Kraus *et al.*, 2021).

Other organelles have been observed to participate in mitochondrial fission. Notably, the endoplasmic reticulum wraps around the mitochondrion and constricts it, marking the sites of fission (Friedman *et al.*, 2011). This event is also spatially coordinated with mtDNA replication (Murley *et al.*, 2013; Lewis *et al.*, 2016), a feature conserved from yeast to human which mimics the binary fission of bacteria. This mechanism would allow for the proper segregation of mtDNA copies between mitochondria and highlights the critical role of

mitochondrial dynamics in the segregation and inheritance of mtDNA (Nunnari *et al.*, 1997; Labbé *et al.*, 2014; Jokinen *et al.*, 2016).

Other systems implicated in mitochondrial division include the cytoskeleton (Li *et al.*, 2015), Golgi-derived vesicles (Nagashima *et al.*, 2020) and the lysosome (Wong *et al.*, 2018). Similarly to the DRP1 adaptors, the involvement of other organelles has been observed in a context-specific manner: ER contacts are associated with mtDNA replication and MFF-mediated proliferative division, while lysosomes with FIS1-mediated peripheral divisions likely targeted to degradation (Shen *et al.*, 2014; Lewis *et al.*, 2016; Kleele *et al.*, 2021).

The regulated degradation of mitochondria is closely linked to their dynamics. In addition to local quality-control performed by mitochondrial proteases, some of which are also involved in membrane dynamics (Deshwal *et al.*, 2020), large-scale mitochondrial quality control is thought to be performed by DRP1-mediated processes. On one hand, fragmented damaged mitochondria can be wholly engulfed by autophagosomes, in a process known as mitophagy (Youle & Narendra, 2011; Pickles *et al.*, 2018). On the other hand, minor damage can be sequestered and exported in mitochondria-derived vesicles directly to the lysosome (Neuspiel *et al.*, 2008; Soubannier *et al.*, 2012; McLelland *et al.*, 2014; Sugiura *et al.*, 2014).

The interplay between mtDNA segregation, mitochondrial dynamics and organellar contacts is a highly active field of research. The quick rise of novel technologies in genomics and imaging enables the analysis and exploration of these mechanisms in real-time, with new observations highlighting their complexity and generating further exciting questions and unknowns.

2.2 The powerhouse and beyond

Famously known as the "powerhouse of the cell", mitochondria are the major producers of the biologically available cellular energy source, adenosine triphosphate (ATP), from the oxidative degradation of nutrients (Mitchell, 1961). But considering them as simply a powerhouse would unfairly disregard their key functions as metabolic and signalling hubs in the cell. Mitochondria host many of the pathways required for building the cell, as well as for intra- and intercellular communication and regulation.

The Metabolic Hub

One of the defining features of life is the ability to transform matter and energy from the environment. A living cell is simply a highly intricate system of chemical reactions, orchestrated to fulfil certain functions. This symphony of reactions is called metabolism and mitochondria are at its core, both as the conductor and as a big share of the orchestra (Cable *et al.*, 2021).

The cell uptakes many different molecules from the environment, such as carbohydrates, amino acids, fatty acids and ketone bodies. To enter the series of oxidative reactions that will fuel ATP synthesis in mitochondria, carbohydrates and amino acids are partially oxidised in the cytoplasm, amino acids are also deaminated in mitochondria, and fatty acids are transported through the carnitine shuttle system to enter mitochondrial β-oxidation, all eventually resulting in acetyl-coenzyme A (acetyl-CoA) or other useful intermediates (Nelson & Cox, 2017).

Acetyl-CoA is the canonical fuel for the tricarboxylic acid cycle (TCA) in mitochondria (Szent-Györgyi, 1928; Krebs & Johnson, 1937), a series of reactions capable of efficiently

reducing the co-factors NAD^+ and FAD^+ into NADH and $FADH_2$ respectively, and re-generating the original molecule (oxaloacetate) for condensation with a new acetyl-CoA. The TCA cycle is a central pathway in the cell, not only for catabolism but also as the source of carbon backbones for the biosynthesis of amino acids, nucleotides and other structural and signalling molecules. This also means that acetyl-CoA is not the sole "entry" to the cycle, but rather there is a constant dynamic exchange of metabolites. The TCA cycle is considered one of the most fundamental components of life, and evidence even places it at the origin of life itself. Several TCA cycle intermediates have been detected in meteorites completely devoid of life (Cooper *et al.*, 2011), proposing the fascinating possibility that some of these reactions occur inorganically and life simply optimised or was wholly built around them.

The Respiratory Chain

The reduced cofactors NADH and $FADH_2$ originate from the TCA cycle and other redox reactions (such as β-oxidation) and carry electrons to feed one of the most sophisticated systems in the eukaryotic cell: the respiratory chain.

Hundreds of protein subunits, assembly factors and cofactors are required for the correct formation of the five canonical respiratory complexes embedded in the inner mitochondrial membrane, efficiently named complex I, II, III, IV and V (Hatefi *et al.*, 1962; Ziegler & Doeg, 1962; Orme Johnson *et al.*, 1974). The conformation of the complexes has additional roles: Complex V physically bends the membrane to form the cristae invaginations (Paumard, 2002; Blum *et al.*, 2019) and multiple complexes can assemble into different configurations of supercomplexes to regulate their efficiency and respond to stress (Schägger & Pfeiffer, 2000; Letts *et al.*, 2016).

In an oversimplification of the respiratory chain's function (or oxidative phosphorylation; OXPHOS), the oxidation of NADH and $FADH_2$ (by complex I and II respectively) results in the transfer of electrons to the ubiquinone pool and subsequently across complexes III and IV, the latter catalysing the reduction of an oxygen molecule into two molecules of water. These electron transfers provide the energy for complexes I, III and IV to pump protons from the mitochondrial matrix into the intermembrane space, generating a strong gradient or membrane potential. The final complex V, named ATP synthase, acts as an ion channel allowing those protons to flow back into the matrix, and exploits that flow in a process reminiscent of a watermill to produce phosphorylate adenosine diphosphate into ATP (Mitchell, 1961; Mitchell & Moyle, 1967). ATP production by oxidative phosphorylation is highly efficient, producing an average of 36 molecules of ATP per molecule of glucose (in contrast to anaerobic energy production only yielding 2 net ATP molecules). This efficiency is both causal and required by multicellular life, as a human consumes approximately their body weight in ATP daily (Nelson & Cox, 2017).

In addition to ATP generation, oxidative phosphorylation is connected to other cell processes. The proton gradient is harnessed by protein translocation across the membrane (Neupert & Herrmann, 2007) and the leakage of protons back into mitochondria (or uncoupling) drives thermogenesis (Chouchani *et al.*, 2019), with a recent report proposing the physiological temperature of mitochondria to be as high as 50°C (Chrétien *et al.*, 2018). Some metabolic reactions also interact and require respiratory chain function and the ubiquinone pool, such as the oxidations of pyrimidine precursor dihydroorotate, proline, glycerol-3-phosphate and fatty acids undergoing β-oxidation (Raimondi *et al.*, 2020).

From invader to overlord

Despite millions of years of co-evolution, mitochondria retained much of their non-eukaryotic nature, producing molecules and structures that the rest of the cell would recognise as foreign. This feature is at the centre of many signalling cascades and their relationship to the host cell (Chandel, 2015).

The best-known case occurs during intrinsic apoptosis: the permeabilization of the mitochondrial membrane and the release of cytochrome c (part of the respiratory chain) are the triggering factors for the pathway leading to the programmed death of the cell (Li *et al.*, 1997). Other regulated cell death processes have also been linked to mitochondria-derived effectors, such as necroptosis, pyroptosis and most notably ferroptosis, described in a later section. (Bock & Tait, 2020).

Mitochondria also store and release signalling molecules such as calcium (Duchen, 2000), iron (Rouault & Tong, 2005), reactive oxygen species (ROS) (Chandel *et al.*, 1998; Hamanaka & Chandel, 2010; Hämäläinen *et al.*, 2015), short peptides (Hashimoto *et al.*, 2001; Lee *et al.*, 2015), and the metabolites required for protein post-translational modifications and the epigenetic regulation of the nuclear genome (Anderson & Hirschey, 2012; Matilainen *et al.*, 2017; Gut *et al.*, 2020). Furthermore, mitochondria can physically and metabolically compete with other intracellular parasites (e.g. toxoplasma), hampering their growth and thus protecting cellular homeostasis (Pernas *et al.*, 2018).

Nucleic acids are also released from mitochondria in situations of stress. Mitochondria-derived DNA and RNA are recognised as foreign and can elicit a wide variety of immune reactions from the cell and organism involving interferon responses and innate immunity (Collins *et al.*, 2004; West *et al.*, 2015; Riley & Tait, 2020).

The Iron Maiden

Iron is the most common element on earth by mass and an essential metal for most known living organisms. Its biological usefulness lies in its ability to shift between two thermodynamically stable states in physiological conditions, namely the ferrous ion (Fe^{2+}, electron donor) and ferric ion (Fe^{3+}, electron acceptor). This is exploited by many enzymes to catalyse electron transfer (redox) reactions, using iron in the form of cofactors such as iron-sulphur clusters (Fe-S) and heme groups (Anderson & Vulpe, 2009). Most of the iron in the human body is utilised for oxygen transport by heme-containing proteins (i.e., haemoglobin and myoglobin). Iron is also a critical component of the respiratory chain complexes, which contain many Fe-S clusters and heme groups to accomplish their electron transport function.

Iron enters the cell bound to diferric transferrin and is endocytosed via the transferrin receptors or GAPDH (Harding *et al.*, 1983; Iacopetta & Morgan, 1983; Kumar *et al.*, 2012). Iron is then reduced into soluble Fe^{2+} inside endosomes, transported into the cytoplasm (free Fe^{2+} can also cross the plasma membrane through similar transporters) and eventually stored by ferritins (Anderson & Vulpe, 2009). Cellular iron is almost exclusively found chaperoned or chelated, as free iron is highly reactive. Mitochondria are central players in iron metabolism, both as major users and sites of Fe-S cluster and heme group assembly. Iron enters the mitochondria through mitoferrins (Shaw *et al.*, 2006) and is stored by a local ferritin pool (Levi *et al.*, 2001).

Fe-S clusters are considered relics of ancient metabolism and remain the catalytic core of many enzymatic reactions. They are assembled inside mitochondria in eukaryotic cells, an apparently essential feature: even unicellular organisms that have lost mitochondria during evolution have kept "mitosomes" where Fe-S are built (Tovar *et al.*, 2003; Stehling & Lill, 2013). Furthermore, defects in frataxin, a protein involved in this pathway, can lead to a neurodegenerative mitochondrial disease known as Friedreich's ataxia (Campuzano *et al.*, 1996).

Heme biosynthesis on the other hand starts and ends in mitochondria: the first and rate-limiting step entails the condensation of succinyl-CoA and glycine by the aminolevulinic acid synthase (ALAS) into delta-aminolevulinic acid, which is then exported into the cytoplasm to join the multi-step pathway into coproporphyrinogen III. The product is then imported back to mitochondria to be oxidised and finally become heme by chelating Fe^{2+} via the mitochondrial ferrochelatase. The mechanism of heme export to the cytosol remains unknown (Ajioka *et al.*, 2006)

Iron handling is as useful as it is risky for the cell. Free Fe^{2+} is spontaneously oxidised into the insoluble and biologically inactive Fe^{3+} (Fenton, 1894). Alongside the loss of bioavailable iron, the aforementioned Fenton and Fenton-like reactions produce hydroxyl and hydroperoxyl radicals as products (Fenton, 1894; Haber & Weiss, 1932), which are reactive oxygen species greatly damaging for cellular homeostasis. For instance, ROS can trigger lipid radical chain reactions, auto-amplifying processes that unless regulated can cause complete membrane destruction (Reis & Spickett, 2012).

When the accumulation of lipid peroxides fatally exceeds the cellular antioxidant capacity, a form of iron-dependent regulated cell death known as ferroptosis occurs (Dixon *et al.*, 2012). Ferroptosis is different in mechanism from other forms of programmed cell death and has been recently associated with a wide range of pathological processes (Cao & Dixon, 2016; Jiang *et al.*, 2021). As the major site of ROS production and iron handling, mitochondria actively participate in ferroptotic signalling from many fronts, hosting both driving and inhibitory pathways and possibly also acting as a primary trigger (Battaglia *et al.*, 2020; Wang *et al.*, 2020).

2.3 Sneaky Little Genomes

Mitochondrial DNA

Deoxyribonucleic acid (DNA) is the double-helical molecule that carries the inheritable genetic instructions of life (Hershey & Chase, 1952; Franklin & Gosling, 1953). Many basic biology lectures would state that DNA is contained in the nucleus of the eukaryotic cell, but this is factually wrong. Approximately 99% of human DNA is indeed in the form of massive linear DNA molecules called chromosomes in the nucleus, but that ~1% left is instead inside mitochondria. Reminiscent of their bacterial origins, mitochondrial DNA (mtDNA) are circular molecules and are present in many copies within each cell and mitochondrion (Nass & Nass, 1963; Nass, 1966). Human mtDNA is 16,569 base pairs long and encodes for 13 proteins (all of which are subunits of respiratory chain complexes), as well as the RNA required for mitochondrial gene expression: 2 ribosomal RNAs and 22 transfer RNAs (Anderson *et al.*, 1981; Bibb *et al.*, 1981). The two mtDNA strands are referred to as heavy and light strands, with the heavy one being particularly GC-rich and containing most of the

genes. Most of the regulatory elements for transcription and translation are contained within a long non-coding region known as the displacement loop (D-loop) (Falkenberg, 2018).

MtDNA is tightly packed into protein complexes called nucleoids (Bereiter-Hahn & Vöth, 1998; Jacobs *et al.*, 2000; Spelbrink *et al.*, 2001). Super-resolution microscopy techniques (e.g. STED, PALM) measure nucleoids at around 80 nm in diameter and slightly ellipsoidal, with each human nucleoid containing an average of 1.4 mtDNA copies each in cell culture (Brown *et al.*, 2011; Kukat *et al.*, 2011; Bonekamp & Larsson, 2018). The degree of packaging of the nucleoid regulates their accessibility, replicative and transcriptional activity and half-life (Ekstrand *et al.*, 2004; Farge *et al.*, 2014; Brüser *et al.*, 2021). The nucleoid is associated with the inner mitochondrial membrane in a manner partially dependent on its replication machinery (Nass, 1969; Rajala *et al.*, 2014).

MtDNA replication and maintenance

MtDNA is replicated independently of the cell cycle (Clayton, 1982), utilising a distinct machinery from that in charge of nuclear DNA replication. All enzymes involved in mtDNA maintenance are encoded in the nucleus and imported into mitochondria, and the removal of any of them leads to severe mtDNA depletion and embryonic lethality in mice (Tyynismaa & Suomalainen, 2010).

The minimal set of proteins required for mtDNA replication *in vitro* includes both subunits of DNA polymerase gamma, Twinkle helicase and the mitochondrial single-strand binding proteins (Korhonen *et al.*, 2004). Many other proteins are also essential for the regulation and maintenance of mtDNA, as well as other polymerases (e.g. PrimPol and other nuclear polymerases) which appear to be required in DNA stress conditions (Stojkovic *et al.*, 2016; Krasich & Copeland, 2017; Torregrosa-Muñumer *et al.*, 2017).

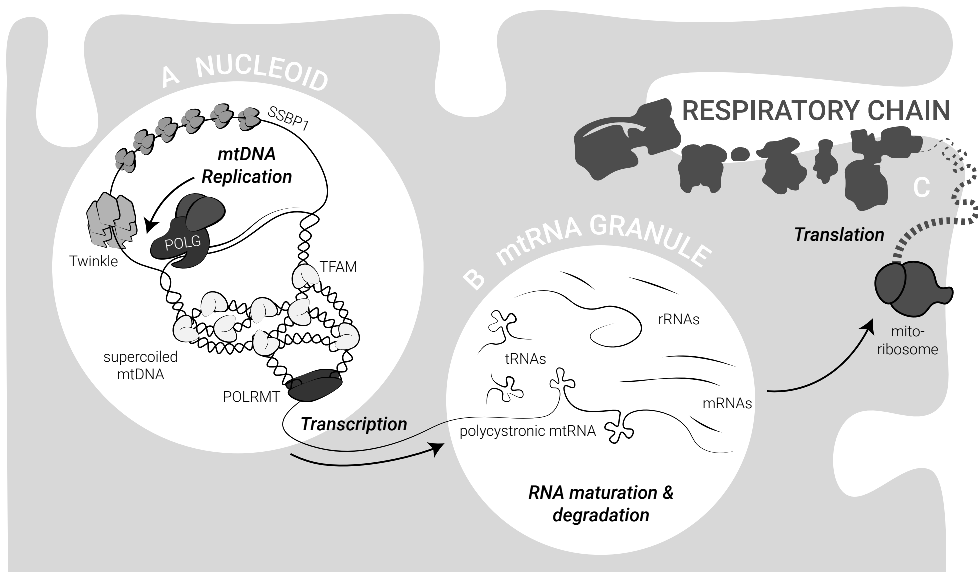

Figure 2: The evolving central dogma of mitochondrial gene expression. **(A)** Nucleoid, containing mtDNA coiled by TFAM binding as well as other nuclear-encoded mtDNA maintenance proteins. On top, the minimal mtDNA replication machinery: DNA polymerase gamma (POLG), Twinkle helicase and mitochondria single-stranded DNA binding proteins (SSBP1). Below, POLRMT-mediated transcription of long polycistronic mtRNA. **(B)** Maturation (excision, post-transcriptional modifications and degradation) of mtRNA in the mitochondrial RNA granule, resulting in mature mRNA and tRNA,

as well as rRNA partially assembled with nuclear-encoded ribosomal subunits. **(C)** Translation of mitochondrial mRNAs by the mitoribosome at the inner membrane, coupled with the insertion and folding of the polypeptides into the inner membrane, to be joined by nuclear-encoded subunits and assembled into respiratory chain complexes.

DNA polymerase gamma is the mtDNA replicase, a heterotrimer including a large catalytic subunit (POLG1) and two accessory subunits that function as processivity factors (POLG2) (Wernette & Kaguni, 1986; Gray & Tai Wai Wong, 1992; Lim *et al.*, 1999; Kaguni, 2004). The functional holocomplex is henceforth referred to as POLG. Besides replication, POLG also exhibits 3'-5' exonuclease and 5'- deoxyribose phosphate lyase activities, enabling proofreading, DNA repair and degradation functions (Fridlender *et al.*, 1972; Olson & Kaguni, 1992; Longley *et al.*, 1998; Kaguni, 2004; Martin & Wood, 2019). Recent reports implicate POLG - particularly its exonuclease domain - in the rapid degradation of linear mtDNA fragments upon double-strand breaks (Nissanka *et al.*, 2018; Peeva *et al.*, 2018) and of the whole mtDNA during nucleotide starvation in yeast (Medeiros *et al.*, 2018).

POLG has notably high fidelity, with an error frequency of around $2x10^{-6}$ per nucleotide (or one mutation every 18 human mtDNA molecules) (Johnson & Johnson, 2001), while even the most accurate nuclear polymerases (polymerase ε and polymerase δ) present error rates in the 10^{-5} range (Korona *et al.*, 2011). Misincorporation decreases POLG polymerisation speed in the thousand-fold range with the notable exception of G:T base formation, which is only 38 times slower than the canonical A:T pairing (Johnson & Johnson, 2001) and thus the most likely misincorporation to occur.

Twinkle is the homo-hexameric protein complex that unwinds double-stranded mtDNA for replication in the 5'-3' direction (Korhonen *et al.*, 2003; Ziebarth *et al.*, 2007). Its highly conserved sequence is evolutionarily related to the T7 bacteriophage primase/helicase T7gp4, consistent with the viral origins of many mtDNA maintenance proteins (Spelbrink *et al.*, 2001; Ziebarth *et al.*, 2007).

Encoded by the *TWNK* gene (formerly *C10orf2* or *PEO1*), Twinkle is essential for mtDNA replication and maintenance. It receives its peculiar name from the "starry night" appearance of its immunofluorescent visualisation (Spelbrink *et al.*, 2001), forming nucleoid-like structures firmly associated with the inner membrane even in the absence of mtDNA (Rajala *et al.*, 2014). Twinkle amounts can directly modulate mtDNA copy number in cells and tissues, suggesting it as a licensing factor for mtDNA replication (Tyynismaa *et al.*, 2004; Ylikallio *et al.*, 2010).

The other member of the minimal replisome is SSBP1, which binds single-stranded mtDNA and protects it during replication, enhancing Twinkle and therefore replisome processivity (Tiranti *et al.*, 1995; Korhonen *et al.*, 2003).

TFAM (Transcription factor A mitochondrial) is the only other protein known to directly modulate mtDNA amount, as well as the most abundant protein in the nucleoid. In addition to its function as a transcription factor for mtDNA (Fisher *et al.*, 1992), TFAM can wrap and U-bend mtDNA, tightly packaging it as a nucleoid and thus protecting it from external damage (Ngo *et al.*, 2011; Rubio-Cosials *et al.*, 2011). TFAM amounts often correlate with mtDNA amounts, as TFAM availability affects mtDNA accessibility and half-life. (Larsson *et al.*, 1998; Ekstrand *et al.*, 2004; Ylikallio *et al.*, 2010).

mtDNA replication models

Despite the small size and relative simplicity of the mitochondrial genome, the specifics of its replication have been a subject of heated debate for half a century. MtDNA replication is thought to occur asymmetrically (Robberson *et al.*, 1972; Clayton, 1982) and start on dedicated origins for each strand: heavy and light strand origins (O_H and O_L respectively) (Falkenberg, 2018). Unlike in their bacterial relatives which use a dedicated primase, mammalian mtDNA replication requires POLRMT for primer synthesis (Xu & Clayton, 1996; Wanrooij *et al.*, 2008).

The conventional paradigm for mtDNA replication today is termed the strand-displacement model. It proposes that replication begins at O_H and proceeds through the heavy strand until O_L, with the light strand protected by SSBP1 binding. When replication reaches O_L, it forms a stem-loop structure that recruits POLRMT, and thus starts the replication of the single-stranded light strand (Fusté *et al.*, 2010). Once the two strands are completed, they are resolved by mitochondrial nucleases for segregation (Macao *et al.*, 2015; Nicholls *et al.*, 2018). An alternative model, the so-called RITOLS model (RNA incorporated throughout the lagging strand) is similar in principle to the strand-displacement model, only differing in the protection of the single-stranded light strand by RNA fragments which are eventually replaced (Yasukawa *et al.*, 2006). A third model has been suggested to involve symmetrical and two-directional strand-coupled replication (Holt *et al.*, 2000). The presence of evidence for all these models may be explained by their co-existence and occurrence in different cell types and under various conditions or stressors. In addition, double-stranded mtDNA replication intermediates exist and are predominant under replication stress (Torregrosa-Muñumer *et al.*, 2019), and complex multi-stranded replication intermediates have been identified in e.g. human hearts (but not rodent hearts) (Pohjoismäki *et al.*, 2010, 2013b), all suggesting that the human mtDNA replication and resolution system may be yet more intricate than expected.

Mitochondrial Gene expression

Unlike the virus-derived machinery involved in replication, mtDNA gene expression shares features with its bacterial relatives, together with some fascinatingly unique quirks.

MtDNA is heavily packed, and transcription is quite different to that of the nuclear genome: genes have no introns and are transcribed as two long polycistronic strands. They are then processed post-transcriptionally, and the resulting mRNAs lack untranslated regions, modified bases and caps (Pearce *et al.*, 2017).

Mitochondrial transcription can be reconstituted *in vitro* by three key components: two transcription factors (TFAM and TFB2M) and the mitochondrial RNA polymerase (POLRMT) (Falkenberg *et al.*, 2002). Transcription initiates at the heavy- and light-strand promoters in the non-coding region of mtDNA (Chang & Clayton, 1984). The two resulting polycistronic RNAs are then cleaved, modified and folded into messenger, ribosomal and transfer RNAs (Hällberg & Larsson, 2014; Suomalainen & Battersby, 2018).

Mitochondrial nucleoids can undergo replication and transcription simultaneously (Brüser *et al.*, 2021), and mitochondrial RNA (mtRNA) also appears to cluster in discrete structures termed mitochondrial RNA granules (MRGs). These granules behave like fluid condensates (Rey *et al.*, 2020) and are often located next to the nucleoids, regulating the availability, maturation and translation of mtRNA (Jourdain *et al.*, 2016).

Like bacterial translation, mitochondrial translation initiates with a formylated methionine, and each three-nucleotide codon is read by the ribosome as "start", "stop" or an amino acid. A crucial difference lies in the genetic code: the rules of what each codon corresponds to in the sequence. Mitochondria are one of the extremely rare exceptions to the "universal genetic code" that rules most of life (Nelson & Cox, 2017; Pearce *et al.*, 2017).

The amino acids used as building blocks for proteins are conveyed by mitochondrial tRNAs and condensed into a polypeptide in the ribosome, in a process known as translation. Mitochondrial translation occurs at the inner mitochondrial membrane simultaneously to membrane insertion and folding of the nascent protein (Itoh *et al.*, 2021). Aided by an army of chaperones and quality control machinery, this unique process allows for the highly hydrophobic components of the respiratory chain to be correctly and safely introduced into the mitochondrial membrane (Hällberg & Larsson, 2014; Suomalainen & Battersby, 2018).

2.4 Mitochondrial dysfunction

Since the discovery of mitochondria, the list of physiological and pathological processes associated with their function and dysfunction has unceasingly increased. From disorders caused by primary mitochondrial dysfunction (mitochondrial diseases) to common diseases with strong mitochondrial involvement such as Parkinson's disease, diabetes, cancer, obesity and even normal ageing; mitochondria continue to be in the spotlight for novel research and therapeutic interest (McBride *et al.*, 2006; Nunnari & Suomalainen, 2012; Gorman *et al.*, 2016; Russell *et al.*, 2020)

Mitochondrial diseases

Mitochondrial diseases are a heterogeneous group of genetic disorders characterised by the malfunction of mitochondria. Even though mitochondria are essential for almost all cells in the human body, their dysfunction can lead to vastly different clinical presentations. Each defect has its own constellation of tissues affected, ages of onset and severity; and the reason behind this diversity remains largely unknown (Gorman *et al.*, 2016).

Mitochondrial diseases can arise from defects in either genome (nuclear or mitochondrial) and follow any inheritance pattern. MtDNA defects are maternally inherited or sporadic, with rare occurrences of paternal influence (Luo *et al.*, 2018), and nuclear gene defects can be autosomal dominant, autosomal recessive, X-linked or *de novo*. The genes that cause mitochondrial disease are those that directly or indirectly affect the mitochondrial functions: mtDNA maintenance and integrity, transcription, translation, import and assembly of mitochondrial proteins and complexes, mitochondrial dynamics and turnover, and even metabolic enzymes involved in fuelling mitochondrial processes such as mtDNA replication (Nunnari & Suomalainen, 2012; Ylikallio & Suomalainen, 2012; Gorman *et al.*, 2016; Russell *et al.*, 2020).

MtDNA maintenance disorders

As described in section 2.3, mtDNA maintenance involves many nuclear-encoded proteins. The malfunction of these often leads to damage in mtDNA (e.g., deletions and rearrangements) or its depletion by instability, increased turnover, or inability to replicate. As a result, mtDNA maintenance disorders are some of the most common causes of mitochondrial disease, and some of the most diverse in presentation.

Among the first mtDNA maintenance diseases reported is PEO (progressive external ophthalmoplegia), a late-onset progressive myopathy associated with mtDNA deletions (Moraes *et al.*, 1989; Zeviani *et al.*, 1989), later shown to also affect the heart and the brain (Suomalainen *et al.*, 1992). Interestingly, the disease-causing defect was later mapped to three different proteins crucial for mtDNA maintenance: twinkle helicase (Suomalainen *et al.*, 1995; Spelbrink *et al.*, 2001), the adenine nucleotide translocator ANT1 (Kaukonen *et al.*, 1999, 2000) and DNA polymerase gamma (Van Goethem *et al.*, 2001).

The genetic background of PEO and other mtDNA maintenance diseases has expanded, but the original reports nicely exemplify the main disease-causing groups of genes: proteins directly involved in mtDNA replication, developed below, and enzymes affecting the mitochondrial deoxynucleoside triphosphate (dNTP) pool, described in a later section.

Mutations in the *POLG1* gene are the most common cause of mtDNA maintenance disease and typically cause neurological disorders. They have been associated with late-onset autosomal dominant or recessive forms of PEO (Van Goethem *et al.*, 2001), a childhood-onset mtDNA depletion syndrome affecting liver and brain known as Alpers-Huttenlocher syndrome (Naviaux & Nguyen, 2004), and ataxia-neuropathies such as the mitochondrial recessive ataxia syndrome (MIRAS) (Hakonen *et al.*, 2005). Parkinsonism and early menopause have also been associated with POLG defects (Luoma *et al.*, 2004).

The diverse presentation of POLG disorders achieved another level of complexity with the discovery that patients with identical MIRAS mutations may present remarkably different diseases, ranging from adolescent-onset ataxia with seizures to milder adult neuropathy (Rantamäki *et al.*, 2001; Hakonen *et al.*, 2005; Winterthun *et al.*, 2005). Consistent with its often neurological presentation, *POLG* transcription is regulated by a central nervous system -specific genomic region (Nikkanen *et al.*, 2018) driving POLG expression to specific neural populations, and co-expressed with a microRNA (Mir-9-3). The complex regulation of *POLG* together with the involvement of Mir-9 in stem cell maintenance and metabolic rewiring (Bonev *et al.*, 2012; Selcuklu *et al.*, 2012; Coolen *et al.*, 2013) could indicate neural- and stem cell-specific vulnerabilities for *POLG* defects and provide insight into the causes for POLG tissue-specificity.

Twinkle is another underlying cause of several different diseases. That includes autosomal dominant PEO (Suomalainen *et al.*, 1995), Alpers-Huttenlocher syndrome (Hakonen *et al.*, 2007), Perrault syndrome (Morino *et al.*, 2014) and infantile-onset spinocerebellar ataxia (IOSCA) (Nikali *et al.*, 2005; Hakonen *et al.*, 2008).

2.5 Building blocks and Currency

Nucleotide Metabolism

Nucleotides are multifaceted biomolecules by excellence. They are the energy currency of a myriad of enzymatic reactions, key players in almost all cell signalling cascades and, if that was not enough, they are the literal building blocks of all genetic material in the cell (Nelson & Cox, 2017). Deoxynucleoside 5´ triphosphates (dNTPs) are the reduced and activated nucleotides destined for DNA synthesis, providing both the carbon skeleton and the chemical energy for polymerisation (Reichard, 1988; Mathews, 2006). In animal cells, dNTPs can be either synthesised *de novo* from amino acids and other simple metabolites and then reduced by the ribonucleotide reductase (Thelander & Reichard, 1979; Nordlund &

Reichard, 2006), or recycled from other intermediates and degradation products by the cytosolic or mitochondrial salvage pathways (Wang, 2010).

The concentration and the relative balance of dNTPs have been long known to be critical for cellular genetic and metabolic homeostasis: they directly modulate nuclear DNA replication and fidelity, further influencing DNA repair and genome integrity, cell cycle regulation, oncogenesis, viral infection and many other vital cell processes (Reichard, 1988; Chabes *et al.*, 2003; Wheeler *et al.*, 2005; Mathews, 2014).

Reflecting the wide diversity of roles nucleotides play in the cell, defects in nucleotide metabolism enzymes associate with many different developmental, metabolic, neurological, and immunological diseases (Nyhan, 2005). They can also cause secondary metabolic deficiencies due to their role as co-factors, e.g. guanine-derived tetrahydrobiopterin is required for dopamine synthesis, making dopaminergic neurons susceptible to guanine metabolic defects and causing dystonia-tremor diseases (Lesch & Nyhan, 1964; Ichinose *et al.*, 1994; Göttle *et al.*, 2014; Kuukasjärvi, Landoni *et al.*, 2021).

Nucleotides in mitochondrial disease

The importance of nucleotide metabolism for the mitochondrial genome was also largely unveiled by investigating the genetics of human disease. A defect in the cytosolic degradation enzyme thymidine phosphorylase was discovered to cause mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), a multi-organ mitochondrial disease characterised by dTTP accumulation, dNTP imbalance and mtDNA depletion and deletions (Nishino *et al.*, 1999; López *et al.*, 2009). It soon became evident that mtDNA synthesis is the critical consumer of dNTPs in post-mitotic cells, and so defects in the pathways fuelling mtDNA replication post-mitotically cause mtDNA depletion and mitochondrial disease. The mitochondrial salvage pathway proteins thymidine kinase 2 (TK2) and deoxyguanosine kinase (DGUOK) cause recessive myopathic and hepatocerebellar mtDNA depletion syndromes respectively (Mandel *et al.*, 2001; Saada *et al.*, 2001; Galbiati *et al.*, 2006), with deoxythymidylate kinase recently being reported as a likely new member of the group (Lam *et al.*, 2019). The small subunit of the ribonucleotide reductase expressed in post-mitotic cells RRM2B also causes severe multi-organ mtDNA depletion syndrome (Bourdon *et al.*, 2007), highlighting the importance of *de novo* dNTP synthesis to mtDNA maintenance. TK2 and RRM2B defects have also been linked to other mitochondrial diseases such as juvenile MNGIE (Shaibani *et al.*, 2009) and PEO (Tyynismaa *et al.*, 2009, 2012). A few other genes have been associated with mtDNA depletion syndrome, most of which are consistently directly or indirectly involved in nucleotide metabolism and transport (Elpeleg *et al.*, 2005; Spinazzola *et al.*, 2006; Ylikallio & Suomalainen, 2012; Besse *et al.*, 2015; Thompson *et al.*, 2016; Viscomi & Zeviani, 2017).

Measuring dNTPs

Unravelling the importance of dNTP balance required the ability to measure them, a historically challenging endeavour. The cellular concentration of dNTPs is especially low, and the amount of biological material for molecular research is often limiting. In addition, dNTP concentrations vary enormously between cell types, cell cycle stage and subcellular compartment; and are orders of magnitude lower than the concentration of other similar nucleosides (Shewach, 1992; Gandhi & Samuels, 2011; Wheeler & Mathews, 2011; Pancsa *et al.*, 2022).

Methodologies using High-Performance Liquid Chromatography (HPLC) followed by ultraviolet or mass spectrometry detection have been used in the past (Decosterd et al., 1999; Di Pierro et al., 1995), as well as boronate chromatography and gradient HPLC with an anion-exchange column to separate and avoid the interference of excess ribonucleotides (Shewach, 1992). While the HPLC method is robust, its use in biomedical research has been encumbered by the large amounts of material required for its performance, often incompatible with minute biological samples or experiments requiring subcellular fractionation.

As it often occurs in molecular biology, human-made tools can rarely compete with millions of years of evolutionary optimisation. So, if one aims to differentiate dNTPs from excess ribonucleosides, DNA polymerases are highly proficient for precisely that job. The modern state-of-the-art dNTP quantitation relies on the accuracy of a DNA polymerase to incorporate dNTPs into DNA templates and the quantification of the product. Before the availability of synthetic oligonucleotides, the assay used DNA polymers isolated from calf thymus and the Klenow fragment of the *E. coli* polymerase I as an enzyme (Solter & Handschumacher, 1969). As expected, the possibility of designing synthetic DNA sequences drastically improved the accuracy and sensitivity of the method (Sherman & Fyfe, 1989), along with the use of high-fidelity thermostable polymerases to further reduce NTP misincorporation (Ferraro *et al.*, 2010).

Modern oligonucleotide templates ensure the proportional enzymatic incorporation of a specific dNTP to be measured and a radioactive dNTP in excess (tritium-labelled dATP or dTTP). The resulting radioactive signal can be compared to a standard curve of known dNTP concentrations, extrapolating the exact dNTP amount in the sample. Further improvements have focused on the optimisation of the template sequences and polymerase reaction components, as well as adapting the extraction method to different cells, tissues and subcellular compartments (Martí *et al.*, 2012).

2.6 Ageing & progeroid syndromes

Ageing is one of those concepts which are known to all yet notoriously hard to specifically define. The passage of time leads to the structural and functional decline in a living organism, causing its eventual death. This decline is a consequence of the gradual accumulation of damage at all levels, molecular to organismal, and typically burdens the capacity of the organism to adapt to its environment and increases its likelihood to die. Ageing is characterised by cellular and molecular hallmarks (e.g. genomic instability, stem cell attrition, defective nutrient sensing, cellular senescence) as well as the macroscopic characteristics we can all recognise (hair greying, skin wrinkling, muscle and bone wearying, decreased cognitive abilities and higher risk for cancer and other diseases) (López-Otín *et al.*, 2013).

Progeria and progeroid syndromes are rare disorders that mimic the clinical features of physiological ageing, but in an accelerated fashion, first described already over 130 years ago (Hutchinson, 1886; Gilford, 1904; Carrero *et al.*, 2016). Genetics and mouse models have been valuable to shed light on the molecular background of premature ageing and highlight its shared molecular features and potential relevance to normal ageing (Gordon *et al.*, 2014). A common mechanism for premature ageing is compromised nuclear DNA, either by defects in DNA repair machinery or nuclear architecture (Carrero *et al.*, 2016). This threat

critically affects somatic stem cell maintenance and leads to a collection of symptoms that bear a striking similarity to those of normal ageing.

While the vast majority of premature ageing models follow the abovementioned mechanism there is a notable outlier, which involves mitochondrial DNA.

The Mitochondrial Theory of Ageing arose from the observation that mtDNA mutations accumulate during ageing, proposing a mechanism whereby the slow generation of mtDNA mutations through time would cause progressive defects in mitochondrial respiratory chain function, in turn increasing ROS production (Harman, 1972). ROS would then provoke oxidative tissue deterioration and further mtDNA damage, resulting in a vicious cycle underlying ageing-related degeneration.

To test this model, two separate groups simultaneously generated the **mtDNA Mutator mouse**, carrying a D257A amino acid change in murine POLG ($Polg^{D257A}$) (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). This change inactivates the exonuclease domain which is required for proofreading misincorporations during replication as well as other maintenance functions (See section DNA Polymerase Gamma).

The mice present mtDNA mutation accumulation in tissues but develop normally. They show the first signs of premature ageing after 6 months and die at around 13-15 months of age due to severe anaemia (~50% of the lifespan of a healthy mouse of the same inbred line). Other symptoms include many hallmarks of ageing: hair greying, reduced subcutaneous fat, osteoporosis, kyphosis, and sarcopenia. Most symptoms are exclusive to $Polg^{D257A}$ homozygotes, since a single wildtype POLG copy appears to be sufficient to maintain low mutagenesis and perform other functions (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Szczepanowska & Trifunovic, 2015).

Similar to other ageing and progeroid models, Mutator mice present defects in the somatic stem cell compartment with neural, spermatogonial and hematopoietic stem cells particularly affected (Chen *et al.*, 2009; Norddahl *et al.*, 2011; Ahlqvist *et al.*, 2012, 2015a; Wahlestedt *et al.*, 2014), with a general loss of stemness and cell-specific differentiation defects such as defective iron loading during erythroid differentiation (Ahlqvist *et al.*, 2015a). Mutator stem cells have been shown to be especially sensitive to redox balance, and while antioxidants have been shown to rescue the self-renewal and reprogramming defects in pluripotent stem cell cultures, identical doses were toxic for e.g. neural stem cells (Hämäläinen *et al.*, 2015). This highlights the complex role of ROS in physiological signalling and the differential sensitivity of tissues and cell types.

Questions and concerns

While the "mtDNA mutations cause ageing" causality seems straightforward, further studies on the Mutator mouse and other models have surfaced a few concerns and alternative mechanistic viewpoints. Firstly, the original mitochondrial theory of ageing was not fully supported by the findings in Mutators. Oxidative stress was not detectable in post-mitotic tissues and mtDNA mutations accumulated linearly rather than exponentially (Trifunovic *et al.*, 2005), directly contradicting the hypothesis of a vicious cycle. In addition, the mtDNA point mutation load in $Polg^{D257A}$ heterozygotes, which showed no ageing phenotype, was remarkably higher than that of aged mice (Vermulst *et al.*, 2007), questioning their causal role for ageing.

The Mutator mouse has been an invaluable model to uncover other functions of the exonuclease domain of POLG: notably, the degradation of linear mtDNA fragments (Nissanka *et al.*, 2018), as well as increased processivity and roles in ligation generating control region multimers (Williams *et al.*, 2010; Macao *et al.*, 2015). These observations also signify that mtDNA point mutations cannot be conclusively assumed to be the sole culprit for the premature ageing in Mutators.

Mitochondrial dysfunction has been considered a hallmark of ageing for decades (López-Otín *et al.*, 2013). Nonetheless, while a myriad of different models exists of mild and severe mitochondrial dysfunction, none of them show similar signs of premature ageing as Mutators, and neither do humans with mitochondrial disease (Gorman *et al.*, 2016). Even models presenting with high ROS production show increased DNA damage and cancer incidence but no progeroid symptoms (Yang *et al.*, 2007), and the correlation between ROS and longevity in the literature is irregular (Schriner *et al.*, 2005; Yang *et al.*, 2007).

When one looks at premature ageing models, the mtDNA Mutator mice also stand as the exception, as an outsider to the common mechanism of nuclear DNA instability (Carrero *et al.*, 2016). This whilst sharing many of the molecular and macroscopic characteristics. What makes Mutators unique among mitochondrial dysfunction models and connects them to progeroid models remains an outstanding question in both the mitochondrial and ageing fields.

2.7 Treatments for mitochondrial dysfunction

Few curative treatments exist for mitochondrial diseases, and the existing approaches are mostly symptomatic. Nonetheless, new developments in recent years have brought hope to the cause, enabled by a general paradigm shift originating from fundamental research. The field is moving away from the assumption that energy deficiency underlies all mitochondrial disease, and understanding the complex interplay of metabolic and signalling pathways which are affected and potentially targetable (Chung *et al.*, 2021). Below, I summarise some of the key research in this field of particular relevance for this thesis. Importantly, because of the wide variability of mitochondrial disease manifestations, the physiological responses and mechanisms are different. It is thus unlikely that a single treatment will be beneficial for all mitochondrial diseases, and each discovery must be assessed in the context of the pathomechanism it affects. For a thorough review of the specific approaches and ongoing clinical trials, see (Garone & Viscomi, 2018; Russell *et al.*, 2020; Ramón *et al.*, 2021; Tinker *et al.*, 2021).

Genetic interventions & transplants

The insertion of the healthy version of a disease-causing gene into a patient (e.g. using a viral vector) has proven good efficacy in preclinical and some clinical trials, with approaches targeting the liver (Di Meo *et al.*, 2012; Bottani *et al.*, 2014; Torres-Torronteras *et al.*, 2014), eyes (Wan *et al.*, 2016; Sarzi *et al.*, 2018) or brain (Di Meo *et al.*, 2017).

Other genetic approaches include the use of mitochondria-targeted nuclease-derived proteins to modulate heteroplasmy by elimination or editing of the mutant mtDNA (Gammage *et al.*, 2018; Bacman & Moraes, 2020; Zekonyte *et al.*, 2021; Silva-Pinheiro *et al.*, 2022), the genetic or pharmacological regulation of mitochondrial dynamics genes (Civiletto *et al.*, 2015; Karaa *et al.*, 2018; Rocha *et al.*, 2018), and the revolutionary and controversial

mitochondrial replacement therapies. The latter allow for the generation of an embryo carrying the nuclear genetic material of the parents but the mitochondrial DNA of a healthy donor (Hyslop *et al.*, 2016; Kang *et al.*, 2016), circumventing the maternal inheritance of the disease.

Although not a genetic intervention *per se*, the transplantation of donor tissue carrying the healthy version of a gene has also proven to be a robust strategy against often fatal enzyme deficiencies. Notably bone marrow transplants for MNGIE (Hirano *et al.*, 2006; Halter *et al.*, 2011), and liver transplant for ethylmalonic encephalopathy (Dionisi-Vici *et al.*, 2016) and MNGIE (De Giorgio *et al.*, 2016; D'Angelo *et al.*, 2020; Kripps *et al.*, 2020).

Control of oxidative damage

As previously mentioned, mitochondria are major producers of ROS, as a by-product of respiration and amplified upon respiratory chain dysfunction (Murphy, 2009). ROS have been traditionally perceived as undeniably damaging, making antioxidants the most commonly used drugs for mitochondrial disease, with several clinical trials ongoing for novel developments. Despite their common use and large market, the preclinical and clinical data backing their efficacy is very much limited and often anecdotal (Hart *et al.*, 2005; Meier *et al.*, 2012; Garone & Viscomi, 2018). Since ROS play an important role in mitochondrial signalling and stem cell maintenance, antioxidant approaches should be cautious not to disrupt the physiological functions of redox balance and potentially cause more harm than good (Holmström & Finkel, 2014; Hämäläinen *et al.*, 2015; Dogan *et al.*, 2018).

Limiting oxygen availability recently surfaced as a highly unconventional yet effective method for mitochondrial disease therapy. Hypoxia and oxygen-depriving interventions such as severe anaemia and carbon monoxide exposure can drastically ameliorate the phenotype of a model of neurological mitochondrial disease (Jain *et al.*, 2016, 2019; Ferrari *et al.*, 2017). This surprising and exciting therapeutic possibility also suggests that unused oxygen plays a critical role in the pathogenesis of certain disorders.

Enhancement of mitochondrial biogenesis

The mitochondrial amount in the cell is coordinated to its metabolic needs, balanced between biogenesis and degradation (Nunnari & Suomalainen, 2012). Stimulation of mitochondrial biogenesis has shown promising results, often by boosting NAD+ content with its precursors (e.g. nicotinamide riboside or niacin), inhibition of NAD-consuming enzymes, or pharmacological induction by bezafibrate as originally proposed by the Moraes laboratory in 2008 (Dillon *et al.*, 2012; Yatsuga & Suomalainen, 2012; Cerutti *et al.*, 2014; Khan *et al.*, 2014; Pirinen *et al.*, 2020).

Rapamycin, a drug inhibiting (and naming) the anabolic master regulator mTORC1, enables mitochondrial recycling among its many roles and shows beneficial effects in a wide range of models, including murine mitochondrial myopathy (Khan *et al.*, 2017), mouse and fly models of Leigh syndrome (respiratory complex I defects) (Johnson *et al.*, 2013; Wang *et al.*, 2016; Felici *et al.*, 2017) and TK2-related mtDNA depletion in mice (Siegmund *et al.*, 2017). In contrast, rapamycin was not effective and even harmful for murine encephalopathic models like coenzyme Q deficiency and astrocyte-specific Twinkle knock-out (Barriocanal-Casado *et al.*, 2019; Ignatenko *et al.*, 2020), indicating the alleviatory potential of rapamycin is specific to certain mitochondrial defects and their associated stress responses (Garone & Viscomi, 2018; Suomalainen & Battersby, 2018).

High-fat and low-carbohydrate diets known as ketogenic diets have been used since the 1920s to treat drug-resistant epilepsy (Veech, 2004), and can alleviate the seizure frequency in mitochondrial diseases such as POLG-linked Alpers-Huttenlocker syndrome and children carrying defects of the mitochondrial respiratory chain complexes presenting with epilepsy (Kang *et al.*, 2007; Joshi *et al.*, 2009). Ketogenic diets can induce mitochondrial biogenesis through a variety of transcriptional and metabolic mechanisms, including the inhibition of mTORC1 (Bough *et al.*, 2006; McDaniel *et al.*, 2011; Danial *et al.*, 2013). Consistently, a ketogenic diet can slow down the disease progression in the myopathic Deletor mice carrying a mutant Twinkle, enhancing mitochondrial biogenesis and improving mitochondrial morphology and function (Ahola-Erkkilä *et al.*, 2010). In mitochondrial myopathy patients, a strict ketogenic diet had different effects: it caused the targeted degradation of the most affected muscle fibres leaving healthy fibres unaffected and showing mild long-term improvements (Ahola *et al.*, 2016), reminiscent to *in vitro* data from heteroplasmic cultures where a ketogenic treatment can enrich for cells carrying healthy mtDNA (Santra *et al.*, 2004). Ketosis has also been reported to exacerbate the spongiotic encephalopathy of astrocyte-specific Twinkle knock-out mice (Ignatenko *et al.*, 2020), further highlighting the importance for species- and disease-specific testing and treatments.

Improvement of mitochondrial DNA homeostasis

A particularly successful therapeutic approach has been the bypass of a metabolic defect by supplementation of metabolites in that pathway. Notably, the use of deoxynucleosides to rescue mtDNA depletion caused by nucleotide metabolic defects. The preclinical results were encouraging for several defects: mtDNA levels could be recovered in cellular models of pharmacological and genetic mtDNA depletion (Bulst *et al.*, 2009, 2012; Cámara *et al.*, 2014), as well as in mouse models of TK2-deficiency which also showed significant improvements in lifespan and mitochondrial function in the affected organs (Garone *et al.*, 2014; Lopez-Gomez *et al.*, 2017). Based on this, compassionate use of the treatment was attempted in patients with TK2 defects, typically presenting with an devastating disease which rapidly leads to myopathy and childhood death (Saada *et al.*, 2001; Garone *et al.*, 2018). The outcome of these trials has been remarkable, with the 5 early-onset patients studied presenting marked improvements in survival and muscle function compared with the historical record, and the other 11 patients with later onsets showing stabilisation or improvement of their symptomatology (Domínguez-González *et al.*, 2019).

Modulation of mtDNA copy number

In addition to the straightforward logic of restoring mtDNA amount when its synthesis is defective, the total mtDNA amount has been further interpreted to influence disease severity. As described previously, only two proteins are known to directly regulate mtDNA copy number: TFAM by increasing mtDNA half-life, and Twinkle by enhancing mtDNA replication (Ekstrand *et al.*, 2004; Tyynismaa *et al.*, 2004; Ylikallio *et al.*, 2010).

Twinkle overexpression has been shown to be protective against genetic and ischemic heart insults (Pohjoismäki *et al.*, 2013a; Tanaka *et al.*, 2013; Ikeda *et al.*, 2015; Inoue *et al.*, 2016). On the other hand, the overexpression of TFAM appears to ameliorate the phenotype of an mtDNA heteroplasmic disease model (Filograna *et al.*, 2019) and improve the infertility phenotype of the Mutator mouse, without reports of the physiological consequences in other tissues and mouse physiology (Jiang *et al.*, 2017). Based on these observations, the

boosting of total mtDNA amount as a beneficial therapeutic target for human disease was proposed (Filograna *et al.*, 2021). The human evidence for high mtDNA copy number as a beneficial factor for disease has so far been correlative, showing mild and often contradictory changes in mtDNA related to age, disease severity and penetrance, mostly from blood cells. MtDNA amount changes in cancer are also a focus, where both low and high amounts appear to correlate with worse outcomes (Mi *et al.*, 2015; Filograna *et al.*, 2021).

While intriguing, the conclusion of "more mtDNA is better" may be too simplistic and still lacks definite causal data. Outstandingly, the overexpression of either TFAM or Twinkle is known to inhibit mtDNA transcription and cause progressive respiratory chain deficiency (Ylikallio *et al.*, 2010; Farge *et al.*, 2014), further emphasising both the intricacy and the sensitivity of the system being disturbed.

Challenges and outlooks

A significant challenge for mitochondrial disease therapy development is the rarity and severity of the diseases, exacerbated by the diversity of clinical presentation even upon identical genotypes. This encumbers the arrangement of traditional randomised clinical trials, which require large and relatively homogeneous groups of patients. This is especially challenging in rapidly fatal childhood diseases like mtDNA depletion syndromes, where the current treatment is provided under compassionate use. Recent global efforts have been pursued to fully describe the natural history of some of these disorders (Garone *et al.*, 2018; Keshavan & Rahman, 2018; Keshavan *et al.*, 2020). These reports provide evidence on the typical progression of the disease and meaningful and standardised outcome measures, to enable the development of novel clinical trial settings that minimise the risk of the participants and maximise their access to potentially life-saving treatment.

Another challenge is awareness. Like many rare disorders, mitochondrial diseases are often under- or misdiagnosed, mistaken with similar common afflictions, or simply unknown by the clinician. Moreover, regular treatments for common diseases could be harmful to a mitochondrial disease patient. Such is the case for valproate, a normally safe anti-epileptic drug that can trigger sudden liver failure in patients with POLG mutations (Van Goethem *et al.*, 2004; Stewart *et al.*, 2010). The rapid advancements and lowering of costs in genomic analyses aid to mitigate these issues (Carroll *et al.*, 2014) and recent calculations report that sequencing young neurological patients early on is already faster, more efficacious and more cost-effective than traditional diagnostic paths (Aaltio *et al.*, 2022).

Finally, the broad range of mitochondrial disease presentations suggests that different pathomechanisms, factors and/or tissue sensitivities are involved (Ylikallio & Suomalainen, 2012; Gorman *et al.*, 2016; Suomalainen & Battersby, 2018), and thus the effectiveness of promising therapies is likely to be just as varied. While certain therapies might affect shared responses and alleviate the symptomatology, it is unlikely that a one-for-all cure exists. Instead, treatments should be carefully tested for each disease and genetic defect, and disease-tailored approaches given support. While reports of unexpected harmful effects or negative results exist (Ahola *et al.*, 2016; Purhonen *et al.*, 2018; Blázquez-Bermejo *et al.*, 2019; Ignatenko *et al.*, 2020), the current scientific publishing system seldom supports such publications, so it can be assumed that many more attempts have failed inaudibly. It is crucial to take this into account and, whilst collectively fighting for healthier scientific publishing, be extremely careful when extrapolating results from a different species, disease, age, or genetic background.

3 AIMS OF THE STUDY

The general aim of this thesis is to investigate the impact of mtDNA integrity and amount in development and ageing, and their interaction with metabolism and disease. Specifically, the aims were as follows:

I. To decipher the molecular mechanism behind the stem cell defect in the mtDNA Mutator mouse and reconcile it with other premature ageing models.

II. To investigate the consequences of increased mtDNA replication and amount in combination with a mutagenic mtDNA polymerase.

III. To develop the state-of-the-art methodology for the effective measurement of deoxynucleoside triphosphates.

4 MATERIALS & METHODS

Due to the wide physiological scope of this thesis work, the list of methodologies is large, many methods benefitting from the expertise and talent of collaborators and co-authors. Table 1 below presents a summary of the main methodologies employed, and their details are presented in each of the original publications. In addition, the most crucial methods for this thesis are presented in more detail below.

4.1 List of Methodologies

Method	Article
Genetically modified mouse models	I, II
Tissue/cell culture	I, II, III
Nucleic acid purification (DNA & RNA)	I, II
Quantitative PCR (mtDNA, cDNA)	I, II
Protein extraction & immunoblot	I
Histological stains & analysis	I, II
Immunofluorescence	I, II
Fluorescent/confocal microscopy	I, II
Image analysis and quantification	I, II
Electron microscopy	II
dNTP quantitation (metabolite extraction & radioactive-labelled polymerase amplification)	I, III

Nucleotide analogue incorporation	I, II
DNA gel analysis (topological and 2-dimensional)	I, II
Flow cytometry	I
Analysis and integration of omics data (transcriptomics, metabolomics, proteomics, mtDNA deep-sequencing)	I, II
Statistical analyses	I, II, III

4.2 Ethical statements and licenses

All animal work was performed following the European Union Directives and the 3R principle and approved by the National Animal Review Board and regional State Administrative Agency for Southern Finland (permits: ESAVI/689/04.10.07/2015 & ESAVI/3686/2021).

4.3 Mouse models

MtDNA Mutator mice

The mtDNA Mutator mouse carrying a D257A amino acid change in the *Polg* gene was obtained from Prof. Prolla's group (Kujoth *et al.*, 2005). To avoid the inheritance of mtDNA mutations, the strain was maintained by crossing heterozygous males with wildtype 6BL/Rcc females. Heterozygous females were used exclusively to generate experimental groups with homozygous offspring, thus minimising the inheritance of mtDNA mutations to a single generation.

Twinkle overexpressor mice

The mice overexpressing murine Twinkle cDNA were generated in our lab (Tyynismaa *et al.*, 2004), leading to an increased mtDNA copy number. The transgene, located within an intron of the *Tmprss11d* gene, was expressed under a β-actin promoter (Ylikallio *et al.*, 2010).

Mutator Twinkle-overexpressor mice

We crossed Twinkle-overexpressing females with heterozygous $Polg^{D257A}$ males to obtain Twinkle-overexpressing $Polg^{D257A}$ heterozygous pups. The resulting offspring was then inter-crossed, generating litters containing $Polg^{D257A}$ wildtype, heterozygous and homozygous mice, with and without the Twinkle transgene.

4.4 Quantitation of dNTP pools

The isolation and quantitation of dNTP pools were performed using a novel radio-labelled solid-phase polymerase-based method [III & (Landoni *et al.*, 2021)].

Shortly, the metabolites were isolated from biological samples or mitochondrial pellets by physical homogenisation and cold 60% methanol extraction as described in (Martí *et al.*, 2012), ensuring the boiling is performed previous to the precipitation. Then, the solvent was vacuum evaporated, and the precipitate was redissolved in water.

The measurement was carried out in four separate reactions, one per canonical dNTP. A biotin-labelled template oligonucleotide specific for each dNTP was covalently bound to the streptavidin-coated wells (Figure 3.1) and the reaction mix added, which included a DNA polymerase and its buffer, a reducing agent, the extract, a DNA primer, and a radioactive dNTP in excess (usually tritium-labelled dATP or dTTP). The templates were designed to incorporate radioactive nucleotides proportionally to the target dNTP (Figure 3.2). By quantifying the radioactive counts from the newly synthesised DNA fragments (released by sodium hydroxide) and comparing them to those of a standard curve of reactions of known dNTP concentration, we could extrapolate the exact concentration of each dNTP (Figure 3.3).

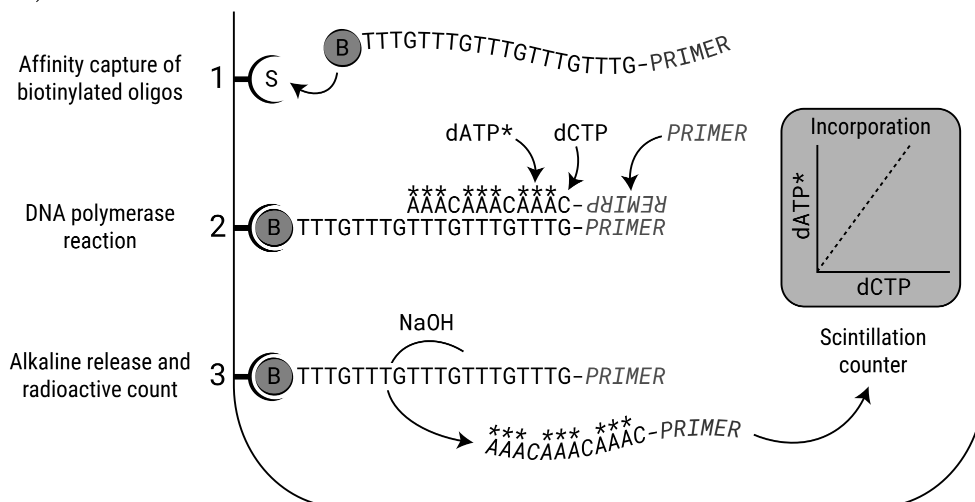

Figure 3: Graphical summary of the novel dNTP quantitation method, with dCTP as an example. **(1)** Affinity capture of the dCTP-specific biotinylated oligo into a streptavidin-coated plate. **(2)** DNA polymerase reaction in its optimised buffer, allowing for the proportional incorporation of the measured dNTP and the excess radioactive dNTP, dCTP and tritium-labelled dATP respectively in this case. **(3)** Sodium hydroxide denaturation and solubilisation of the newly synthesised strand and transfer of the solution into scintillation liquid for radioactive counting. Abbreviations: S, streptavidin; B, biotin. A*, tritium-labelled adenosine.

4.5 Statistical analyses

Statistical analyses and graphical representation were performed with MS Excel and R (R studio and ggplot2). Due to the relatively small number of observations, scatterplot representations were chosen to clearly visualise the distributions and allow the reader to directly judge the data. As most results compare the distribution between two groups (e.g. wildtype vs Mutator), pairwise comparisons are often performed and reported with a p-value from Student's t-test (Livingston, 2004). The details from each analysis can be found in the figure legends and in (I-III).

Larger datasets requiring specialised analyses were performed using established pipelines for each: RNA sequencing data was analysed using DESeq2 in R (Love *et al.*, 2014), metabolomics on MetaboAnalyst (Chong *et al.*, 2018), and mtDNA sequencing data were processed as in (Isokallio & Stewart, 2021). In addition, a myriad of freely-available online tools was employed for omics data filtering and exploration (Eden *et al.*, 2009; Raudvere *et al.*, 2019; Szklarczyk *et al.*, 2019; Ge *et al.*, 2020).

5 RESULTS

5.1 Disrupted cell cycle and replication stalling in Mutator mouse stem cells

Previous research from our lab and others revealed that the mtDNA Mutator mice, carrying a defective POLG, present attrition of their stem cell compartments, a typical feature of premature ageing mouse models (Carrero *et al.*, 2016). This included self-renewal and maturation defects in both somatic stem cells and induced pluripotent stem cells (iPSC) (Chen *et al.*, 2009; Norddahl *et al.*, 2011; Ahlqvist *et al.*, 2012; Wahlestedt *et al.*, 2014; Hämäläinen *et al.*, 2015). Premature ageing has not been observed in other mitochondrial dysfunction models or diseases, so why and how this particular insult causes premature ageing has been an outstanding question in the field.

Using iPSC as an *in vitro* model for highly replicative cells, we sought to clarify the molecular mechanisms behind the Mutator stem cell defect, starting with the proliferation phenotype. When compared to wildtype cells, Mutator iPSC showed a disturbed cell cycle (Figure 4A) and a deceleration of nuclear DNA replication (Figure 4B), as measured by propidium iodide staining and the incorporation of thymidine analogues (CldU & IdU) into DNA strands respectively. A well-characterised consequence of replication fork stalling is DNA double-strand breaks (Kaushal & Freudenreich, 2019). The increased phosphorylation levels of known DNA damage markers in the cells were consistent with this premise (Figure 4C).

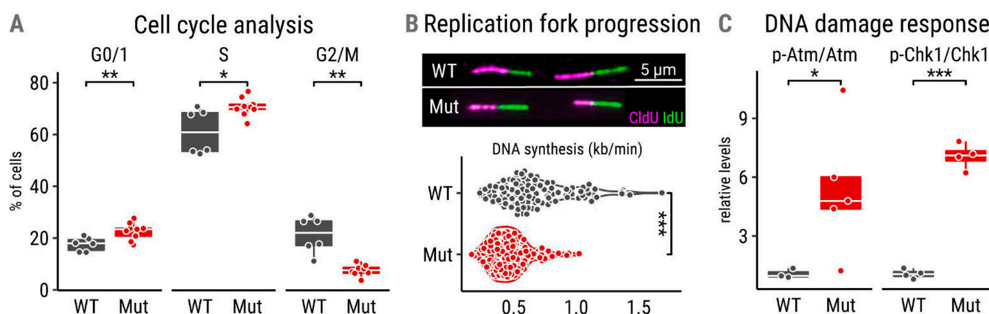

Figure 4: Mutator iPSC cell cycle and nuclear DNA replication disruption (A) Cell cycle analysis of iPSC by propidium iodide staining and fluorescent flow cytometry. **(B)** Nuclear replication fork progression speed analysis on DNA fibres by quantification of nucleotide analogue incorporation. **(C)** Quantification of phosphorylated and total ATM-kinase and Chk1-kinase immunoblots. P values from a two-tailed t-test with $p<0.05$ as a significance threshold. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

As a mitochondrial polymerase, the POLG defect was expected to only affect mtDNA replication (Krasich & Copeland, 2017). However, our results excitingly and unexpectedly indicated that the mutator allele can challenge nuclear DNA replication and integrity.

5.2 Nuclear DNA damage in Mutator stem cells *in vitro* and *in vivo*

To corroborate the DNA damage observation, we studied the phosphorylation of histone H2AX (γH2AX), an established marker for DNA double-strand breaks (Mah *et al.*, 2010). Immunostaining and flow cytometry quantification of γH2AX revealed a significant increase of double-strand breaks in iPSC (Figure 5A).

We then sought to test these results *in vivo* by studying DNA damage in mouse testes, in the highly proliferative sperm cell progenitors. Male infertility is a known phenotype of mutators (Trifunovic *et al.*, 2004) and patients carrying certain *POLG1* mutations (Rovio *et al.*, 2001; Luoma *et al.*, 2004). In addition to the expected disruption of the seminiferous tubule ultrastructure in Mutators, co-staining of γH2AX and PCNA (a marker for proliferating cells) revealed a striking increase in DNA damage foci, specifically in replicating cells (Figure 5B).

Taken together, the results suggest an intriguing process by which an insult to mitochondrial DNA can threaten nuclear DNA stability.

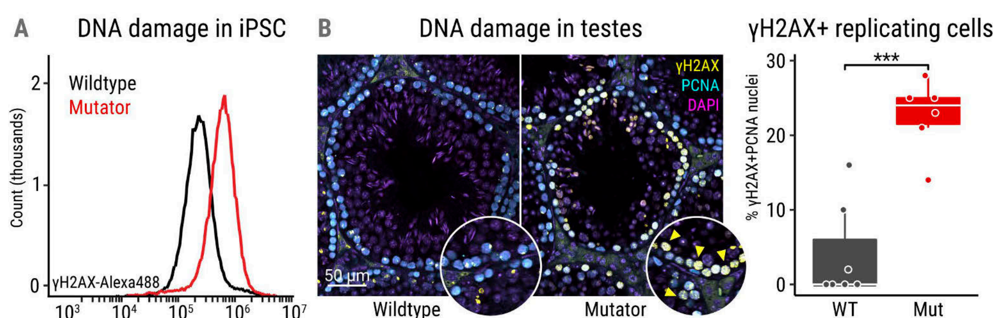

Figure 5: Nuclear DNA double-strand breaks *in vitro* and *in vivo* (A) γH2AX staining quantification per cell by flow cytometry, representative histogram, three independent experiments, P = 0.0002. **(A)** Representative images and quantification of γH2AX (yellow, marking double-strand breaks) and PCNA (cyan, marking replicating cells) staining in testes. Arrowheads indicating γH2AX foci. P values from a two-tailed t-test with $p<0.05$ as a significance threshold. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

5.3 mtDNA over-replication and nucleotide metabolic shift

A classical cause for nuclear replication stress and cell-cycle stalling is the incorrect or imbalanced availability of dNTPs at the replication fork (Reichard, 1988; Mathews, 2006), the building blocks shared by both genomes. In addition, we and others have reported that primary mitochondrial defects can affect whole-cellular dNTP metabolism (Bao *et al.*, 2016; Dalla Rosa *et al.*, 2016; Nikkanen *et al.*, 2016). We thus asked whether nucleotide metabolism could be the causal link between the defects.

RNA sequencing analysis of iPSC revealed an intriguing pattern: the cytosolic pathways for dNTP synthesis appeared to be downregulated, which was consistent with the predicted nucleotide depletion in the nucleus. However, the mitochondrial salvage pathway was induced (Figure 6A), suggesting the prioritisation of mtDNA replication despite a whole-cellular deficiency.

To further confirm this, we quantified the total cellular dNTP pools, as well as those from isolated mitochondria. In agreement with the transcriptomic data and the nuclear dNTP deficiency hypothesis, the total dNTP pools were generally lower in Mutator iPSC, particularly dTTP concentrations (Figure 6B).

The opposite trend was detected in mitochondrial dNTP pools, with higher concentrations of dNTPs in Mutator mitochondria than wildtype (Figure 6B). This organelle-specific regulation of dNTPs is rather unexpected, as mitochondrial and cytosolic dNTP pools are thought to closely correlate in normal replicating cells (Gandhi & Samuels, 2011).

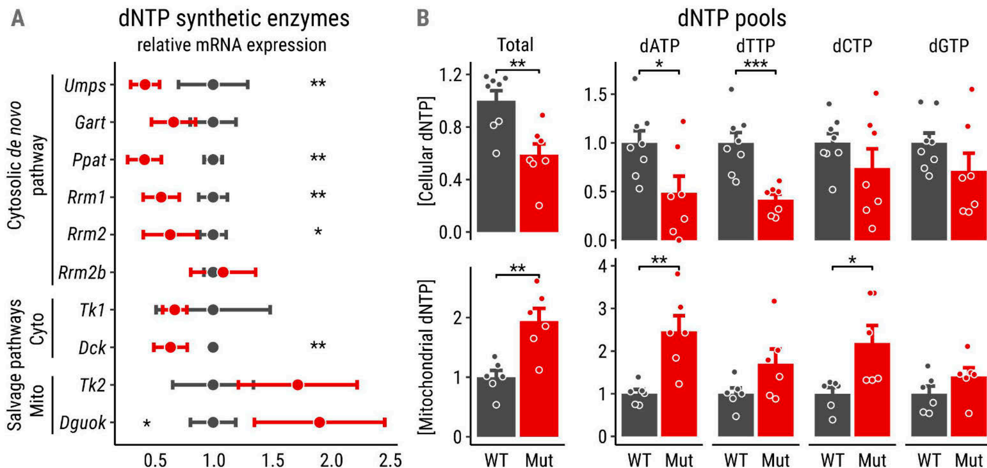

Figure 6: Mitochondrial restructuring of nucleotide metabolism (A) Relative expression levels of nucleotide metabolic enzymes, classified as *de novo* and two salvage pathways. Mutator in red, wildtype controls in grey. **(B)** Relative whole-cellular (top) and mitochondrial (bottom) dNTP pools. P values from a two-tailed t-test with $p<0.05$ as a significance threshold. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

The intriguing dNTP rewiring was suggestive of an augmented requirement of nucleotides in mitochondria, which could be caused by an increased mtDNA replication frequency by the more rapid Polg^{D257A} (Macao *et al.*, 2015). We tested this by labelling newly synthesised mtDNA with the thymidine analogue BrdU, and quantifying its signal against an mtDNA hybridization probe, obtaining the ratio between new and total mtDNA. This "South-Western" analysis revealed that Polg^{D257A} leads to increased mtDNA replication frequency (Figure 7), implying an increased consumption of dNTPs in mitochondria. Interestingly, the change in replication also occurred without significant variation in total mtDNA copy number, hinting at yet undiscovered turnover mechanisms.

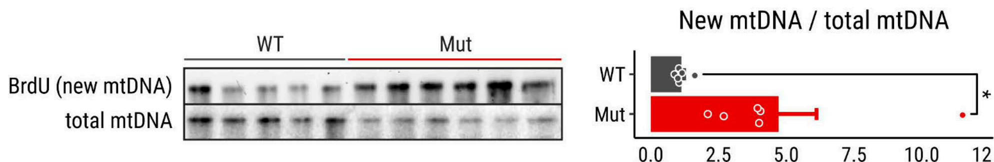

Figure 7: Increased mtDNA processivity by exo-deficient POLG. South-western blot measuring total mtDNA and incorporation of the nucleotide analogue BrdU, and quantification of the BrdU/total mtDNA ratio indicating novel mtDNA replication. P values from a two-tailed t-test with $p<0.05$ as a significance threshold. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

The data indicated that an unknown sensing mechanism in mitochondria, possibly related to dNTP consumption or mtDNA replication, was miscommunicating dNTP availability to the nucleus. In turn, this signal would be triggering a deleterious adaptation where mitochondrial dNTPs were prioritised, and cytosolic biosynthesis was hampered.

5.4 TFAM overexpression can rescue the Mutator stem cell DNA damage

To further assess the causality between mtDNA replication and nuclear damage in iPSC, we overexpressed the mtDNA-binding and transcription factor TFAM. TFAM is known to increase mtDNA packaging, thus inhibiting/decelerating its replication (Farge *et al.*, 2014; Brüser *et al.*, 2021). Short-term TFAM overexpression resulted in the reduction of γH2AX signal in the nucleus of Mutator iPSC to wildtype levels (Figure 8A), strongly supporting a causative link between mtDNA replication and double-strand breaks in the nuclear genome.

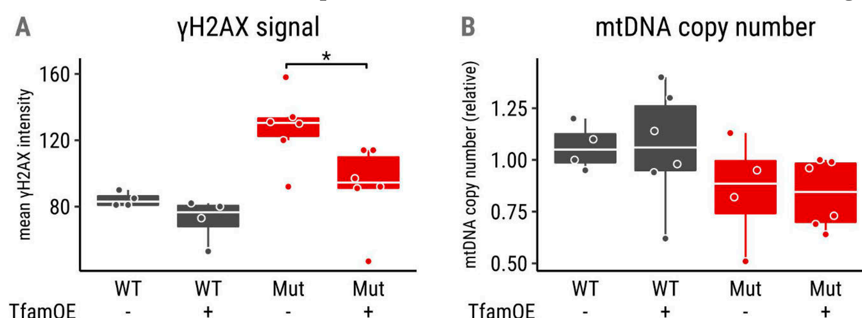

Figure 8: TFAM rescues DNA damage without changing mtDNA copy number. **(A)** Mean intensities of γH2AX flow cytometry in arbitrary units from control and *TFAM*-overexpressing iPSC. **(B)** Relative mtDNA copy number of control and *TFAM*-overexpressing iPSC. P values from a two-tailed t-test with $p<0.05$ as a significance threshold. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

This result joins the body of evidence associating TFAM overexpression with beneficial outcomes for mitochondrial defects (Jiang *et al.*, 2017; Filograna *et al.*, 2019). In contrast with the published interpretation, the effects cannot be attributed to an increase in total mtDNA amount (Figure 8B). Instead, it suggests the involvement of mtDNA replication modulation and/or other TFAM functions in the mechanism.

5.5 A unifying mechanism for mouse premature ageing

Based on the observed phenomena we propose a model in which, in addition to accumulating point mutations, the increased processivity of Polg^{D257A} increases replication and/or dNTP consumption. This in turn affects dNTP homeostasis in replicating cells, causing a nuclear dNTP deficiency and consequential replication stalling and DNA damage.

Taken together, the data redefine the mutator mouse as a secondary nuclear DNA damage model, thus reconciling the mechanisms for murine premature ageing under nuclear genomic instability (Carrero *et al.*, 2016; Schumacher & Vijg, 2019). Additionally, it reopens the necessity of conclusive experimental evidence for the contribution of mtDNA mutations in ageing.

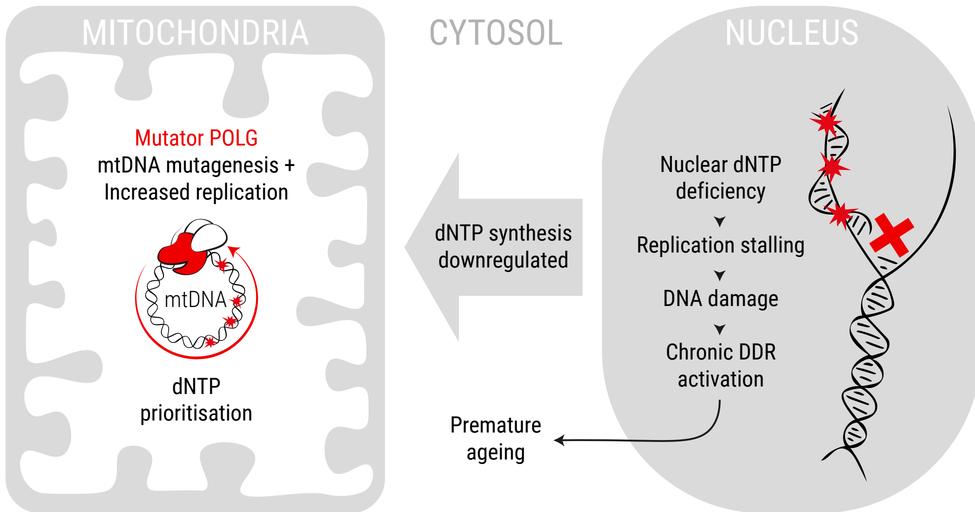

Figure 9: Model for mtDNA mutator premature ageing mechanism. Graphical summary of the proposed model for exonuclease-deficient Polg -mediated progeroid syndrome in replicating cells. In addition to mtDNA mutagenesis, the POLG defect causes increased mtDNA replication. This leads to an imbalance in nucleotide metabolism, whereby mitochondrial dNTP pools are prioritised, and non-mitochondrial pathways are downregulated. The consequential dNTP deficiency in the nucleus leads to nuclear replication fork stalling and triggers the cascade of DNA damage and its responses that characterises all progeroid syndromes.

5.6 Overexpressing Twinkle helicase increases mtDNA copy number by replication

Our results from replicating cells strongly supported the increased mtDNA replication activity by Polg^{D257A} to be causative for the nuclear genomic damage. To test this connection further, we designed a mouse crossing that would further increase the replication rate of mtDNA.

Twinkle is essential for mtDNA replication initiation, so its expression correlates with the number of mtDNA copies and amount of mtDNA replication (Tyynismaa *et al.*, 2004; Ylikallio *et al.*, 2010). Therefore, by overexpressing Twinkle in mutators, expected to further increase mtDNA replication, and hypothesise that if increased replication is indeed deleterious, the mice would present with an acceleration of the progeroid phenotype.

By crossing the Mutator line with the Twinkle-overexpressor (TwOE) line, we obtained litters with all Mutator (Polg^{D257}) genotypes: *Polg*$^{wt/wt}$, *Polg*$^{wt/D257A}$ and *Polg*$^{D257A/D257A}$ with and without the Twinkle transgene (Figure 10A).

The Twinkle-overexpressing groups presented a marked increase in both Twinkle mRNA (Figure 10B) and a 2-3-fold increase in mtDNA copy number (Figure 10C). Quantification of nucleoid replication by EdU incorporation and DNA immunostaining from embryo-derived fibroblasts also confirmed the expected higher proportion of replicating nucleoids and their increase in size caused by Twinkle-overexpression (Figure 10D-F), corroborating that the system is working as anticipated.

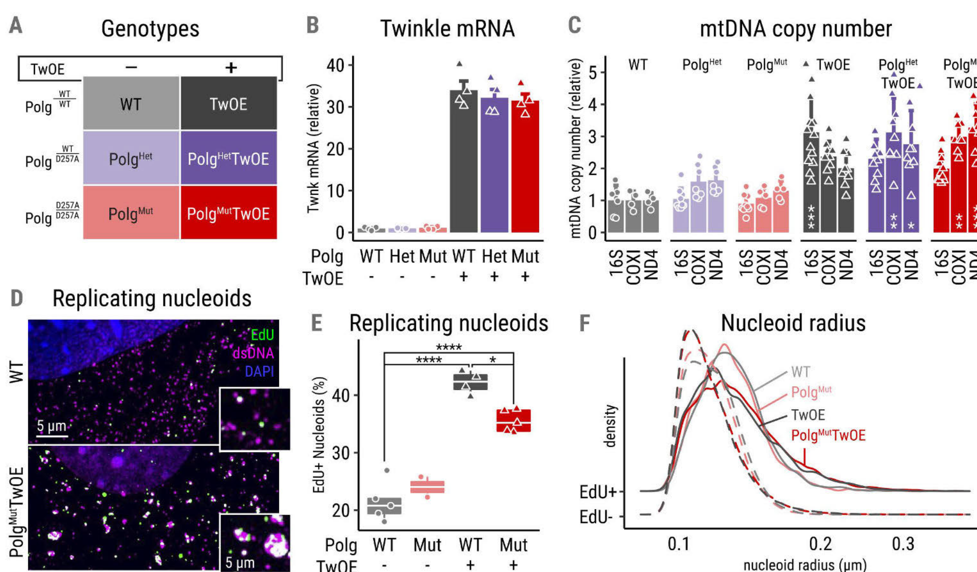

Figure 10: Increasing mtDNA copy number by replication in mice. **(A)** Array of genotypes in the cohort, showing the three possible Polg^{D257A} genotypes and their Twinkle-overexpressing counterparts. **(B)** Relative amount of Twinkle mRNA in the heart at birth. **(C)** MtDNA copy number in the heart at one week, probing three genes across mtDNA (16S, COXI and ND4) against the nuclear gene RBM15, relative to wildtype control. P values from Kruskal-Wallis/Dunn's test with Bonferroni correction, comparing each group to wildtype controls. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). **(D)** Representative fluorescent microscope image of embryonic fibroblasts stained with an antibody against double-stranded DNA (dsDNA, nucleus and nucleoids, magenta), EdU (replicating mtDNA, green) and DAPI (nucleus). **(E)** Quantification of nucleoids in replication (EdU+) compared to total nucleoid number. P values from ANOVA/Tukey's HSD test; * ($p < 0.05$), **** ($p < 0.0001$). **(F)** Quantified nucleoid radius distribution by genotype and by replication status (EdU+ as full lines, EdU- as dashed lines), showing bigger nucleoids in replicating nucleoids of Twinkle-overexpressing genotypes.

5.7 Enhanced replication in the mutator background causes fatal neonatal heart failure

Both parental lines present late-onset phenotypes: Mutator homozygotes show symptoms at 6 to 8 months of age (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005), while Twinkle-overexpressors have no visible phenotype and normal lifespan with mild OXPHOS deficiency only after 2 years (Ylikallio *et al.*, 2010; Pohjoismäki *et al.*, 2013a). Consistently, our experimental litters were born normal (Figure 11A).

Strikingly, however, the double transgenic mice rapidly developed a clear growth defect during the first week of life, dying shortly after (Figure 11B & J). The growth defect was associated with enlarged hearts (Figure 11C—E & K), which echocardiographic analysis revealed to be severe heart failure due to dilated cardiomyopathy (Figure 11F-I).

Structurally, the hearts presented severe infarction-like pathology with endomyocardial fibrosis, vacuolation, degenerating myofibrils, enlarged cardiomyocytes and larger and less abundant nuclei, suggestive of a cardiomyocyte maturation defect (Figure 11 & III).

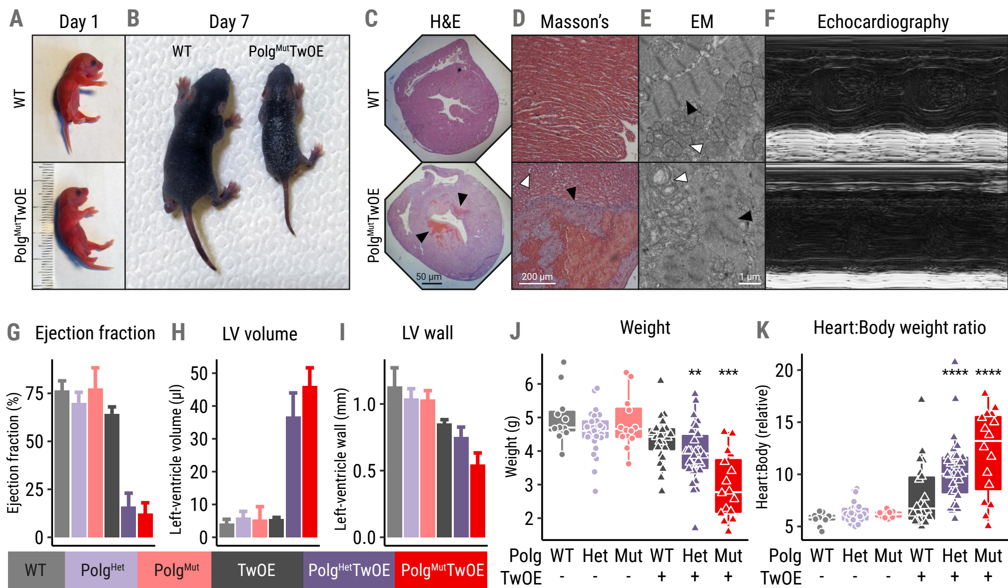

Figure 11: Twinkle overexpression in combination with mutator POLG associates with neonatal heart failure. **(A-B)** Representative images of WT and Polg^{Mut}TwOE mice at postnatal day 1 (P1, A) and P8 (B), showing the progressive growth defect. **(C-F)** Representative images of P7 heart characterisation: **(C)** Haematoxylin/eosin staining showing infarction-like pathology (black arrowheads). **(D)** Masson's trichrome staining showing myocardial disarray, vacuolation (white arrowheads) and interstitial fibrosis (black arrowheads). **(E)** Electron micrograph depicting the complete degradation of the myofibrillar structure (black arrowheads), and the disruption of mitochondrial membrane ultrastructure (white arrowheads). **(F-I)** Echocardiography and its quantification, indicating dilated cardiomyopathy and severe heart failure in Polg^{Het}TwOE and Polg^{Mut}TwOE. **(J)** Weight of the mice in grams at P7. **(K)** Heart-to-body weight ratio at P7. P values from Kruskal-Wallis/Dunn's test with Bonferroni correction, comparing each group to wildtype controls unless specifically indicated. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$).

5.8 The cardiac failure is dose-responsive to Polg^{D257A} alleles and affects other organs

Mice carrying a single Polg^{D257A} (heterozygotes) present virtually no phenotype, as a single exonuclease-proficient *Polg* allele is sufficient to fulfil most functions and maintain a low mutation and deletion load (Vermulst *et al.*, 2007; Edgar & Trifunovic, 2009). In contrast, the Twinkle-overexpressing Polg^{D257A} heterozygotes (Polg^{Het}TwOE) in our cohort had a considerable growth and heart phenotype, the severity and incidence of which were dose-responsive to the number of Polg^{D257A} alleles (Figure 11J-K).

To evaluate whether the previously unveiled mutator mechanism could play an accelerated role in this disorder, we assessed proliferation and nuclear DNA damage from replicating (testes, skin) and post-mitotic (heart) tissues. No differences were detected in proliferation markers or γH2AX foci (II), implying that the pathomechanism in the neonatal heart was different and more acute than the chronic nuclear DNA stress observed in the replicating cells of older mutators (I-II).

Of other organ systems, heart function is closely connected with that of lung and liver, and they can affect one another when dysfunctional (Kee & Naughton, 2010; Møller & Bernardi, 2013). Indeed, the double transgenic mice showed severely fatty liver and disrupted alveoli

in the lungs. Nonetheless, further analysis revealed the onset of the damage is posterior to that of the heart and neither lung nor liver present changes in mtDNA copy number at one week of age, all suggesting that their damage is likely secondary to the cardiac failure (II). A mild decrease in haemoglobin and glucose concentrations was also detected in blood (II), the first of which overlaps with the mutator phenotype. However, as they are known consequences of a failing heart (Stanley *et al.*, 2005; Anand & Gupta, 2018), they are also likely secondary.

5.9 Increasing mtDNA replication intensifies the likelihood of mtDNA damaging events

As the major consequence of the proofreading-deficient Polg^{D257A}, we asked whether increased replication could be exacerbating the damage to mtDNA (mutagenesis or other) and possibly causing the defect.

We first characterised the landscape of mtDNA point mutations, the best-known feature of mutators. Using deep-sequencing on enriched mtDNA we found that Twinkle overexpression did significantly increase the mutation rate in both Polg^{Mut}TwOE and Polg^{Het}TwOE mice compared to their respective Polg^{Mut} and Polg^{Het} controls (Figure 12A). However, the extent of the increase could not explain the severity of the cardiac phenotype. Notably, because the mutation load of asymptomatic Polg^{Mut} was much higher than that of Polg^{Het}TwOE with failing hearts. Additionally, the mutation heteroplasmy levels were comparably low across all genotypes (Figure 12B). The majority of mutations enriched in Polg^{D257A}-carriers were T or A misincorporations (C:G>T:A and A:T>T:A) (Figure 12C), indicating also that the bulk of mutations arose directly from POLG function (Johnson & Johnson, 2001).

The quantitation of different genes across mtDNA revealed potential disruptions in mtDNA integrity, as the relative amounts of the target genes were variable among the Twinkle-overexpressing genotypes (Figure 1C). Indeed, DNA gel analysis confirmed this observation and revealed other mtDNA maintenance irregularities. The accumulation of linear fragments spanning between the two replication origins of mtDNA is a known occurrence in mutator homozygotes, as its degradation requires the exonuclease activity of POLG (Nissanka *et al.*, 2018). Consistent with enhanced replication, we observed an increase of those species, as well as other rare events of replication interruption (Figure 12D). For further detailed data please see (II).

In summary, our data reflect that an increase in replication events augments the probability of mtDNA damaging incidents to occur, leading to the accumulation of their products (point mutations, linear deletions and other abnormal mtDNA species). Nonetheless, the lack of significant mtDNA disruptions detected in Polg^{Het}TwOE despite their clear disease presentation suggests that mtDNA damage is unlikely to be the primary cause of the defect.

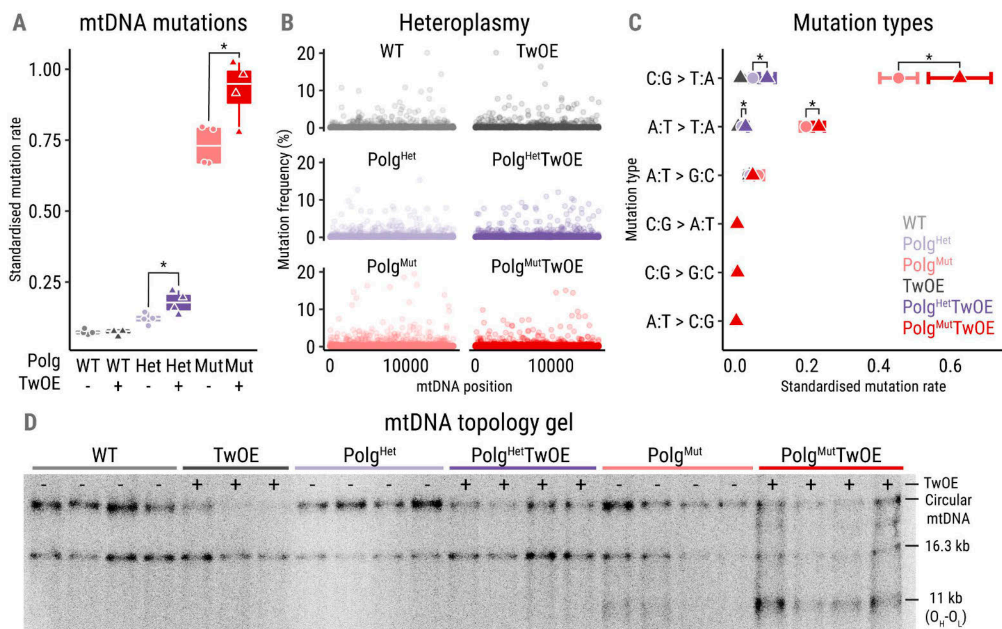

Figure 12: Twinkle overexpression exacerbates the mtDNA damage associated with Mutator POLG. **(A)** Standardised mutation rate quantified from mtDNA deep sequencing; total number of mtDNA mutations detected standardised to the occurrence of each base. **(B)** Detected point mutations across mtDNA and their frequency (heteroplasmy) as a percentage of the total reads for that locus. **(C)** Mean standardised mutation rate by substitution type, with SEM. **(D)** MtDNA topology; Southern hybridization analysis showing the different topological species of mtDNA present in the sample. P values from a two-tailed t-test contrasting groups with identical POLG genotype. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

5.10 Multi-omic analysis reveals perinatal signalling and metabolic remodelling

To unbiasedly dissect the molecular signals triggering the heart failure, we performed omics analyses from perinatal hearts. The first week of life is an extraordinarily active stage for the heart, as it must rapidly adapt to oxidative metabolism and to a remarkable increase in size and energetic demand (Pohjoismäki & Goffart, 2017; Lalowski *et al.*, 2018). To capture a full snapshot of the pathways affected, we performed metabolomic and proteomic analyses from the hearts at day 1. The proteomic analysis resulted in a large number of significantly affected hits, where all the critical processes required for normal heart maturation were burdened (Figure 13A-C).

The analysis revealed a typical mitochondrial integrated stress response (ISR^{mt}) signature, a recently described pseudo-anabolic reaction to different mitochondrial stressors and diseases (Tyynismaa *et al.*, 2010; Dogan *et al.*, 2014; Nikkanen *et al.*, 2016; Suomalainen & Battersby, 2018; Forsström *et al.*, 2019). It is characterised by a strong induction of *de novo* serine biosynthesis (PHGDH, PSPH, PSAT1), the mitochondrial folate cycle (MTHFD2, MTHFD1L) and asparagine and proline metabolism (ASNS, PYCR1), among others. Cytosolic translation was also remarkably induced, indicated by the upregulation of cytosolic aminoacyl tRNA synthetases, amino acid transporters, and the subunits and assembly factors of the ribosome.

The mitochondrial translation machinery, on the other hand, appeared downregulated. In fact, ~50% of all detected mitochondrial proteins classified by MitoCarta3.0 (Rath *et al.*, 2021) were significantly affected, most of which downregulated (Figure 13C). Mitochondrial respiratory chain subunits were among the most affected, the subunits of complex I, III and IV in particular; denoting defective oxidative phosphorylation (Figure 13D), alongside a downregulation of coenzyme Q biosynthesis. Fatty acid oxidation was another crucial adaptation for mature cardiomyocytes which was downregulated or unable to be induced.

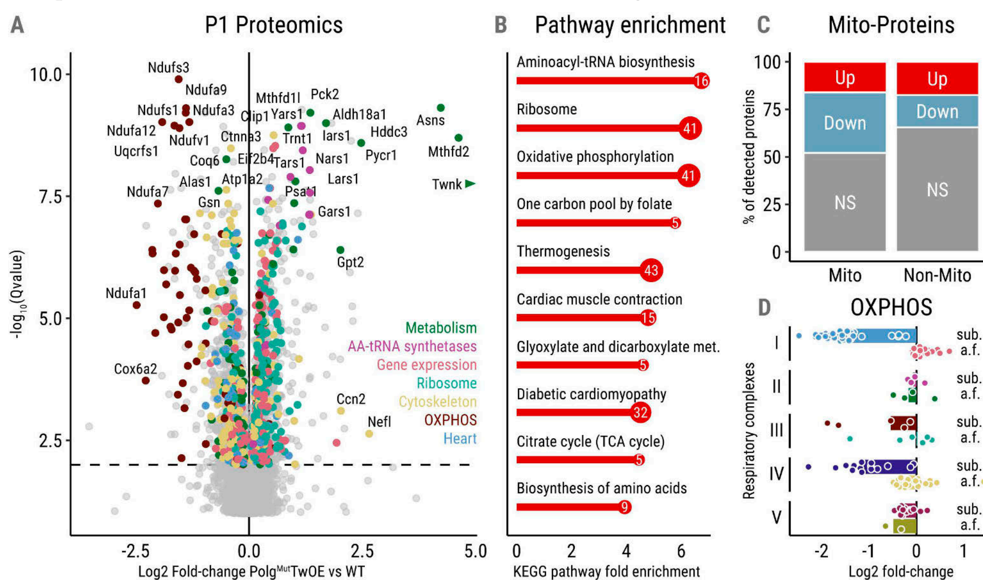

Figure 13: Proteomic changes in newborns. **(A)** Volcano plot depicting the fold-change and statistical significance of the proteomic changes between WT and Polg^{Mut}TwOE groups. The dotted line indicates the significance threshold at Q<0.01 and changed hits are categorised into groups. *Twnk* arrowhead indicates the position of the cropped datapoint. **(B)** Enriched KEGG pathways among the proteomic hits, fold enrichment on the x-axis and log10 p-value in the lollipop plot. **(C)** Percentage of changed hits classified according to MitoCarta. **(D)** Changes in respiratory chain subunits (sub.) and assembly factors (a.f.) by respiratory complex.

Interestingly, genes classified by the KEGG database (Kanehisa *et al.*, 2016) as involved in cardiomyocyte contraction and the cytoskeleton were downregulated, and markers of cardiomyopathy were induced. This suggested that the cardiac damage signalling is occurring before the onset of visible damage and either acutely induced upon birth or already occurring prenatally.

In contrast to the proteomic results, the metabolomic analysis revealed just a handful of significant hits (Figure 14A-B). Upon pathway contextualisation, a common pattern emerged: the deficiency of metabolites requiring cysteine for their synthesis such as coenzyme A, taurine and glutathione (Stipanuk *et al.*, 2006), alongside the accumulation of metabolites upstream from cysteine, such as cystathionine and D-pantothenate. In addition, we found an enrichment of metabolites strongly dependent on redox balance for their catabolism, such as proline, pipecolic acid and sarcosine (Campbell *et al.*, 1997; Dodt *et al.*, 2000). The results thus highlighted cysteine metabolism as a relevant pathway to address, with a deficiency of metabolites crucial for cell and cardiac function triggered by TwOE (Figure 14B-C).

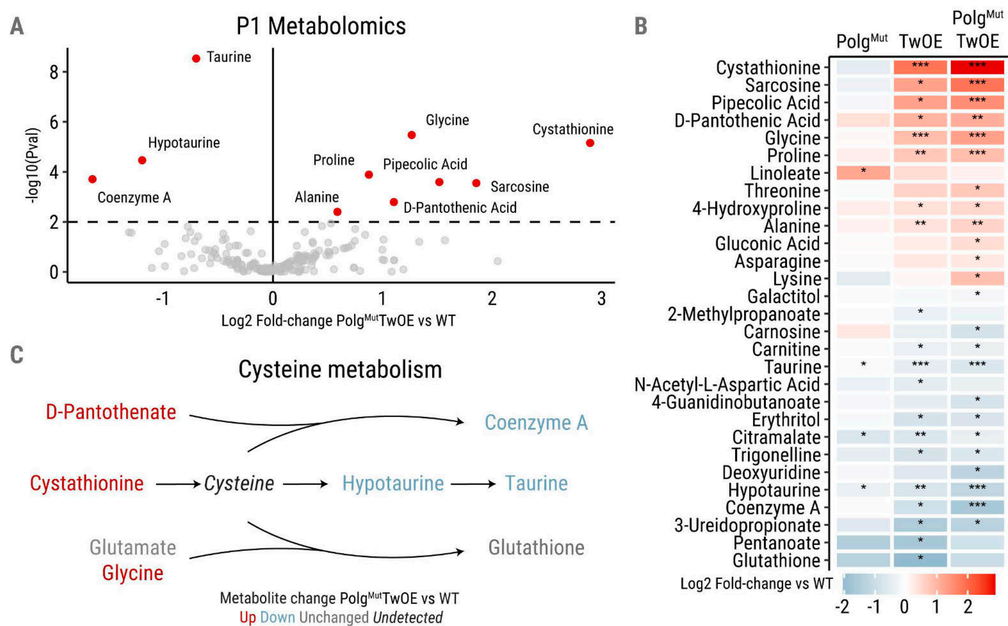

Figure 14: Metabolomic changes in newborns. **(A)** Volcano plot depicting the fold-change and statistical significance of the metabolite's change between WT and Polg^{Mut}TwOE groups. The dotted line indicates the significance threshold at p<0.01 and changed hits are highlighted in red and named. **(B)** Heatmap displaying the Log2 fold change of the significantly affected metabolites across all measured genotypes. P values from a two-tailed t-test as stars: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). **(C)** Simplified cysteine metabolic pathway integrating and depicting some of the significant changes from the metabolomic analysis.

We then asked whether some of these changes were already affected prenatally and could be hampering normal cardiomyocyte metabolic development. Thus, we collected hearts at embryonic day 16.5 and analysed the transcriptome of all genotypes in the cohort.

The resulting profile (Figure 15A-B) revealed a more modest response than the proteomic analysis, characterised by a clear upregulation of the ISR^{mt} and cytosolic tRNA synthetases, with ATF5 as the induced and likely effector of the response. Genes involved in heart contraction and extracellular collagen matrix maintenance were enriched among the downregulated genes, indicating changes to myocardial structure were already taking place during late embryogenesis. Thrillingly, the magnitude of the stress response closely correlated with the severity of the heart damage, seemingly triggered by Twinkle-overexpression and dose-responsive to *Polg*^{D257A} alleles (Figure 15C-D).

While the potential damaging or protective roles of ISR^{mt} remain a subject of debate (Suomalainen & Battersby, 2018; Forsström *et al.*, 2019; Kaspar *et al.*, 2021), its induction does indicate that mitochondrial stress signalling is occurring prenatally and before the onset of macroscopic damage, in a pattern that is consistent with the defect's incidence and severity.

Figure 15: Transcriptomic changes in E16.5 embryos. (A) Volcano plot depicting the fold-change and statistical significance of the transcriptomic changes between WT and Polg^{Mut}TwOE groups. The dotted line marks the significance threshold at $p<0.01$ and changed hits are categorised into groups. *Twnk* arrowhead indicates the position of the cropped datapoint. **(B)** Enriched KEGG pathways among the transcriptomic hits, fold enrichment on the x-axis and log10 p-value in the lollipop plot. **(C)** Example plots of the landscape of change in ISR^{mt}-related genes across all genotypes. **(D)** Venn diagram of the overlap in significantly changed genes across the genotypes. Specific groups highlighted and detailed as lists.

5.11 Ferroptotic cell death induced by mitochondrial dysfunction in the heart

The intriguing dose-responsiveness of the molecular and macroscopic defects to Polg^{D257A} alleles prompted us to seek a possible susceptibility factor that may respond only to Polg^{D257A} dosage. In other words, what increases proportionally with Polg^{D257A} that could make cardiomyocytes vulnerable to damage arising from increased replication. To find such a candidate, we returned to the transcriptomic data and searched for transcripts significantly affected in both Polg^{Mut} controls and Polg^{Mut}TwOE mice. This analysis revealed a clear strong hit: *Hddc3* (encoding for MESH1) (Figure 16A), a recently described cytosolic NADPH phosphatase that drives ferroptosis (Ding *et al.*, 2020). *Hddc3* was among the two differentially expressed genes shared by all Polg^{D257A} carriers (Figure 15D), and its expression showed a near-perfect dose-responsive expression pattern to Polg^{D257A} and complete independence to Twinkle in all genotypes, and a similar upregulation in Mutators at the protein level (Figure 16B-C).

As a newly discovered process of regulated cell death (Cao & Dixon, 2016; Chen *et al.*, 2021), ferroptosis is not as readily recognised by *in silico* prediction software as well-established pathways. Nonetheless, comparing manually curated databases (Kanehisa *et al.*, 2016; Zhou & Bao, 2020) with our proteomics data revealed a strong proportion of ferroptotic markers to be significantly affected in the neonatal hearts (Figure 16D-E). This included a pattern of mostly upregulated markers and downregulated inhibitors of ferroptosis. Combined with the metabolomic hits indicating increased utilisation of cysteine towards glutathione and dysregulation of redox-dependent reactions, the data suggests a response to increased lipid ROS (Figure 14B & 16E).

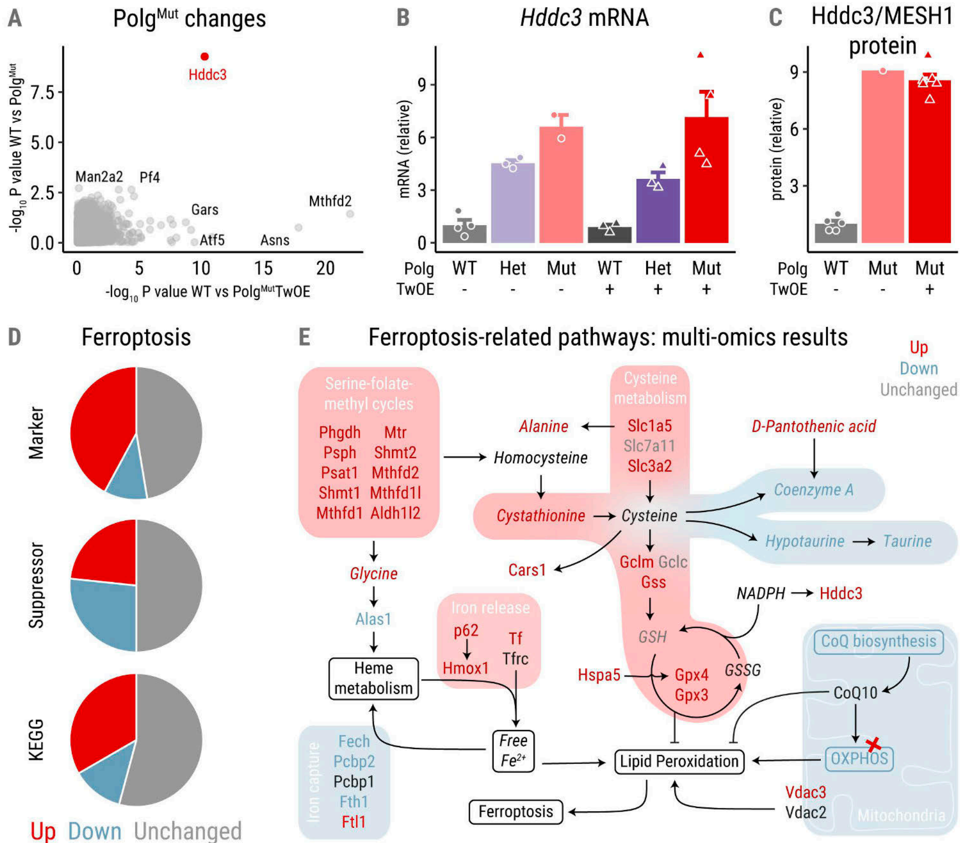

Figure 16: Ferroptosis involvement in Polg^{Mut}TwOE molecular pathogenesis. **(A)** Scatterplot comparing the statistical significance of the change in Polg^{Mut} and Polg^{Mut}TwOE groups (against WT), indicating *Hddc3* as a unique similarly changed gene candidate. **(B-C)** Relative amounts of *Hddc3*/MESH1 mRNA (B) and protein (C) in different genotypes, showing dose-responsiveness to Polg^{D257A} alleles. **(D)** Pie charts showing the proportion of proteins marked as ferroptosis marker or suppressor (FerrDb), or in the KEGG ferroptosis pathway, found significantly (Qval < 0.01) upregulated (red) or downregulated (blue) or non-significantly changed (grey) in Polg^{Mut}TwOE. **(E)** Main pathways involved in ferroptosis, overlaid with the change detected by proteomics (normal font) and metabolomics (italics) with the same colour scheme as (D) and black as not measured/detected.

Taken together, the identification of *Hddc3* responsiveness to Polg^{D257A} and the integration of omics analyses fulfils the criteria for ferroptotic pathway induction and indicate that Polg^{D257A} carriers are primed to ferroptosis in the heart. While *Hddc3* upregulation is likely innocuous or even beneficial alone, its combination with TwOE and/or ISR^{mt} can trigger ferroptosis, hamper normal cardiomyocyte metabolic maturation and lead to heart failure.

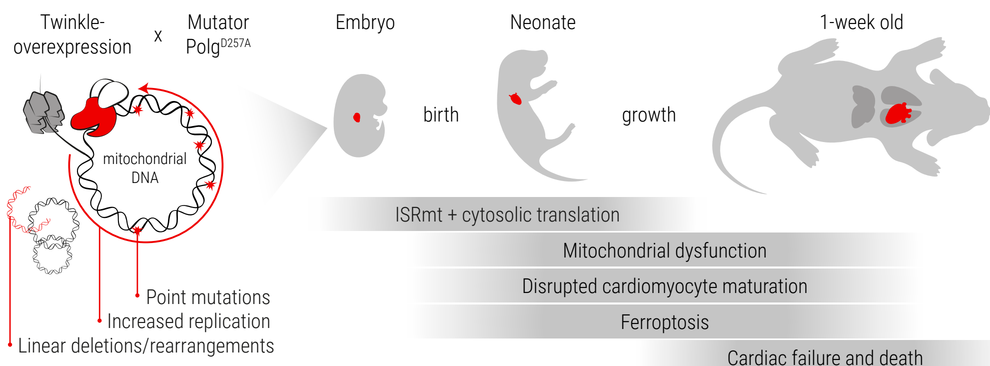

Figure 17: Graphical summary of the Polg^{Mut}TwOE outcomes. On the left, the protein players acting on mtDNA: Twinkle helicase being overexpressed, and exonuclease-deficient POLG replicating. In red, the molecular consequences of the alteration: point mutation accumulation, enhanced replication and generation of linear deletions and other mtDNA rearrangements. On the right, the timeline of affected systems and detected onsets, with the damaged organ systems highlighted (heart in red, lungs and liver in dark grey).

5.12 Development of a new dNTP quantitation method

The third project in the thesis arose from the technical requirements of project I. As previously described, the state-of-the-art methodology for dNTP pool measurement has long been a radio-labelled polymerase-based reaction, widely used in the fields of nucleotide metabolism and mitochondrial disease research. Despite its high sensitivity, several features had room for improvement. In addition, the field had been hampered by the commercial discontinuation of specific materials required, motivating the further development of the method. We thus decided to develop an adaptation of the method, inspired by a previously published minisequencing protocol (Suomalainen & Syvänen, 1996, 2003) used to determine mtDNA heteroplasmy and allele frequencies from pooled DNA samples.

The major innovation was the adaptation to a solid-phase setting, permitting the performance of the reaction and washing steps in the same well and enabling the use of multi-channel and automated equipment, allowing for parallelisation and markedly accelerating the method's performance. In addition, other improvements were made in the extraction process (optimisation of the boiling and centrifugation steps), new biotinylated oligos and longer primer, the use of a high-fidelity thermostable polymerase with its optimised buffer. Many laborious steps were in consequence combined or omitted, such as a separate primer annealing incubation and the transfer of individual reaction mixtures onto filter papers and their manual handling.

Along with significantly reducing the time and manual work required, the method diminished the potential sources of error and sample degradation arising from extra volumetric measurements, physical changes of the mixture and manual handling, and minimised the operator's exposure to hazardous and radioactive compounds.

The resulting new protocol is described in (III) and developed further in (Landoni *et al.*, 2021). It produced replicable results from an array of cell lines (Figure 18A), as well as from animal tissues and fractionated mitochondrial pools [(Landoni *et al.*, 2021) & Figure 18B], and generated comparable results to the traditional methodology, with a tendency to higher absolute amounts possibly due to the optimised handling of the unstable metabolites (Figure 18B).

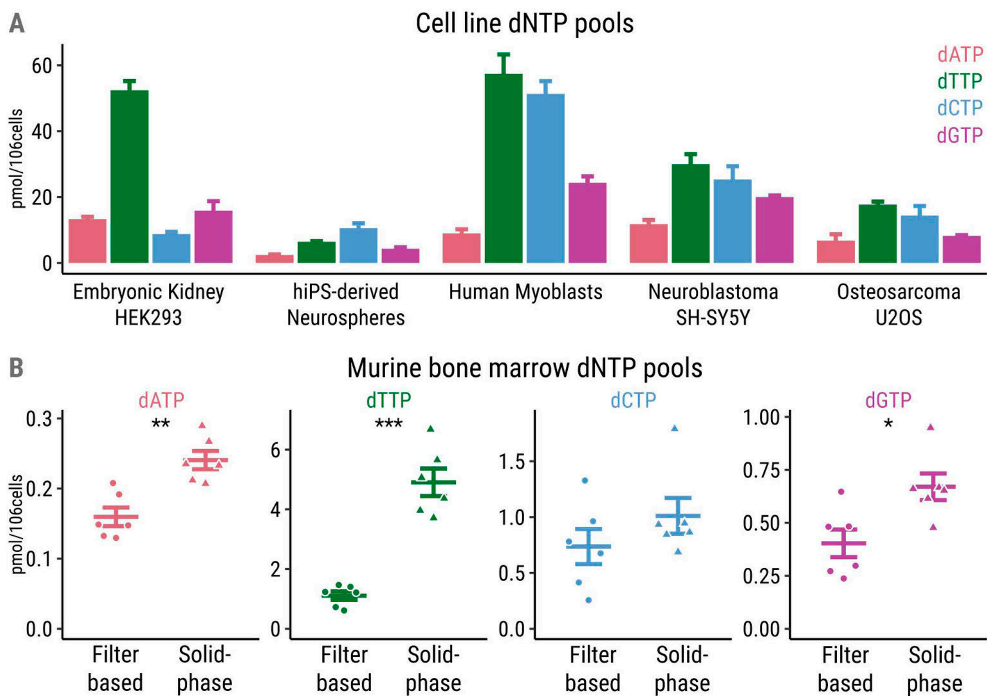

Figure 18: Validation of the novel dNTP quantitation method. (A) Quantitation of dNTP pools in an array of cell lines, data presented as mean bars with standard deviation range, three technical and two biological replicates per bar. (B) Comparison between dNTP quantitation in isolated murine bone marrow between the traditional method (filter-based) and the novel one (solid-phase). Each data point represents the mean of an individual, from two technical replicates. P values from a two-tailed t-test with $p<0.05$ as a significance threshold. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

6 DISCUSSION

Mitochondrial dysfunction is implicated in the pathogenesis of a vast number of human diseases as well as age-related degeneration (López-Otín *et al.*, 2013; Gorman *et al.*, 2016). The Mutator mouse, carrying a defective POLG and presenting with a progeroid syndrome (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005) was long interpreted to be the confirmation for the causal role of mtDNA mutagenesis in ageing, as proposed 30 years prior (Harman, 1972). However, accumulating data on the molecular phenomena taking place in Mutators have revealed additional consequences of $Polg^{D257A}$, such as increased POLG processivity and large-scale mtDNA disruptions (Williams *et al.*, 2010; Ahlqvist *et al.*, 2015b; Macao *et al.*, 2015; Szczepanowska & Trifunovic, 2015; Nissanka *et al.*, 2018), which remove mtDNA mutagenesis as the sole possible culprit. Mutators also showed little molecular evidence supporting the original model, with linear mutation accumulation (i.e. no exponential vicious cycle) and lack of significant disruptions in OXPHOS function and ROS signalling in liver, heart or muscle (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Ahlqvist *et al.*, 2012). Paradoxically, other POLG defects and mitochondrial models and diseases with high mtDNA mutational burden can exhibit harsher OXPHOS dysfunction in tissues but do not present signs of premature ageing (Tyynismaa & Suomalainen, 2009; Gorman *et al.*, 2016). Altogether, the published evidence indicates that OXPHOS deficiency alone is insufficient to cause progeroid symptoms and suggests the existence of an additional mechanism taking place in Mutators.

The Mutator pathogenesis originates in the progenitor/stem cell compartment (Chen *et al.*, 2009; Norddahl *et al.*, 2011; Ahlqvist *et al.*, 2012; Wahlestedt *et al.*, 2014). They share this feature with other premature ageing mouse models, typically caused by defects that threaten nuclear DNA integrity (Barlow *et al.*, 1996; Gu *et al.*, 1997; De Boer *et al.*, 2002; Carrero *et al.*, 2016). Our results demonstrate that in the replicating cells of Mutators, the POLG defect causes a nucleotide metabolic rewiring, secondary nuclear replication stress and DNA damage. The data also locates mitochondria at the centre of nucleotide metabolism regulation, as a possible sensor of nucleotide availability and usage which can affect the maintenance of both genomes.

The evidence raises the exciting possibility of a shared mechanism between Mutators and other premature ageing models: nuclear DNA stress in proliferating cells causes the attrition of the progenitor/stem cell compartment and thus threatens normal tissue homeostasis (Vijg & Suh, 2013; Carrero *et al.*, 2016; Wheaton *et al.*, 2017; Schumacher & Vijg, 2019). We propose that the abovementioned genomic instability mechanism, rather than mtDNA mutagenesis, is the effector of the progeroid phenotype in Mutators. This provides an answer to the long-standing debate in the field regarding the lack of premature ageing in mitochondrial disease and unifies the pathogenesis of murine progeroid syndromes.

The discovery also further emphasises that Mutators cannot be considered proof of the contribution of mtDNA mutations to ageing, and therefore that evidence supporting the mitochondrial theory of ageing is still lacking.

The new mechanism was well received into the ageing paradigms (Baumann, 2019; Schumacher & Vijg, 2019), but it did elicit some debate (Sharma *et al.*, 2020). Particularly, the nucleotide measurement was criticised, in a report that whole Mutator mouse embryos do not show dNTP pool changes compared to wildtypes. This was a curious observation,

however the authors failed to generate comparable data, hampering any accurate interpretation. Specifically, Sharma *et al.* utilised an HPLC-based methodology for dNTP quantitation, which is robust but lacks the sensitivity required for mitochondrial dNTP quantitation which was fundamental in our endeavour. Moreover, they study whole E13.5 embryos as their material. Embryos are indeed composed of mostly proliferating cells, but they are highly heterogeneous and regulated systems of numerous different replicating and differentiating cell types. The concentrations of dNTPs vary enormously across cell cycle phases, cell types and developmental stages (Gandhi & Samuels, 2011; Mathews, 2014; Pancsa *et al.*, 2022), so data from a whole embryo will be a mere average of innumerable different populations. Embryonic dNTP pools are typically studied from isolated embryonic fibroblasts to improve sample homogeneity, which we also originally reported to have more modest but similar changes as iPSC (I).

In summary, Chabes *et al.* failed to detect a difference in dNTP concentrations using a less sensitive methodology, from heterogeneous biological material different to the one we studied, and limited their analysis to total dNTP pools (Sharma *et al.*, 2020). This unfortunately cannot be accurately compared to our quantitation of subcellular dNTP pools from stem cell cultures, resulting in cell-type-, genotype- and organelle-specific significant changes in different directions which could not be caused by a systematic methodological flaw.

Our dNTP quantitation method builds on the state-of-the-art protocol developed for decades focusing on improving the specificity and sensitivity to very low dNTP pools (Traut, 1994; Ferraro *et al.*, 2010; Martí *et al.*, 2012). Its adaptation to the highly sensitive solid-phase platform (Suomalainen & Syvänen, 2003) enabled a significant reduction of the processing steps, together with the possibility to run all replicates and standards in the same plate (I & Landoni *et al.*, 2021). Deoxynucleotides are extremely unstable compounds and we have detected measurable and significant degradation of dNTP pools even upon deep-frozen overnight storage, so the rapid and effective handling of the sample is critical and will improve accuracy. Our protocol prioritises the parallel collection of samples to be compared, and minimises storage and handling, aspects which are often overlooked in other reported methodologies.

We addressed the concerns from Sharma *et al.* on this important topic in a response publication (Hämäläinen *et al.*, 2020), including additional validation data requested on the polymerase-based method and highlighting our predisposition to collaborate in the generation of relevant and comparable data. It is noteworthy that the authors' concerns were focused on dNTP quantitation, and do not challenge the conclusions of the original report regarding the mechanisms of the mtDNA Mutator progeroid syndrome (Sharma *et al.*, 2020).

Seeking to further explore the relationship between increased mtDNA replication and premature ageing, we boosted replication initiation by overexpressing Twinkle helicase in Mutator mice and their littermates, resulting in mice with higher mtDNA copy number (Tyynismaa *et al.*, 2004; Ylikallio *et al.*, 2010). This resulted in an unexpected and fatal heart failure phenotype during the first week of age in mice carrying the $Polg^{D257A}$ defect. The pathology appears to be triggered by a large-scale proteomic and metabolomic remodelling stalling the heart's normal postnatal maturation, consequently causing organismal death. The relevance of this intriguing phenotype and the insight it provides into the role of mtDNA replication and integrity in early cardiac maturation touch many fields, discussed below.

Heart disease is the leading cause of death worldwide, but the molecular mechanisms behind cardiomyocyte loss remain poorly understood. The Polg^{Mut}TwOE mice present a unique opportunity to study and decipher the development of early-onset heart disease *in vivo*, which phenotypically mimics severe human childhood mitochondrial cardiomyopathies typically caused by defects in mtDNA gene expression (Götz *et al.*, 2011; Carroll *et al.*, 2013; Vasilescu *et al.*, 2018; Jackson *et al.*, 2019). Characterising the molecular processes behind the Polg^{Mut}TwOE phenotype and attempting to rescue it may provide invaluable information for the development of therapeutic possibilities to alleviate and treat such devastating diseases.

Ferroptosis, and mitochondria-mediated ferroptosis in particular, appear to be pivotal in the pathogenesis of heart defects such as doxorubicin-induced cardiomyopathy or ischemia-reperfusion heart injury (Conrad & Proneth, 2019; Fang *et al.*, 2019; Tadokoro *et al.*, 2020). More recently, mtDNA stress and DGUOK deficiency (causing mtDNA depletion) have also been linked to ferroptotic death (Guo *et al.*, 2021; Li *et al.*, 2021). Mitochondrial function appears to be required for ferroptosis induction (Gao *et al.*, 2019), but whether primary mitochondrial dysfunction by itself can trigger ferroptosis is under debate (Battaglia *et al.*, 2020; Wang *et al.*, 2020). Our data joins the body of evidence associating heart failure to mitochondrial ferroptosis signalling (as highlighted in figure 16E). The detection of *Hddc3* responsiveness to Polg^{D257A} alleles suggested a sensitisation of the cardiac cells to ferroptosis (Ding *et al.*, 2020). This was further supported by the induction of Gpx4, its regulators and glutathione synthetic enzymes, the central system responsible for lipid ROS and ferroptosis mitigation (Cao & Dixon, 2016), alongside the metabolomic findings showing consistent changes in cysteine metabolism and the prioritisation of glutathione biosynthesis.

Consistent with the iron-dependency of ferroptosis (Dixon *et al.*, 2012), iron metabolism is also dysregulated in Polg^{Mut}TwOE hearts, with its transport and storage mostly down and iron releasing systems upregulated. Notably, the heme catabolic enzyme Hmox1 (or HO-1) has been reported as the major perpetrator of iron release in ferroptosis-mediated cardiac cell death (Tadokoro *et al.*, 2020). Hmox1 is strongly upregulated in Polg^{Mut}TwOE hearts, while the rate-limiting heme synthetic (and iron-chelating) enzymes Alas1 and Fech are downregulated, suggesting the unopposed release of heme iron (Ajioka *et al.*, 2006; Fang *et al.*, 2019). Interestingly, Hmox and Alas1 are also the main responders to glutathione depletion in cells (Wang *et al.*, 2021), further emphasising the involvement of cysteine/glutathione deficiency in these hearts.

Mitochondria and the respiratory chain are both major sinks of iron (as heme and Fe-S clusters) and the main producers of ROS, especially when the respirasome is dysfunctional (Murphy, 2009). The general shut down of respiratory chain subunits suggests iron-containing cofactors remain unbound, and OXPHOS dysfunction will directly lead to oxidative stress and lipid peroxidation. In addition, cofactor Q10 biosynthesis is strongly downregulated, likely leading to a deficiency of Q10 both as part of OXPHOS and as a crucial ferroptosis mitigator (Bersuker *et al.*, 2019; Doll *et al.*, 2019).

The first week of postnatal maturation is a period of rapid growth, inhibition of glycolytic and proliferative metabolism, and adaptation to new energy sources and oxidative metabolism (Pohjoismäki *et al.*, 2013b; Pohjoismäki & Goffart, 2017; Lalowski *et al.*, 2018). Furthermore, it is the critical period for the polyploidization of mature cardiomyocytes and the loss of cardiac regenerative capacity (Soonpaa *et al.*, 1996; Uygur & Lee, 2016). Our results indicate that mitochondrial DNA replication stress can hamper normal cardiac

metabolic maturation, resulting in a proteomic landscape resembling that of embryonic hearts, triggering ferroptosis signalling, and leading to the failure of the heart. In addition, the data raise the question of whether this pathway is shared by human mitochondrial childhood heart diseases. If so, they suggest the potential usefulness of ferroptosis inhibitors or scavengers in the prevention and treatment of such devastating disorders, an exciting possibility to be explored further.

The analysis of mtDNA integrity did not provide information directly relevant to understanding the pathology, as the mice carrying a single Polg^{D257A} defect were similarly affected upon Twinkle overexpression but without detectable mtDNA disruptions. Nonetheless, the detected effects on mtDNA integrity and replication provide important insight into the interaction between Twinkle and POLG *in vivo* and add to the body of knowledge deciphering the intriguing process of mtDNA replication.

We originally predicted a mild acceleration of the progeroid symptoms, as the increased consumption of dNTPs by enhanced replication could worsen the damage to nuclear DNA in stem cells. However, Polg^{Mut}TwOE mice did not present signs of an early Mutator phenotype (except a mild decrease in hemoglobinemia), affected nucleotide metabolism, or nuclear DNA damage signalling. While surprising, this is perhaps logical as the Mutator phenotype is the consequence of slow, progressive, and chronic stress in both genomes. The disruptions taking place in Polg^{Mut}TwOE mice are acute, severe, and likely cause their death before any of the gradual damage is detectable.

Finally, the Polg^{Mut}TwOE mice also serve as models for increased mtDNA copy number in disease, analogous to the published TFAM-overexpressing Mutators (Jiang *et al.*, 2017). TFAM overexpression appears to be curative for certain models of mtDNA mutations (Filograna *et al.*, 2019, 2021) and even rescues the nuclear DNA damage phenotype in Mutator iPSC (I), although it does so without mtDNA copy number variation in the latter. Despite other reports interpreting mtDNA copy number as the sole potential cause, our results suggest the mechanism of TFAM action may be different, likely related to packaging and mtDNA replication inhibition (Ylikallio *et al.*, 2010; Farge *et al.*, 2014; Hämäläinen *et al.*, 2019; Brüser *et al.*, 2021). The Polg^{Mut}TwOE mice further emphasise this, displaying how an active increase in mtDNA copy number in a background of mitochondrial dysfunction can be highly deleterious. Remarkably, despite Polg^{D257A} heterozygotes being mostly asymptomatic, they were similarly susceptible to Twinkle overexpression as their homozygote siblings. This warns that a comparable intervention may not only worsen a symptomatic condition but also trigger a defect in an otherwise healthy individual.

In stark contrast to our results, TwOE has been shown to be protective for the heart against ischemic and genetic defects (Pohjoismäki *et al.*, 2013a; Tanaka *et al.*, 2013; Ikeda *et al.*, 2015; Inoue *et al.*, 2016), while triggering a mild respiratory chain dysfunction in old mice without affecting their lifespan (Ylikallio *et al.*, 2010; Pohjoismäki *et al.*, 2013a). In combination with our data, this suggests that increased mtDNA replication alongside other potential functions of Twinkle can be curative, but its combination with certain stressors like accelerated processivity can be highly deleterious during early life. While the exact mechanisms behind the consequences of mtDNA copy number modulation are clarified, the field should proceed with extreme caution when proposing and considering mtDNA boosting approaches to treat mitochondrial dysfunction or other diseases.

7 CONCLUSIONS

This thesis explores the surprising variety of molecular and physiological consequences of disrupting normal mtDNA replication for the organism.

Firstly, it reconciles the pathomechanism of the mtDNA Mutator mouse with that of other progeroid syndromes. The apparent simplicity and clarity of Mutators made them one of the most prolific models in the fields of mitochondrial dysfunction and ageing. Nonetheless, accumulating data highlighted its inconsistencies with the established paradigm, as well as revealed additional consequences of the defective POLG. The data in this thesis provide a probable answer to those uncertainties and add new data and perspective to the thousands of papers discussing the model. Even if the mtDNA Mutator mouse is not a disease model *per se*, it has been crucial to understand the diverse consequences of mitochondrial dysfunction and discover the many roles of POLG and its exonuclease domain. Our data joins the long list of discoveries arising from Mutators, clarifying and unifying the causes of premature ageing and opening the door to new questions regarding the role of mtDNA mutations and replication in cellular signalling and metabolic regulation.

The thesis also presents a development on the state-of-the-art dNTP quantitation method, leveraging a methodology published two decades ago to circumvent the somewhat absurd challenge of commercial discontinuation of materials. The process nicely exemplified the stepwise nature of scientific progress and endured the scrutiny of the scientific community through traditionally published commentaries, personal discussion and appraisal, and even deliberation over social media.

The final project of this thesis attempted to further confirm the previous claims, while also serving as an opportunity to test a novel paradigm: that more mitochondrial DNA is beneficial against mitochondrial dysfunction. The data demonstrates that increasing mtDNA amount by replication (overexpressing Twinkle) can have unexpected and highly damaging consequences in mice with mild or severe mtDNA defects (heterozygote and homozygote Mutators respectively). It presents the detailed characterisation of those consequences: functional, genetic, and molecular. The results indicate that the interaction of two stress pathways arising from the manipulation of mtDNA replication is halting normal cardiomyocyte maturation, stalling metabolic development in a pseudo-embryonic state, and rendering the hearts vulnerable to oxidative damage, which together likely cause the failure of the system.

The first significance of the data is to caution about the danger and unpredictability of manipulating such an intricate system, and the need for a deeper understanding before the translation of any mtDNA-boosting therapy to the clinic.

The second set of conclusions is significantly more optimistic. The surprisingly severe pathology presents an equally unexpected opportunity: that of studying early cardiomyopathy *in vivo* and dissecting the contributions of each involved protein and metabolic pathway in the process. Future research in this direction will reveal if the $Polg^{Mut}$TwOE heart disease has similarities and relevance for the pathogenesis of human cardiomyopathies and whether therapeutic targets could be identified. In the meantime, I am tempted to predict that the mechanisms unveiled will drive us closer, even if ever so slightly, to understanding and curing these devastating disorders, an endeavour I am proud and grateful to have contributed to.

Finally, this thesis is a testament to three crucial parts of science: serendipity, curious scepticism, and fundamental research. On serendipity and randomness, every single project presented is at least partially a consequence of an unexpected event or result which was wielded for the better. Despite careful planning, being attentive and open to change and adapt in light of new data is what brought us to the most interesting results.

Secondly, the importance of fundamental research. Although in the shadow of applied and impactful results, none of the discoveries we talk about today would be possible without the herculean efforts of fundamental scientists. All the models included in this thesis fall in that category, as they do not directly reflect human disease. Nonetheless, what we can learn from them has an impact that goes beyond themselves, pushing our knowledge further and building the shoulders for future ground-breaking discoveries to stand on.

Lastly, with curious scepticism, I refer to the importance of constructively challenging what we think we know. Inspired by new information and puzzling patterns and inconsistencies, this thesis revisits several reputable paradigms, testing and developing them into something that is hopefully closer to the truth. Even the most established models and theories are simply human interpretations of data, and science only evolves as these concepts survive or adapt to the contest of new evidence. As a human endeavour, science is a prisoner to all our defects, simple world views, biases, greed, and ambition, as well as a problematic history. We must actively strive to fix those issues and constantly challenge and improve the structures, ensuring they adapt to the rapidly evolving and highly interconnected world of new, diverse, and nuanced knowledge we live in.

8 ACKNOWLEDGEMENTS

The series of wonderful people and extremely fortunate events that led to me writing these words is quite lengthy, and I am infinitely grateful for each and every one of them. I will attempt to be as concise as possible – but also knowing this is the only section some of you will read, I will not let you go too quickly either.

The work in this thesis was performed in Biomedicum Helsinki, at the Stem Cells and Metabolism Research Program, between the years 2018 and 2022. The work was financially supported by the University of Helsinki Funds and the Biomedicum Helsinki, Päivikki ja Sakari Sohlberg, Maud Kuistila, Alfred Kordelin and Orion Science Foundations; as well as by the general grants to the research group from the Sigrid Juselius and Jane and Aatos Erkko Foundations, the Academy of Finland, the European Research Council (ERC), and the University of Helsinki.

First, I wish to thank Prof. Anu Suomalainen Wartiovaara, Anu. I could not have asked for a better mentor. You hired me as a nerdy teenager from the other side of the world with nothing but curiosity to offer, and you completely changed my life. Despite my lack of experience, from day one you made me feel that I mattered, that my ideas and voice were valid, and that everyone else's were, too. You always pushed me to explore and learn new things, trusted me to go overseas and talk about your lab's work, and dared me to think outside the box brainstorming the craziest ideas. You constantly challenged me and allowed me to challenge you back. I admire you hugely, and I am so grateful and honoured I had the opportunity to learn from you all these years. Sydämelliset kiitokset.

I want to thank the members of my thesis committee, Assoc. Prof. Pekka Katajisto & Docent Cory Dunn, for their caring, constructive, and challenging comments during our discussions. I thoroughly enjoyed our meetings. I also want to thank the external reviewers of my thesis, Adj. Prof. Jaakko Pohjoismäki and Prof. Carlos Moraes, for their time, expertise and encouraging comments, as well as Prof. Eero Mervaala for kindly accepting the role of the Faculty representative. And, of course, I am grateful to Assoc. Prof. Nika Danial for doing me the great honour of being my opponent. I am very much looking forward to our discussion.

How and why I ended up in Helsinki is a question I have answered innumerable times, and I will spare you most details. But I wouldn't be here without the life-changing experiences that were the Biology and Chemistry Olympiads, Expedición Ciencia and the Millennium Youth Camp, which introduced me to a multitude of wonderful and inspiring nerds, many of whom remain my most cherished friends. The latter also introduced me to Finland and to a certain Kirmo Wartiovaara, who somehow via genetically engineering plants and teen vampire odontology convinced me that moving here was a good idea. Kirmo you were so very right, and I will forever be grateful for your massive impact on my life. I also want to thank Technology Academy Finland and LUMA Centre Finland for supporting these and many other wonderful opportunities for young people to find their paths.

Next, I want to thank the people I had the joy to work with in these and many other projects, all collaborators and co-authors, from whom I have learned enormously. I am privileged to have my name sitting next to others like Riikka Martikainen, Joni Nikkanen, Liya Wang, Diego Balboa, Jim Stewart, Steffi Goffart, Riikka Kivelä, Kati Ahlqvist, Anna Kuukasjärvi, Tuomas Laalo, and many others, to whom I owe a great deal of gratitude. I also wish to

thank Howard Jacobs, Jodi Nunnari, Brendan Battersby, Henna Tyynismaa, Timo Otonkoski and Vanessa Fuller for providing invaluable mentorship, leadership, and advice.

I could not be happier to have been part of ASW lab, in its many shapes throughout the years. This lab has been my second home for almost a decade, inhabited by a bunch of hilarious, chaotic, diverse, and often delusional little humans who always support one another and speak up for what they believe in. I sincerely hope this lab spirit never goes away. Joni Nikkanen was my first mentor, who taught me much of what I know. Our countless hours working side by side and shared obsession for optimisation will forever mark me as a scientist. Then came some independence and with it a wild ride of bouncing around projects, learning to play with proteins with Liliya Euro both *in silico* and *in lab*, exciting immunofluorescence with Riikka Äänismaa, hectic cloning with Gulayse Ince Dunn and great times teaching and learning from Anna Kuukasjärvi, Tuomas Laalo and Paulína Nemcová. The technical heroes that keep the lab running: Markus Innilä, Tuula Manninen, Sonja Jansson, Babette Hollmann, Satu Malinen, Kirsi Mattinen, it has been an honour to learn from you and share many lovely times, I am very grateful for your invaluable contributions to the data in this thesis. The wonderful breakfasts, lunches, coffee breaks, office laughs and perjantai pullot with Jana Buzková, Saara Forsström, Riikka Äänismaa, Olesia Ignatenko, Nahid Khan, Kaisa Sarkkinen and the rest of ASW lab (past and present) have fuelled my time here, thank you all for being awesome. The gratitude extends to the STEMM research program, which despite the long pandemic still managed to introduce me to great people, wonderful collaborations, seminars, pizzas, and parties. I also want to thank the fantastic work and communication from the mouse facility staff, especially Amy Platt and Aurora Hämäläinen.

And while the lab has been a wonderful place to be, I would not have survived PhD without the horde of friends that kept me sane every step of the way, coming and going from Finland but always close to my heart (even if I am terrible at keeping in touch). Wilhelmiina Tiainen, thank you for being the perfect first Finn to meet, and a fantastic friend for over a decade. In come the exchange students, yearly cohorts of fantastic people with whom I shared the most wonderful adventures; Hannah Tonry, Justine Tavera, Julia Keil, Dominik Böhm, Giulia Cappelletto, Letizia Santivetti, Francesco Ferraresso and many others, thank you all for the craziest memories I will never forget. And while some came and went, others were always there as we fought together to endure university life and Klusteri nights, deciphering assignments in various languages and levels of clarity, and karaoke-ing the stress away. Thank you Brittany Rose, Juulia Talvitie, Heta Nieminen, Paloma Ruiz, Nidia Obscura, Max Pohjanpelto, Caterina Vitale. I truly couldn't have done it without you.

And there are some friends you are lucky enough to find along the way, who infinitely improve your life through hikes, saunas, circus, parties, dinners, co-working days, political projects, PhD peer support and bizarre and fascinating conversations. Thank you, Niko Johansson, Kalle Vikman, Maxime Grandin, Michael Saikali, James Albasi, Todd Elliott, Ida Berg, Sarri Nykänen, Jonne Lintunen, Ilari Sahi, Leevi Lappi, Otso Peippo, Pooja Manjunath, Iina Saarinen, Henri Marjomaa. Bo Liljeberg, thank you for always being there when I needed you the most, with good food and quality company to balance out my chaotic life. Together with Tom Barsby, José Arrias, Nazlı Eskici and Simon Andersson, thank you also for providing a strange and wonderful escape from reality by pretending to fight goblins and crawl dungeons as a stormy blind old lady or a shady hummingbird with anger management issues.

To the gang, thank you for being my chosen PhD family. Rocío Maldonado, Sami Jalil, Nazlı Eskici, Cecilia Cannarozzo, Karlo Skube, Swagat Pradhan, Christian Stadelmann, Pavel Sitkin. I will truly miss our ability to enjoy the simplest of things: a hammock by the lake, a sweet dessert, or the sound of a bridge. Thank you for being amazing, and may we keep laughing for many years to come. Ro, Sami, gracias por ser y construir conmigo ese pedacito de Argentina en Finlandia que no sabía que tanto necesitaba.

Niko Johansson, Max Pohjanpelto, Olesia Ignatenko and Marcell Harhai are also thanked for volunteering as tributes to proofread and comment on the thesis draft, this book is infinitely better and more coherent as a result of your great, diverse and critical eyes.

This is getting long, I know, and now it's getting multilingual – buckle up.

Je veux remercier la famille Ciamous, ma famille du cœur : Corinne, Jean-Marc, Emmanuelle, Olivier, Jeremy et Marinette Ciamous, Yves et Nicole Lartigue. Merci pour m'avoir ouvert vos portes et vos bras dans votre famille, et ainsi avoir ouvert mon esprit au grand monde dehors l'Argentine. Corinne, un après-midi tu m'as convaincu que déménager en Finlande ce n'était pas un truc des fous, mais une grande opportunité tout à fait possible. Je suis là grâce à toi et cette petite conversation. Merci à tous, vous avez changé ma vie plusieurs fois et j'ai hâte d'être vachement plus proche de vous bientôt !

Marcell Harhai. My partner, my team. I cannot possibly imagine these last 3 years without you. From isolating together for weeks at the time to long months at the distance, and who knows what new challenges will arise in the couple of months until this is printed, you have been an invaluable support every step of the way. You are my companion, my best friend, my quest sidekick, my head counsellor, my noise machine, my devil's advocate, and my absolute favourite person. I am unbelievably lucky to have you by my side, and so excited for the adventures that await us ahead. Köszönöm szépen, szeretlek.

Finalmente, mi familia. Tengo la suerte de haber nacido rodeado de gente loca y maravillosa que me enseñó a cuidarnos, a querernos, y a disfrutar y celebrar la vida, con abuelos que me transmitieron legados invaluables. Gracias a todos por aceptarme y apoyarme, y siempre recibirme con los brazos abiertos. Andrea Martín, Guillermo Landoni y Franco Landoni, la familia hundida. No me quedan palabras para describir lo mucho que los quiero y lo agradecido que estoy por su apoyo y confianza desde que tengo memoria. Uno pensaría que vivir a 13 000 km de distancia por casi una década haría que uno se distancie, pero cada año me siento más cerca de ustedes y valoro y honro más sus enseñanzas. La confianza, la risa, el disfrute de la vida, el apoyarnos entre nosotros, el optimismo pelotudo, el respeto a las piedras (tanto literales como metafóricas). Apolo, el quinto miembro de nuestra familia, gracias por ser nuestro protector y fiel compañero, tanto en la barranca como escribiendo esta tesis en la pile a la tardecita. Estoy infinitamente agradecido por tenerlos y orgulloso de todo lo que han logrado. Escribo estas palabras sentado en el quincho del maravilloso hogar que crearon en Alpa Corral, donde luchando contra monstruos y vacas, comiendo rico y explorando la naturaleza con ustedes, me convertí en la persona que soy. Los amo. Gracias.

9 REFERENCES

Aaltio J., Hyttinen V., Kortelainen M., Frederix G.W.J., Lönnqvist T., Suomalainen A., *et al.* (2022) 'Cost-effectiveness of whole-exome sequencing in progressive neurological disorders of children: WES cost-effectiveness in children's encephalopathies', *Eur. J. Paediatr. Neurol.*, 36, pp. 30–36.

Ahlqvist K.J., Hämäläinen R.H., Yatsuga S., Uutela M., Terzioglu M., Götz A., *et al.* (2012) 'Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in polg mutator mice', *Cell Metab.* 2012/01/10, 15(1), pp. 100–109.

Ahlqvist K.J., Leoncini S., Pecorelli A., Wortmann S.B., Ahola S., Forsström S., *et al.* (2015a) 'MtDNA mutagenesis impairs elimination of mitochondria during erythroid maturation leading to enhanced erythrocyte destruction', *Nat. Commun.*, 6(1), p. 6494.

Ahlqvist K.J., Suomalainen A., & Hämäläinen R.H. (2015b) 'Stem cells, mitochondria and aging', *Biochim. Biophys. Acta - Bioenerg.*, 1847(11), pp. 1380–1386.

Ahola-Erkkilä S., Carroll C.J., Peltola-Mjösund K., Tulkki V., Mattila I., Seppänen-Laakso T., *et al.* (2010) 'Ketogenic diet slows down mitochondrial myopathy progression in mice', *Hum. Mol. Genet.* 2010/02/20, 19(10), pp. 1974–1984.

Ahola S., Auranen M., Isohanni P., Niemisalo S., Urho N., Buzkova J., *et al.* (2016) 'Modified Atkins diet induces subacute selective ragged-red-fiber lysis in mitochondrial myopathy patients', *EMBO Mol. Med.*, 8(11), pp. 1234–1247.

Ajioka R.S., Phillips J.D., & Kushner J.P. (2006) 'Biosynthesis of heme in mammals', *Biochim. Biophys. Acta - Mol. Cell Res.*, 1763(7), pp. 723–736.

Anand I.S. & Gupta P. (2018) 'Anemia and Iron Deficiency in Heart Failure: Current Concepts and Emerging Therapies', *Circulation*, 138(1).

Anderson G.J. & Vulpe C.D. (2009) 'The Cellular Physiology of Iron', *Iron Defic. Overload*, pp. 3–29.

Anderson K.A. & Hirschey M.D. (2012) 'Mitochondrial protein acetylation regulates metabolism', *Essays Biochem.*, 52(1), pp. 23–35.

Anderson S., Bankier A.T., Barrell B.G., De Bruijn M.H.L., Coulson A.R., Drouin J., *et al.* (1981) 'Sequence and organization of the human mitochondrial genome', *Nature*, 290(5806), pp. 457–465.

Arnaudo E., Shanske S., DiMauro S., Schon E.A., Moraes C.T., Schon E.A., *et al.* (1991) 'Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy', *Lancet*, 337(8740), pp. 508–510.

Bacman S.R. & Moraes C.T. (2020) 'Mitochondrial DNA Base Editing: Good Editing Things Still Come in Small Packages', *Mol. Cell*, 79(5), pp. 708–709.

Bao X.R., Ong S.E., Goldberger O., Peng J., Sharma R., Thompson D.A., *et al.* (2016) 'Mitochondrial dysfunction remodels one-carbon metabolism in human cells', *Elife*, 5(JUN2016), p. e10575.

Barlow C., Hirotsune S., Paylor R., Liyanage M., Eckhaus M., Collins F., *et al.* (1996) 'Atm-deficient mice: A paradigm of ataxia telangiectasia', *Cell*, 86(1), pp. 159–171.

Barriocanal-Casado E., Hidalgo-Gutiérrez A., Raimundo N., González-García P., Acuña-Castroviejo D., Escames G., *et al.* (2019) 'Rapamycin administration is not a valid therapeutic strategy for every case of mitochondrial disease', *EBioMedicine*, 42, pp. 511–523.

Battaglia A.M., Chirillo R., Aversa I., Sacco A., Costanzo F., & Biamonte F. (2020) 'Ferroptosis and Cancer: Mitochondria Meet the "Iron Maiden" Cell Death', *Cells*.

Baumann K. (2019) 'mtDNA robs nuclear dNTPs', *Nat. Rev. Mol. Cell Biol.*

Benda C. (1898) 'Über die Spermatogense der Vertebraten und höherer Evertebraten. 2. Teil. Die Histiogenese der Spermien', *Arch. für Physiol.*, pp. 393–398.

Bereiter-Hahn J. & Vöth M. (1998) 'Distribution and dynamics of mitochondrial nucleoids in animal cells in culture', *EBO — Exp. Biol. Online Annu. 1996/97*, pp. 58–77.

Bersuker K., Hendricks J.M., Li Z., Magtanong L., Ford B., Tang P.H., *et al.* (2019) 'The CoQ oxidoreductase FSP1 acts parallel to GPX4 to inhibit ferroptosis', *Nature*, 575(7784).

Besse A., Wu P., Bruni F., Donti T., Graham B.H., Craigen W.J., *et al.* (2015) 'The GABA transaminase, ABAT, is essential for mitochondrial nucleoside metabolism', *Cell Metab.*, 21(3), pp. 417–427.

Bibb M.J., Van Etten R.A., Wright C.T., Walberg M.W., & Clayton D.A. (1981) 'Sequence and gene organization of mouse mitochondrial DNA', *Cell*, 26(2 PART 2), pp. 167–180.

Blázquez-Bermejo C., Molina-Granada D., Vila-Julià F., Jiménez-Heis D., Zhou X., Torres-Torronteras J., *et al.* (2019) 'Age-related metabolic changes limit efficacy of deoxynucleoside-based therapy in thymidine kinase 2-deficient mice', *EBioMedicine*, 46, pp. 342–355.

Blum T.B., Hahn A., Meier T., Davies K.M., & Kühlbrandt W. (2019) 'Dimers of mitochondrial ATP synthase induce membrane curvature and self-assemble into rows', *Proc. Natl. Acad. Sci. U. S. A.*, 116(10), pp. 4250–4255.

Bock F.J. & Tait S.W.G. (2020) 'Mitochondria as multifaceted regulators of cell death', *Nat. Rev. Mol. Cell Biol.*, 21(2), pp. 85–100.

De Boer J., Andressoo J.O., De Wit J., Huijmans J., Beems R.B., Van Steeg H., *et al.* (2002) 'Premature aging in mice deficient in DNA repair and transcription', *Science (80-.).*, 296(5571).

Bonekamp N.A. & Larsson N.G. (2018) 'SnapShot: Mitochondrial Nucleoid', *Cell*, pp. 388-388.e1.

Bonev B., Stanley P., & Papalopulu N. (2012) 'MicroRNA-9 modulates hes1 ultradian oscillations by forming a double-negative feedback loop', *Cell Rep.*, 2(1), pp. 10–18.

Bottani E., Giordano C., Civiletto G., Di Meo I., Auricchio A., Ciusani E., *et al.* (2014) 'AAV-mediated liver-specific MPV17 expression restores mtdna levels and prevents diet-induced liver failure', *Mol. Ther.*, 22(1), pp. 10–17.

Bough K.J., Wetherington J., Hassel B., Pare J.F., Gawryluk J.W., Greene J.G., *et al.* (2006) 'Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet', *Ann. Neurol.*, 60(2), pp. 223–235.

Bourdon A., Minai L., Serre V., Jais J.P., Sarzi E., Aubert S., *et al.* (2007) 'Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion', *Nat. Genet.*, 39(6), pp. 776–780.

Brown T.A., Tkachuk A.N., Shtengel G., Kopek B.G., Bogenhagen D.F., Hess H.F., *et al.* (2011) 'Superresolution Fluorescence Imaging of Mitochondrial Nucleoids Reveals Their Spatial Range, Limits, and Membrane Interaction', *Mol. Cell. Biol.*, 31(24), pp. 4994–5010.

Brüser C., Keller-Findeisen J., & Jakobs S. (2021) 'The TFAM-to-mtDNA ratio defines inner-cellular nucleoid populations with distinct activity levels', *Cell Rep.*, 37(8), p. 110000.

Bulst S., Abicht A., Holinski-Feder E., Müller-Ziermann S., Koehler U., Thirion C., *et al.* (2009) 'In vitro supplementation with dAMP/dGMP leads to partial restoration of mtDNA levels in mitochondrial depletion syndromes', *Hum. Mol. Genet.*, 18(9), pp. 1590–1599.

Bulst S., Holinski-Feder E., Payne B., Abicht A., Krause S., Lochmüller H., *et al.* (2012) 'In vitro supplementation with deoxynucleoside monophosphates rescues mitochondrial DNA depletion', *Mol. Genet. Metab.*, 107(1–2), pp. 95–103.

Cable J., Pourquié O., Wellen K.E., Finley L.W.S., Aulehla A., Gould A.P., *et al.* (2021) 'Metabolic decisions in development and disease—a Keystone Symposia report', *Ann. N. Y. Acad. Sci.*, 1506(1), pp. 55–73.

Cámara Y., González-Vioque E., Scarpelli M., Torres-Torronteras J., Caballero A., Hirano M., *et al.* (2014) 'Administration of deoxyribonucleosides or inhibition of their catabolism as a pharmacological approach for mitochondrial DNA depletion syndrome', *Hum. Mol. Genet.*, 23(9), pp. 2459–2467.

Campbell H.D., Webb G.C., & Young I.G. (1997) 'A human homologue of the Drosophila melanogaster sluggish-A (proline oxidase) gene maps to 22q11.2, and is a candidate gene for type-I hyperprolinaemia', *Hum. Genet.*, 101(1), pp. 69–74.

Campuzano V., Montermini L., Moltò M.D., Pianese L., Cossée M., Cavalcanti F., *et al.* (1996) 'Friedreich's ataxia: Autosomal recessive disease caused by an intronic GAA triplet repeat expansion', *Science (80-.).*, 271(5254), pp. 1423–1427.

Cao J.Y. & Dixon S.J. (2016) 'Mechanisms of ferroptosis', *Cell. Mol. Life Sci.*, pp. 2195–2209.

Carrero D., Soria-Valles C., & López-Otín C. (2016) 'Hallmarks of progeroid syndromes: Lessons from mice and reprogrammed cells', *DMM Dis. Model. Mech.*, 9(7), pp. 719–735.

Carroll C.J., Isohanni P., Pöyhönen R., Euro L., Richter U., Brilhante V., *et al.* (2013) 'Whole-exome sequencing identifies a mutation in the mitochondrial ribosome protein MRPL44 to underlie mitochondrial infantile cardiomyopathy', *J. Med. Genet.* 2013/01/15, 50(3), pp. 151–159.

Carroll C.J., Brilhante V., & Suomalainen A. (2014) 'Next-generation sequencing for mitochondrial disorders', *Br. J. Pharmacol.*, 171(8), pp. 1837–1853.

Cerutti R., Pirinen E., Lamperti C., Marchet S., Sauve A.A., Li W., *et al.* (2014) 'NAD+-dependent activation of Sirt1 corrects the phenotype in a mouse model of mitochondrial disease', *Cell Metab.*, 19(6), pp. 1042–1049.

Chabes A., Georgieva B., Domkin V., Zhao X., Rothstein R., & Thelander L. (2003) 'Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase', *Cell*, 112(3), pp. 391–401.

Chandel N.S., Maltepe E., Goldwasser E., Mathieu C.E., Simon M.C., & Schumacker P.T. (1998) 'Mitochondrial reactive oxygen species trigger hypoxia-induced transcription', *Proc. Natl. Acad. Sci. U. S. A.*, 95(20), pp. 11715–11720.

Chandel N.S. (2015) 'Evolution of Mitochondria as Signaling Organelles', *Cell Metab.*, 22(2), pp. 204–206.

Chang D.D. & Clayton D.A. (1984) 'Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA', *Cell*, 36(3), pp. 635–643.

Chen M.L., Logan T.D., Hochberg M.L., Shelat S.G., Yu X., Wilding G.E., *et al.* (2009) 'Erythroid dysplasia, megaloblastic anemia, and impaired lymphopoiesis arising from mitochondrial dysfunction', *Blood.* 2009/09/08, 114(19), pp. 4045–4053.

Chen X., Comish P.B., Tang D., & Kang R. (2021) 'Characteristics and Biomarkers of Ferroptosis', *Front. Cell Dev. Biol.*

Chong J., Soufan O., Li C., Caraus I., Li S., Bourque G., *et al.* (2018) 'MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis', *Nucleic Acids Res.*, 46(W1), pp. W486–W494.
Chouchani E.T., Kazak L., & Spiegelman B.M. (2019) 'New Advances in Adaptive Thermogenesis: UCP1 and Beyond', *Cell Metab.*, 29(1), pp. 27–37.
Chrétien D., Bénit P., Ha H.H., Keipert S., El-Khoury R., Chang Y.T., *et al.* (2018) 'Mitochondria are physiologically maintained at close to 50 °C', *PLoS Biol.*, 16(1), p. e2003992.
Chung C.Y., Valdebenito G.E., Chacko A.R., & Duchen M.R. (2021) 'Rewiring cell signalling pathways in pathogenic mtDNA mutations', *Trends Cell Biol.*, 0(0).
Civiletto G., Varanita T., Cerutti R., Gorletta T., Barbaro S., Marchet S., *et al.* (2015) 'Opa1 overexpression ameliorates the phenotype of two mitochondrial disease mouse models', *Cell Metab.*, 21(6), pp. 845–854.
Clayton D.A. (1982) 'Replication of animal mitochondrial DNA', *Cell.* 1982/04/01, 28(4), pp. 693–705.
Collins L.V., Hajizadeh S., Holme E., Jonsson I.M., & Tarkowski A. (2004) 'Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses', *J. Leukoc. Biol.*, 75(6), pp. 995–1000.
Collins T.J., Berridge M.J., Lipp P., & Bootman M.D. (2002) 'Mitochondria are morphologically and functionally heterogeneous within cells', *EMBO J.*, 21(7), pp. 1616–1627.
Conrad M. & Proneth B. (2019) 'Broken hearts: Iron overload, ferroptosis and cardiomyopathy', *Cell Res.*, 29(4), pp. 263–264.
Coolen M., Katz S., & Bally-Cuif L. (2013) 'miR-9: A versatile regulator of neurogenesis', *Front. Cell. Neurosci.*, 7(NOV), p. 220.
Cooper G., Reed C., Nguyen D., Carter M., & Wang Y. (2011) 'Detection and formation scenario of citric acid, pyruvic acid, and other possible metabolism precursors in carbonaceous meteorites', *Proc. Natl. Acad. Sci. U. S. A.*, 108(34), pp. 14015–14020.
D'Angelo R., Boschetti E., Amore G., Costa R., Pugliese A., Caporali L., *et al.* (2020) 'Liver transplantation in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE): clinical long-term follow-up and pathogenic implications', *J. Neurol.*, 267(12), pp. 3702–3710.
Dalla Rosa I., Cámara Y., Durigon R., Moss C.F., Vidoni S., Akman G., *et al.* (2016) 'MPV17 Loss Causes Deoxynucleotide Insufficiency and Slow DNA Replication in Mitochondria', *PLoS Genet.*, 12(1).
Danial N.N., Hartman A.L., Stafstrom C.E., & Thio L.L. (2013) 'How does the ketogenic diet work? Four potential mechanisms', in *J. Child Neurol.*
Deshwal S., Fiedler K.U., Langer T., Langer T., & Langer T. (2020) 'Mitochondrial Proteases: Multifaceted Regulators of Mitochondrial Plasticity', *Annu. Rev. Biochem.*
Dillon L.M., Hida A., Garcia S., Prolla T.A., & Moraes C.T. (2012) 'Long-Term Bezafibrate Treatment Improves Skin and Spleen Phenotypes of the mtDNA Mutator Mouse', *PLoS One*, 7(9).
Ding C.K.C., Rose J., Sun T., Wu J., Chen P.H., Lin C.C., *et al.* (2020) 'MESH1 is a cytosolic NADPH phosphatase that regulates ferroptosis', *Nat. Metab.*, 2(3), pp. 270–277.
Dionisi-Vici C., Diodato D., Torre G., Picca S., Pariante R., Giuseppe Picardo S., *et al.* (2016) 'Liver transplant in ethylmalonic encephalopathy: A new treatment for an otherwise fatal disease', *Brain*, 139(4), pp. 1045–1051.
Dixon S.J., Lemberg K.M., Lamprecht M.R., Skouta R., Zaitsev E.M., Gleason C.E., *et al.* (2012) 'Ferroptosis: An Iron-Dependent Form of Nonapoptotic Cell Death', *Cell*, 149(5), pp. 1060–1072.
Dodt G., Kim D.G., Reimann S.A., Reuber B.E., McCabe K., Gould S.J., *et al.* (2000) 'L-Pipecolic acid oxidase, a human enzyme essential for the degradation of L-pipecolic acid, is most similar to the monomeric sarcosine oxidases', *Biochem. J.*, 345(3), pp. 487–494.
Dogan S.A., Pujol C., Maiti P., Kukat A., Wang S., Hermans S., *et al.* (2014) 'Tissue-specific loss of DARS2 activates stress responses independently of respiratory chain deficiency in the heart', *Cell Metab.*, 19(3), pp. 458–469.
Dogan S.A., Cerutti R., Benincá C., Brea-Calvo G., Jacobs H.T., Zeviani M., *et al.* (2018) 'Perturbed Redox Signaling Exacerbates a Mitochondrial Myopathy', *Cell Metab.*, 28(5), pp. 764-775.e5.
Doll S., Freitas F.P., Shah R., Aldrovandi M., da Silva M.C., Ingold I., *et al.* (2019) 'FSP1 is a glutathione-independent ferroptosis suppressor', *Nature*, 575(7784).
Domínguez-González C., Madruga-Garrido M., Mavillard F., Garone C., Aguirre-Rodríguez F.J., Donati M.A., *et al.* (2019) 'Deoxynucleoside Therapy for Thymidine Kinase 2–Deficient Myopathy', *Ann. Neurol.*, 86(2), pp. 293–303.
Duchen M.R. (2000) 'Mitochondria and Ca2+ in cell physiology and pathophysiology', *Cell Calcium*, 28(5–6), pp. 339–348.
Eden E., Navon R., Steinfeld I., Lipson D., & Yakhini Z. (2009) 'GOrilla: A tool for discovery and visualization of enriched GO terms in ranked gene lists', *BMC Bioinformatics*, 10.
Edgar D. & Trifunovic A. (2009) 'The mtDNA mutator mouse: Dissecting mitochondrial involvement in aging.', *Aging (Albany. NY).*, 1(12), pp. 1028–1032.

Ehses S., Raschke I., Mancuso G., Bernacchia A., Geimer S., Tondera D., *et al.* (2009) 'Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1', *J. Cell Biol.*, 187(7), pp. 1023–1036.

Ekstrand M.I., Falkenberg M., Rantanen A., Park C.B., Gaspari M., Hultenby K., *et al.* (2004) 'Mitochondrial transcription factor A regulates mtDNA copy number in mammals', *Hum. Mol. Genet.*, 13(9), pp. 935–944.

Elpeleg O., Miller C., Hershkovitz E., Bitner-Glindzicz M., Bondi-Rubinstein G., Rahman S., *et al.* (2005) 'Deficiency of the ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial DNA depletion', *Am. J. Hum. Genet.* 2005/05/07, 76(6), pp. 1081–1086.

Falkenberg M., Gaspari M., Rantanen A., Trifunovic A., Larsson N.G., & Gustafsson C.M. (2002) 'Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA', *Nat. Genet.* 2002/06/18, 31(3), pp. 289–294.

Falkenberg M. (2018) 'Mitochondrial DNA replication in mammalian cells: Overview of the pathway', *Essays Biochem.* Edited by Garone, C. and Minczuk, M., 62(3), pp. 287–296.

Fang X., Wang H., Han D., Xie E., Yang X., Wei J., *et al.* (2019) 'Ferroptosis as a target for protection against cardiomyopathy', *Proc. Natl. Acad. Sci. U. S. A.*, 116(7), pp. 2672–2680.

Farge G., Mehmedovic M., Baclayon M., vandenWildenberg S.M.J.L., Roos W.H., Gustafsson C.M., *et al.* (2014) 'In Vitro-reconstituted nucleoids can block mitochondrial DNA replication and transcription', *Cell Rep.*, 8(1), pp. 66–74.

Felici R., Buonvicino D., Muzzi M., Cavone L., Guasti D., Lapucci A., *et al.* (2017) 'Post onset, oral rapamycin treatment delays development of mitochondrial encephalopathy only at supramaximal doses', *Neuropharmacology*, 117, pp. 74–84.

Fenton H.J.H. (1894) 'LXXIII. - Oxidation of tartaric acid in presence of iron', *J. Chem. Soc. Trans.*, 65(65), pp. 899–910.

Ferrari M., Jain I.H., Goldberger O., Rezoagli E., Thoonen R., Chen K.H., *et al.* (2017) 'Hypoxia treatment reverses neurodegenerative disease in a mouse model of Leigh syndrome', *Proc. Natl. Acad. Sci. U. S. A.*, 114(21), pp. E4241–E4250.

Ferraro P., Franzolin E., Pontarin G., Reichard P., & Bianchi V. (2010) 'Quantitation of cellular deoxynucleoside triphosphates', *Nucleic Acids Res.*, 38(6), pp. e85–e85.

Filograna R., Koolmeister C., Upadhyay M., Pajak A., Clemente P., Wibom R., *et al.* (2019) 'Modulation of mtDNA copy number ameliorates the pathological consequences of a heteroplasmic mtDNA mutation in the mouse', *Sci. Adv.*, 5(4), p. eaav9824.

Filograna R., Mennuni M., Alsina D., & Larsson N.G. (2021) 'Mitochondrial DNA copy number in human disease: the more the better?', *FEBS Lett.*, 595(8), pp. 976–1002.

Fisher R.P., Lisowsky T., Parisi M.A., & Clayton D.A. (1992) 'DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein', *J. Biol. Chem.*, 267(5), pp. 3358–3367.

Forsström S., Jackson C.B., Carroll C.J., Kuronen M., Pirinen E., Pradhan S., *et al.* (2019) 'Fibroblast Growth Factor 21 Drives Dynamics of Local and Systemic Stress Responses in Mitochondrial Myopathy with mtDNA Deletions', *Cell Metab.*, 30(6), pp. 1040-1054.e7.

Franklin R.E. & Gosling R.G. (1953) 'Molecular configuration in sodium thymonucleate', *Nature*, 171(4356), pp. 740–741.

Frezza C., Cipolat S., Martins de Brito O., Micaroni M., Beznoussenko G. V., Rudka T., *et al.* (2006) 'OPA1 Controls Apoptotic Cristae Remodeling Independently from Mitochondrial Fusion', *Cell*, 126(1), pp. 177–189.

Fridlender B., Fry M., Bolden A., & Weissbach A. (1972) 'A new synthetic RNA-dependent DNA polymerase from human tissue culture cells (HeLa-fibroblast-synthetic oligonucleotides-template-purified enzymes).', *Proc. Natl. Acad. Sci. U. S. A.*, 69(2), pp. 452–455.

Friedman J.R., Lackner L.L., West M., DiBenedetto J.R., Nunnari J., & Voeltz G.K. (2011) 'ER tubules mark sites of mitochondrial division', *Science (80-.).*, 334(6054), pp. 358–362.

Fusté J.M., Wanrooij S., Jemt E., Granycome C.E., Cluett T.J., Shi Y., *et al.* (2010) 'Mitochondrial RNA Polymerase Is Needed for Activation of the Origin of Light-Strand DNA Replication', *Mol. Cell*, 37(1), pp. 67–78.

Galbiati S., Bordoni A., Papadimitriou D., Toscano A., Rodolico C., Katsarou E., *et al.* (2006) 'New mutations in TK2 gene associated with mitochondrial DNA depletion', *Pediatr. Neurol.* 2006/03/01, 34(3), pp. 177–185.

Gammage P.A., Moraes C.T., & Minczuk M. (2018) 'Mitochondrial Genome Engineering: The Revolution May Not Be CRISPR-Ized', *Trends Genet.*, 34(2), pp. 101–110.

Gandhi V. V. & Samuels D.C. (2011) 'A review comparing deoxyribonucleoside triphosphate (dNTP) concentrations in the mitochondrial and cytoplasmic compartments of normal and transformed cells', *Nucleosides, Nucleotides and Nucleic Acids*, 30(5), pp. 317–339.

Gandre-Babbe S. & Van Der Bliek A.M. (2008) 'The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells', *Mol. Biol. Cell*, 19(6).

Gao M., Yi J., Zhu J., Minikes A.M., Monian P., Thompson C.B., *et al.* (2019) 'Role of Mitochondria in Ferroptosis', *Mol. Cell*, 73(2), pp. 354-363.e3.
Garone C., Garcia-Diaz B., Emmanuele V., Lopez L.C., Tadesse S., Akman H.O., *et al.* (2014) 'Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency', *EMBO Mol. Med.*, 6(8), pp. 1016–1027.
Garone C., Taylor R.W., Nascimento A., Poulton J., Fratter C., Domínguez-González C., *et al.* (2018) 'Retrospective natural history of thymidine kinase 2 deficiency', *J. Med. Genet.*, 55(8), pp. 515–521.
Garone C. & Viscomi C. (2018) 'Towards a therapy for mitochondrial disease: An update', *Biochem. Soc. Trans.*, 46(5), pp. 1247–1261.
Ge S.X., Jung D., Jung D., & Yao R. (2020) 'ShinyGO: A graphical gene-set enrichment tool for animals and plants', *Bioinformatics*, 36(8), pp. 2628–2629.
Gilford H. (1904) 'Progeria, a form of senilism', *J. Am. Med. Assoc.*, XLIII(13), pp. 894–895.
De Giorgio R., Pironi L., Rinaldi R., Boschetti E., Caporali L., Capristo M., *et al.* (2016) 'Liver transplantation for mitochondrial neurogastrointestinal encephalomyopathy', *Ann. Neurol.*, 80(3), pp. 448–455.
Van Goethem G., Dermaut B., Löfgren A., Martin J.J., & Van Broeckhoven C. (2001) 'Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions', *Nat. Genet.*, 28(3), pp. 211–212.
Van Goethem G., Luoma P., Rantamäki M., Al Memar A., Kaakkola S., Hackman P., *et al.* (2004) 'POLG mutations in neurodegenerative disorders with ataxia but no muscle involvement', *Neurology*, 63(7), pp. 1251–1257.
Gordon L.B., Rothman F.G., López-Otín C., & Misteli T. (2014) 'Progeria: A paradigm for translational medicine', *Cell*, 156(3), pp. 400–407.
Gorman G.S., Chinnery P.F., DiMauro S., Hirano M., Koga Y., McFarland R., *et al.* (2016) 'Mitochondrial diseases', *Nat. Rev. Dis. Prim.*, 2(1), p. 16080.
Göttle M., Prudente C.N., Fu R., Sutcliffe D., Pang H., Cooper D., *et al.* (2014) 'Loss of dopamine phenotype among midbrain neurons in Lesch-Nyhan disease', *Ann. Neurol.*, 76(1), pp. 95–107.
Götz A., Tyynismaa H., Euro L., Ellonen P., Hyötyläinen T., Ojala T., *et al.* (2011) 'Exome sequencing identifies mitochondrial alanyl-tRNA synthetase mutations in infantile mitochondrial cardiomyopathy', *Am. J. Hum. Genet.*, 88(5), pp. 635–642.
Gray H. & Tai Wai Wong (1992) 'Purification and identification of subunit structure of the human mitochondrial DNA polymerase', *J. Biol. Chem.*, 267(9), pp. 5835–5841.
Gray M.W., Burger G., & Lang B.F. (1999) 'Mitochondrial evolution', *Science (80-.).*, pp. 1476–1481.
Gu Y., Seidl K.J., Rathbun G.A., Zhu C., Manis J.P., Van Der Stoep N., *et al.* (1997) 'Growth retardation and leaky SCID phenotype of Ku70-deficient mice', *Immunity*, 7(5).
Guo J., Duan L., He X., Li S., Wu Y., Xiang G., *et al.* (2021) 'A Combined Model of Human iPSC-Derived Liver Organoids and Hepatocytes Reveals Ferroptosis in DGUOK Mutant mtDNA Depletion Syndrome', *Adv. Sci.*, 8(10).
Gut P., Matilainen S., Meyer J.G., Pällijeff P., Richard J., Carroll C.J., *et al.* (2020) 'SUCLA2 mutations cause global protein succinylation contributing to the pathomechanism of a hereditary mitochondrial disease', *Nat. Commun.*, 11(1).
Haber F. & Weiss J. (1932) 'Über die Katalyse des Hydroperoxydes', *Naturwissenschaften*, 20(51).
Hackenbrock C.R. (1966) 'Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria.', *J. Cell Biol.*, 30(2), pp. 269–297.
Hakonen A.H., Heiskanen S., Juvonen V., Lappalainen I., Luoma P.T., Rantamäki M., *et al.* (2005) 'Mitochondrial DNA polymerase W748S mutation: A common cause of autosomal recessive ataxia with ancient European origin', *Am. J. Hum. Genet.*, 77(3), pp. 430–441.
Hakonen A.H., Isohanni P., Paetau A., Herva R., Suomalainen A., & Lönnqvist T. (2007) 'Recessive Twinkle mutations in early onset encephalopathy with mtDNA depletion', *Brain*. 2007/10/09, 130(11), pp. 3032–3040.
Hakonen A.H., Goffart S., Marjavaara S., Paetau A., Cooper H., Mattila K., *et al.* (2008) 'Infantile-onset spinocerebellar ataxia and mitochondrial recessive ataxia syndrome are associated with neuronal complex I defect and mtDNA depletion', *Hum. Mol. Genet.*, 17(23), pp. 3822–3835.
Hällberg B.M. & Larsson N.G. (2014) 'Making proteins in the powerhouse', *Cell Metab.*, pp. 226–240.
Halter J., Schüpbach W.M.M., Casali C., Elhasid R., Fay K., Hammans S., *et al.* (2011) 'Allogeneic hematopoietic SCT as treatment option for patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE): A consensus conference proposal for a standardized approach', *Bone Marrow Transplant.*, 46(3), pp. 330–337.
Hämäläinen R.H., Ahlqvist K.J., Ellonen P., Lepistö M., Logan A., Otonkoski T., *et al.* (2015) 'MtDNA Mutagenesis Disrupts Pluripotent Stem Cell Function by Altering Redox Signaling', *Cell Rep.*, 11(10), pp. 1614–1624.

Hämäläinen R.H., Landoni J.C., Ahlqvist K.J., Goffart S., Ryytty S., Rahman M.O., *et al.* (2019) 'Defects in mtDNA replication challenge nuclear genome stability through nucleotide depletion and provide a unifying mechanism for mouse progerias', *Nat. Metab.*, 1(10), pp. 958–965.

Hämäläinen R.H., Landoni J.C., Ahlqvist K.J., Goffart S., Ryytty S., Rahman M.O., *et al.* (2020) 'Reply to: Proofreading deficiency in mitochondrial DNA polymerase does not affect total dNTP pools in mouse embryos', *Nat. Metab.*, 2(8), pp. 676–677.

Hamanaka R.B. & Chandel N.S. (2010) 'Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes', *Trends Biochem. Sci.*, 35(9), pp. 505–513.

Harding C., Heuser J., & Stahl P. (1983) 'Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes.', *J. Cell Biol.*, 97(2), pp. 329–339.

Harman D. (1972) 'The Biologic Clock: The Mitochondria?', *J. Am. Geriatr. Soc.*, 20(4), pp. 145–147.

Hart P.E., Lodi R., Rajagopalan B., Bradley J.L., Crilley J.G., Turner C., *et al.* (2005) 'Antioxidant treatment of patients with Friedreich ataxia: Four-year follow-up', *Arch. Neurol.*, 62(4), pp. 621–626.

Hashimoto Y., Niikura T., Tajima H., Yasukawa T., Sudo H., Ito Y., *et al.* (2001) 'A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and Aβ', *Proc. Natl. Acad. Sci. U. S. A.*, 98(11), pp. 6336–6341.

Hatefi Y., Haavik A.G., Fowler L.R., & Griffiths D.E. (1962) 'Studies on the Electron Transfer System', *J. Biol. Chem.*, 237(8), pp. 2661–2669.

Head B., Griparic L., Amiri M., Gandre-Babbe S., & Van Der Bliek A.M. (2009) 'Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells', *J. Cell Biol.*, 187(7), pp. 959–966.

Helle S.C.J., Feng Q., Aebersold M.J., Hirt L., Grüter R.R., Vahid A., *et al.* (2017) 'Mechanical force induces mitochondrial fission', *Elife*, 6.

Hershey A.D. & Chase M. (1952) 'Independent functions of viral protein and nucleic acid in growth of bacteriophage.', *J. Gen. Physiol.*, 36(1), pp. 39–56.

Hirano M., Martí R., Casali C., Tadesse S., Uldrick T., Fine B., *et al.* (2006) 'Allogeneic stem cell transplantation corrects biochemical derangements in MNGIE', *Neurology*, 67(8), pp. 1458–1460.

Hollenbeck P.J. & Saxton W.M. (2005) 'The axonal transport of mitochondria', *J. Cell Sci.*, 118(23).

Holmström K.M. & Finkel T. (2014) 'Cellular mechanisms and physiological consequences of redox-dependent signalling', *Nat. Rev. Mol. Cell Biol.*, 15(6), pp. 411–421.

Holt I.J., Lorimer H.E., & Jacobs H.T. (2000) 'Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA', *Cell.* 2000/03/18, 100(5), pp. 515–524.

Hutchinson J. (1886) 'Congenital Absence of Hair and Mammary Glands', *Med. Chir. Trans.*, 69, pp. 473–477.

Hyslop L.A., Blakeley P., Craven L., Richardson J., Fogarty N.M.E., Fragouli E., *et al.* (2016) 'Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease', *Nature*, 534(7607), pp. 383–386.

Iacopetta B.J. & Morgan E.H. (1983) 'The kinetics of transferrin endocytosis and iron uptake from transferrin in rabbit reticulocytes', *J. Biol. Chem.*, 258(15), pp. 9108–9115.

Ichinose H., Ohye T., Takahashi E. ichi, Seki N., Hori T. aki, Segawa M., *et al.* (1994) 'Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene', *Nat. Genet.*, 8(3), pp. 236–242.

Ignatenko O., Nikkanen J., Kononov A., Zamboni N., Ince-Dunn G., & Suomalainen A. (2020) 'Mitochondrial spongiotic brain disease: Astrocytic stress and harmful rapamycin and ketosis effect', *Life Sci. Alliance*, 3(9).

Ikeda M., Ide T., Fujino T., Arai S., Saku K., Kakino T., *et al.* (2015) 'Overexpression of TFAM or twinkle increases mtDNA copy number and facilitates cardioprotection associated with limited mitochondrial oxidative stress', *PLoS One*, 10(3).

Inoue T., Ikeda M., Ide T., Fujino T., Matsuo Y., Arai S., *et al.* (2016) 'Twinkle overexpression prevents cardiac rupture after myocardial infarction by alleviating impaired mitochondrial biogenesis', *Am. J. Physiol. - Hear. Circ. Physiol.*, 311(3).

Ishihara N., Eura Y., & Mihara K. (2004) 'Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity', *J. Cell Sci.*, 117(26), pp. 6535–6546.

Isokallio M.A. & Stewart J.B. (2021) 'High-Throughput Detection of mtDNA Mutations Leading to tRNA Processing Errors', in *Methods Mol. Biol.*, pp. 117–132.

Itoh Y., Andréll J., Choi A., Richter U., Maiti P., Best R.B., *et al.* (2021) 'Mechanism of membrane-tethered mitochondrial protein synthesis', *Science (80-.).*, 371(6531), pp. 846–849.

Jackson C.B., Huemer M., Bolognini R., Martin F., Szinnai G., Donner B.C., *et al.* (2019) 'A variant in MRPS14 (uS14m) causes perinatal hypertrophic cardiomyopathy with neonatal lactic acidosis, growth retardation, dysmorphic features and neurological involvement', *Hum. Mol. Genet.*, 28(4).

Jacobs H.T., Lehtinen S.K., & Spelbrink J.N. (2000) 'No sex please, we're mitochondria: A hypothesis on the somatic unit of inheritance of mammalian mtDNA', *BioEssays*.

Jain I.H., Zazzeron L., Goli R., Alexa K., Schatzman-Bone S., Dhillon H., *et al.* (2016) 'Hypoxia as a therapy for mitochondrial disease', *Science (80-.).*, 352(6281), pp. 54–61.

Jain I.H., Zazzeron L., Goldberger O., Marutani E., Wojtkiewicz G.R., Ast T., *et al.* (2019) 'Leigh Syndrome Mouse Model Can Be Rescued by Interventions that Normalize Brain Hyperoxia, but Not HIF Activation', *Cell Metab.*, 30(4), pp. 824-832.e3.

James D.I., Parone P.A., Mattenberger Y., & Martinou J.C. (2003) 'hFis1, a novel component of the mammalian mitochondrial fission machinery', *J. Biol. Chem.*, 278(38), pp. 36373–36379.

Jiang M., Kauppila T.E.S., Motori E., Li X., Atanassov I., Folz-Donahue K., *et al.* (2017) 'Increased Total mtDNA Copy Number Cures Male Infertility Despite Unaltered mtDNA Mutation Load', *Cell Metab.*, 26(2), pp. 429-436.e4.

Jiang X., Stockwell B.R., & Conrad M. (2021) 'Ferroptosis: mechanisms, biology and role in disease', *Nat. Rev. Mol. Cell Biol.*, pp. 266–282.

Johnson A.A. & Johnson K.A. (2001) 'Fidelity of Nucleotide Incorporation by Human Mitochondrial DNA Polymerase', *J. Biol. Chem.*, 276(41), pp. 38090–38096.

Johnson S.C., Yanos M.E., Kayser E.B., Quintana A., Sangesland M., Castanza A., *et al.* (2013) 'MTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome', *Science (80-.).*, 342(6165), pp. 1524–1528.

Jokinen R., Marttinen P., Stewart J.B., Neil Dear T., & Battersby B.J. (2016) 'Tissue-specific modulation of mitochondrial DNA segregation by a defect in mitochondrial division', *Hum. Mol. Genet.*, 25(4), pp. 706–714.

Joshi C.N., Greenberg C.R., Mhanni A.A., & Salman M.S. (2009) 'Ketogenic Diet in Alpers-Huttenlocher Syndrome', *Pediatr. Neurol.* 2009/03/24, 40(4), pp. 314–316.

Jourdain A.A., Boehm E., Maundrell K., & Martinou J.C. (2016) 'Mitochondrial RNA granules: Compartmentalizing mitochondrial gene expression', *J. Cell Biol.*, 212(6), pp. 611–614.

Kaguni L.S. (2004) 'DNA polymerase γ, the mitochondrial replicase', *Annu. Rev. Biochem.*, 73(1), pp. 293–320.

Kamerkar S.C., Kraus F., Sharpe A.J., Pucadyil T.J., & Ryan M.T. (2018) 'Dynamin-related protein 1 has membrane constricting and severing abilities sufficient for mitochondrial and peroxisomal fission', *Nat. Commun.*, 9(1).

Kanehisa M., Sato Y., Kawashima M., Furumichi M., & Tanabe M. (2016) 'KEGG as a reference resource for gene and protein annotation', *Nucleic Acids Res.*, 44(D1), pp. D457–D462.

Kang E., Wu J., Gutierrez N.M., Koski A., Tippner-Hedges R., Agaronyan K., *et al.* (2016) 'Mitochondrial replacement in human oocytes carrying pathogenic mitochondrial DNA mutations', *Nature*, 540(7632), pp. 270–275.

Kang H.C., Lee Y.M., Kim H.D., Lee J.S., & Slama A. (2007) 'Safe and effective use of the ketogenic diet in children with epilepsy and mitochondrial respiratory chain complex defects', *Epilepsia*, 48(1), pp. 82–88.

Karaa A., Haas R., Goldstein A., Vockley J., Douglas Weaver W., & Cohen B.H. (2018) 'Randomized dose-escalation trial of elamipretide in adults with primary mitochondrial myopathy', *Neurology*, 90(14), pp. E1212–E1221.

Kaspar S., Oertlin C., Szczepanowska K., Kukat A., Senft K., Lucas C., *et al.* (2021) 'Adaptation to mitochondrial stress requires CHOP-directed tuning of ISR', *Sci. Adv.*, 7(22).

Kaukonen J., Zeviani M., Comi G.P., Piscaglia M.G., Peltonen L., & Suomalainen A. (1999) 'A third locus predisposing to multiple deletions of mtDNA in autosomal dominant progressive external ophthalmoplegia [5]', *Am. J. Hum. Genet.*, 65(1), pp. 256–260.

Kaukonen J., Juselius J.K., Tiranti V., Kyttala A., Zeviani M., Comi G.P., *et al.* (2000) 'Role of adenine nucleotide translocator 1 in mtDNA maintenance', *Science (80-.).*, 289(5480), pp. 782–785.

Kaushal S. & Freudenreich C.H. (2019) 'The role of fork stalling and DNA structures in causing chromosome fragility', *Genes Chromosom. Cancer*, pp. 270–283.

Kee K. & Naughton M.T. (2010) 'Heart failure and the lung', *Circ. J.*

Keshavan N., Abdenur J., Anderson G., Assouline Z., Barcia G., Bouhikbar L., *et al.* (2020) 'The natural history of infantile mitochondrial DNA depletion syndrome due to RRM2B deficiency', *Genet. Med.*, 22(1), pp. 199–209.

Keshavan N. & Rahman S. (2018) 'Natural history of mitochondrial disorders: A systematic review', *Essays Biochem.*, 62(3), pp. 423–442.

Khan N.A., Auranen M., Paetau I., Pirinen E., Euro L., Forsström S., *et al.* (2014) 'Effective treatment of mitochondrial myopathy by nicotinamide riboside, a vitamin B3', *EMBO Mol. Med.*, 6(6), pp. 721–731.

Khan N.A., Nikkanen J., Yatsuga S., Jackson C., Wang L., Pradhan S., *et al.* (2017) 'mTORC1 Regulates Mitochondrial Integrated Stress Response and Mitochondrial Myopathy Progression', *Cell Metab.*, 26(2), pp. 419-428.e5.

Kleele T., Rey T., Winter J., Zaganelli S., Mahecic D., Perreten Lambert H., *et al.* (2021) 'Distinct fission signatures predict mitochondrial degradation or biogenesis', *Nature*, 593(7859), pp. 435–439.

Korhonen J.A., Pham X.H., Pellegrini M., & Falkenberg M. (2004) 'Reconstitution of a minimal mtDNA replisome in vitro', *EMBO J.* 2004/05/29, 23(12), pp. 2423–2429.

Korhonen J.A., Gaspari M., & Falkenberg M. (2003) 'TWINKLE has 5' → 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein', *J. Biol. Chem.*, 278(49), pp. 48627–48632.

Korona D.A., Lecompte K.G., & Pursell Z.F. (2011) 'The high fidelity and unique error signature of human DNA polymerase ε', *Nucleic Acids Res.*, 39(5).

Koshiba T., Detmer S.A., Kaiser J.T., Chen H., McCaffery J.M., & Chan D.C. (2004) 'Structural basis of mitochondrial tethering by mitofusin complexes', *Science (80-.).*, 305(5685), pp. 858–862.

Krasich R. & Copeland W.C. (2017) 'DNA polymerases in the mitochondria: A critical review of the evidence', *Front. Biosci. - Landmark*, 22(4), pp. 692–709.

Kraus F., Roy K., Pucadyil T.J., & Ryan M.T. (2021) 'Function and regulation of the divisome for mitochondrial fission', *Nature*.

Krebs H.A. & Johnson W.A. (1937) 'Metabolism of ketonic acids in animal tissues', *Biochem. J.*, 31(4), pp. 645–660.

Kripps K.A., Nakayuenyongsuk W., Shayota B.J., Berquist W., Gomez-Ospina N., Esquivel C.O., *et al.* (2020) 'Successful liver transplantation in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)', *Mol. Genet. Metab.*, 130(1), pp. 58–64.

Kujoth C.C., Hiona A., Pugh T.D., Someya S., Panzer K., Wohlgemuth S.E., *et al.* (2005) 'Medicine: Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging', *Science (80-.).*, 309(5733), pp. 481–484.

Kukat C., Wurm C.A., Spåhr H., Falkenberg M., Larsson N.G., & Jakobs S. (2011) 'Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA', *Proc. Natl. Acad. Sci. U. S. A.*, 108(33), pp. 13534–13539.

Kumar S., Sheokand N., Mhadeshwar M.A., Raje C.I., & Raje M. (2012) 'Characterization of glyceraldehyde-3-phosphate dehydrogenase as a novel transferrin receptor', *Int. J. Biochem. Cell Biol.*, 44(1), pp. 189–199.

Kuukasjärvi A., Landoni J.C., Kaukonen J., Juhakoski M., Auranen M., Torkkeli T., *et al.* (2021) 'IMPDH2: a new gene associated with dominant juvenile-onset dystonia-tremor disorder', *Eur. J. Hum. Genet.*, 29(12), pp. 1833–1837.

Labbé K., Murley A., & Nunnari J. (2014) 'Determinants and functions of mitochondrial behavior', *Annu. Rev. Cell Dev. Biol.*, 30(1), pp. 357–391.

Labrousse A.M., Zappaterra M.D., Rube D.A., & Van der Bliek A.M. (1999) 'C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane', *Mol. Cell*, 4(5), pp. 815–826.

Lalowski M.M., Björk S., Finckenberg P., Soliymani R., Tarkia M., Calza G., *et al.* (2018) 'Characterizing the key metabolic pathways of the neonatal mouse heart using a quantitative combinatorial omics approach', *Front. Physiol.*, 9(APR), p. 365.

Lam C. wan, Yeung W.L., Ling T. ki, Wong K. chung, & Law C. yiu (2019) 'Deoxythymidylate kinase, DTYMK, is a novel gene for mitochondrial DNA depletion syndrome', *Clin. Chim. Acta*, 496, pp. 93–99.

Landoni J.C., Laalo T., Goffart S., Kivelä R., Skube K., Nieminen A.I., *et al.* (2022) 'Overactive mitochondrial DNA replisome causes neonatal heart failure via ferroptosis', *bioRxiv*. Cold Spring Harbor Laboratory, p. 2022.04.04.485133.

Landoni J.C., Wang L., & Suomalainen A. (2018) 'Quantitative solid-phase assay to measure deoxynucleoside triphosphate pools', *Biol. Methods Protoc.*, 3(1).

Landoni J.C., Wang L., & Suomalainen A. (2021) 'Whole-Cell and Mitochondrial dNTP Pool Quantification from Cells and Tissues', in *Methods Mol. Biol.*, pp. 143–151.

Lane N. & Martin W. (2010) 'The energetics of genome complexity', *Nature*, 467(7318), pp. 929–934.

Larsson N.G., Wang J., Wilhelmsson H., Oldfors A., Rustin P., Lewandoski M., *et al.* (1998) 'Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice', *Nat. Genet.*, 18(3), pp. 231–236.

Lee C., Zeng J., Drew B.G., Sallam T., Martin-Montalvo A., Wan J., *et al.* (2015) 'The mitochondrial-derived peptide MOTS-c promotes metabolic homeostasis and reduces obesity and insulin resistance', *Cell Metab.*, 21(3), pp. 443–454.

Lesch M. & Nyhan W.L. (1964) 'A familial disorder of uric acid metabolism and central nervous system function', *Am. J. Med.*, 36(4), pp. 561–570.

Letts J.A., Fiedorczuk K., & Sazanov L.A. (2016) 'The architecture of respiratory supercomplexes', *Nature*, 537(7622), pp. 644–648.

Levi S., Corsi B., Bosisio M., Invernizzi R., Volz A., Sanford D., *et al.* (2001) 'A Human Mitochondrial Ferritin Encoded by an Intronless Gene', *J. Biol. Chem.*, 276(27), pp. 24437–24440.

Lewis M.R. & Lewis W.H. (1915) 'Mitochondria (and other cytoplasmic structures) in tissue cultures', *Am. J. Anat.*, 17(3), pp. 339–401.

Lewis S.C., Uchiyama L.F., & Nunnari J. (2016) 'ER-mitochondria contacts couple mtDNA synthesis with Mitochondrial division in human cells', *Science (80-.).*, 353(6296), p. aaf5549.

Li C., Zhang Y., Liu J., Kang R., Klionsky D.J., & Tang D. (2021) 'Mitochondrial DNA stress triggers autophagy-dependent ferroptotic death', *Autophagy*, 17(4), pp. 948–960.

Li P., Nijhawan D., Budihardjo I., Srinivasula S.M., Ahmad M., Alnemri E.S., *et al.* (1997) 'Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade', *Cell*, 91(4).

Li S., Xu S., Roelofs B.A., Boyman L., Jonathan Lederer W., Sesaki H., *et al.* (2015) 'Transient assembly of F-actin on the outer mitochondrial membrane contributes to mitochondrial fission', *J. Cell Biol.*, 208(1).

Lim S.E. & Copeland W.C. (2001) 'Differential Incorporation and Removal of Antiviral Deoxynucleotides by Human DNA Polymerase γ', *J. Biol. Chem.*, 276(26), pp. 23616–23623.

Lim S.E., Longley M.J., & Copeland W.C. (1999) 'The mitochondrial p55 accessory subunit of human DNA polymerase γ enhances DNA binding, promotes processive DNA synthesis, and confers N- ethylmaleimide resistance', *J. Biol. Chem.*, 274(53), pp. 38197–38203.

Livingston E.H. (2004) 'Who was student and why do we care so much about his t-test?', in *J. Surg. Res.*, pp. 58–65.

Longley M.J., Prasad R., Srivastava D.K., Wilson S.H., & Copeland W.C. (1998) 'Identification of 5'-deoxyribose phosphate lyase activity in human DNA polymerase γ and its role in mitochondrial base excision repair in vitro', *Proc. Natl. Acad. Sci. U. S. A.*, 95(21), pp. 12244–12248.

Lopez-Gomez C., Levy R.J., Sanchez-Quintero M.J., Juanola-Falgarona M., Barca E., Garcia-Diaz B., *et al.* (2017) 'Deoxycytidine and Deoxythymidine Treatment for Thymidine Kinase 2 Deficiency', *Ann. Neurol.*, 81(5), pp. 641–652.

López-Otín C., Blasco M.A., Partridge L., Serrano M., & Kroemer G. (2013) 'The hallmarks of aging', *Cell*, 153(6), p. 1194.

López L.C., Akman H.O., García-Cazorla Á., Dorado B., Martí R., Nishino I., *et al.* (2009) 'Unbalanced deoxynucleotide pools cause mitochondrial DNA instability in thymidine phosphorylase-deficient mice', *Hum. Mol. Genet.*, 18(4), pp. 714–722.

Love M.I., Huber W., & Anders S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biol.*, 15(12), pp. 1–21.

Luo S., Valencia C.A., Zhang J., Lee N.C., Slone J., Gui B., *et al.* (2018) 'Biparental inheritance of mitochondrial DNA in humans', *Proc. Natl. Acad. Sci. U. S. A.*, 115(51), pp. 13039–13044.

Luoma P., Melberg A., Rinne J.O., Kaukonen J.A., Nupponen N.N., Chalmers R.M., *et al.* (2004) 'Parkinsonism, premature menopause, and mitochondrial DNA polymerase γ mutations: Clinical and molecular genetic study', *Lancet*, 364(9437), pp. 875–882.

Macao B., Uhler J.P., Siibak T., Zhu X., Shi Y., Sheng W., *et al.* (2015) 'The exonuclease activity of DNA polymerase γ is required for ligation during mitochondrial DNA replication', *Nat. Commun.*, 6(1), p. 7303.

Mah L.J., El-Osta A., & Karagiannis T.C. (2010) 'γh2AX: A sensitive molecular marker of DNA damage and repair', *Leukemia*, 24(4), pp. 679–686.

Mandel H., Szargel R., Labay V., Elpeleg O., Saada A., Shalata A., *et al.* (2001) 'The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA', *Nat. Genet.*, 29(3), pp. 337–341.

Margulis L. (1970) 'Recombination of non-chromosomal genes in Chlamydomonas: Assortment of mitochondria and chloroplasts?', *J. Theor. Biol.*, 26(2), pp. 337–342.

Martí R., Dorado B., & Hirano M. (2012) 'Measurement of mitochondrial dNTP pools', in *Methods Mol. Biol.*, pp. 135–148.

Martijn J., Vosseberg J., Guy L., Offre P., & Ettema T.J.G. (2018) 'Deep mitochondrial origin outside the sampled alphaproteobacteria', *Nature*, 557(7703), pp. 101–105.

Martin S.K. & Wood R.D. (2019) 'DNA polymerase ζ in DNA replication and repair', *Nucleic Acids Res.*, 47(16), pp. 8348–8361.

Mathews C.K. (2006) 'DNA precursor metabolism and genomic stability', *FASEB J.*, 20(9), pp. 1300–1314.

Mathews C.K. (2014) 'Deoxyribonucleotides as genetic and metabolic regulators', *FASEB J.*, 28(9), pp. 3832–3840.

Matilainen O., Quirós P.M., & Auwerx J. (2017) 'Mitochondria and Epigenetics – Crosstalk in Homeostasis and Stress', *Trends Cell Biol.*, 27(6), pp. 453–463.

McBride H.M., Neuspiel M., & Wasiak S. (2006) 'Mitochondria: More Than Just a Powerhouse', *Curr. Biol.*, 16(14).

McDaniel S.S., Rensing N.R., Thio L.L., Yamada K.A., & Wong M. (2011) 'The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway', *Epilepsia*, 52(3).

McLelland G.L., Soubannier V., Chen C.X., McBride H.M., & Fon E.A. (2014) 'Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control', *EMBO J.*, 33(4), pp. 282–295.

Medeiros T.C., Thomas R.L., Ghillebert R., & Graef M. (2018) 'Autophagy balances mtDNA synthesis and degradation by DNA polymerase POLG during starvation', *J. Cell Biol.*, 217(5), pp. 1601–1611.

Meeusen S., McCaffery J.M., & Nunnari J. (2004) 'Mitochondrial fusion intermediates revealed in vitro', *Science (80-.).*, 305(5691), pp. 1747–1752.

Meier T., Perlman S.L., Rummey C., Coppard N.J., & Lynch D.R. (2012) 'Assessment of neurological efficacy of idebenone in pediatric patients with Friedreich's ataxia: Data from a 6-month controlled study followed by a 12-month open-label extension study', *J. Neurol.*, 259(2), pp. 284–291.

Di Meo I., Auricchio A., Lamperti C., Burlina A., Viscomi C., & Zeviani M. (2012) 'Effective AAV-mediated gene therapy in a mouse model of ethylmalonic encephalopathy', *EMBO Mol. Med.*, 4(9), pp. 1008–1014.

Di Meo I., Marchet S., Lamperti C., Zeviani M., & Viscomi C. (2017) 'AAV9-based gene therapy partially ameliorates the clinical phenotype of a mouse model of Leigh syndrome', *Gene Ther.*, 24(10), pp. 661–667.

Mi J., Tian G., Liu S., Li X., Ni T., Zhang L., *et al.* (2015) 'The relationship between altered mitochondrial DNA copy number and cancer risk: A meta-analysis', *Sci. Rep.*, 5.

Mitchell P. (1961) 'Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism', *Nature*, 191(4784), pp. 144–148.

Mitchell P. & Moyle J. (1967) 'Chemiosmotic hypothesis of oxidative phosphorylation', *Nature*, 213(5072), pp. 137–139.

Møller S. & Bernardi M. (2013) 'Interactions of the heart and the liver', *Eur. Heart J.*

Moraes C.T., DiMauro S., Zeviani M., Lombes A., Shanske S., Miranda A.F., *et al.* (1989) 'Mitochondrial DNA Deletions in Progressive External Ophthalmoplegia and Kearns-Sayre Syndrome', *N. Engl. J. Med.*, 320(20), pp. 1293–1299.

Morino H., Pierce S.B., Matsuda Y., Walsh T., Ohsawa R., Newby M., *et al.* (2014) 'Mutations in Twinkle primase-helicase cause Perrault syndrome with neurologic features', *Neurology*, 83(22), pp. 2054–2061.

Murley A., Lackner L.L., Osman C., West M., Voeltz G.K., Walter P., *et al.* (2013) 'ER-associated mitochondrial division links the distribution of mitochondria and mitochondrial DNA in yeast', *Elife*, 2013(2).

Murphy M.P. (2009) 'How mitochondria produce reactive oxygen species', *Biochem. J.*, 417(1), pp. 1–13.

Nagashima S., Tábara L.C., Tilokani L., Paupe V., Anand H., Pogson J.H., *et al.* (2020) 'Golgi-derived PI(4)P-containing vesicles drive late steps of mitochondrial division', *Science (80-.).*, 367(6484), pp. 1366–1371.

Nass M.M. (1966) 'The circularity of mitochondrial DNA.', *Proc. Natl. Acad. Sci. U. S. A.*, 56(4), pp. 1215–1222.

Nass M.M.K. (1969) 'Mitochondrial DNA. I. Intramitochondrial distribution and structural relations of single- and double-length circular DNA', *J. Mol. Biol.*, 42(3).

Nass M.M.K. & Nass S. (1963) 'Intramitochondrial Fibers With DNA Characteristics', *J. Cell Biol.*, 19(3), pp. 593–611.

Nass M.M.K., Nass S., & Afzelius B.A. (1965) 'The general occurrence of mitochondrial DNA', *Exp. Cell Res.*, 37(3), pp. 516–539.

Naviaux R.K. & Nguyen K. V. (2004) 'POLG Mutations Associated with Alpers' Syndrome and Mitochondrial DNA Depletion', *Ann. Neurol.*, 55(5), pp. 706–712.

Nelson D.L. & Cox M.M. (2017) *Lehninger Principles of Biochemistry 7th, W.H. Free. Co.*

Neupert W. & Herrmann J.M. (2007) 'Translocation of proteins into mitochondria', *Annu. Rev. Biochem.*, 76, pp. 723–749.

Neuspiel M., Schauss A.C., Braschi E., Zunino R., Rippstein P., Rachubinski R.A., *et al.* (2008) 'Cargo-Selected Transport from the Mitochondria to Peroxisomes Is Mediated by Vesicular Carriers', *Curr. Biol.*, 18(2), pp. 102–108.

Ngo H.B., Kaiser J.T., & Chan D.C. (2011) 'The mitochondrial transcription and packaging factor Tfam imposes a U-turn on mitochondrial DNA', *Nat. Struct. Mol. Biol.*, 18(11), pp. 1290–1296.

Nicholls T.J., Nadalutti C.A., Motori E., Sommerville E.W., Gorman G.S., Basu S., *et al.* (2018) 'Topoisomerase 3α Is Required for Decatenation and Segregation of Human mtDNA', *Mol. Cell*, 69(1), pp. 9-23.e6.

Nikali K., Suomalainen A., Saharinen J., Kuokkanen M., Spelbrink J.N., Lönnqvist T., *et al.* (2005) 'Infantile onset spinocerebellar ataxia is caused by recessive mutations in mitochondrial proteins Twinkle and Twinky', *Hum. Mol. Genet.*, 14(20), pp. 2981–2990.

Nikkanen J., Forsström S., Euro L., Paetau I., Kohnz R.A., Wang L., *et al.* (2016) 'Mitochondrial DNA Replication Defects Disturb Cellular dNTP Pools and Remodel One-Carbon Metabolism', *Cell Metab.*, 23(4), pp. 635–648.

Nikkanen J., Landoni J.C., Balboa D., Haugas M., Partanen J., Paetau A., *et al.* (2018) 'A complex genomic locus drives mt DNA replicase POLG expression to its disease-related nervous system regions', *EMBO Mol. Med.*, 10(1), pp. 13–21.

Nishino I., Spinazzola A., & Hirano M. (1999) 'Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder', *Science (80-.).*, 283(5402), pp. 689–692.

Nissanka N., Bacman S.R., Plastini M.J., & Moraes C.T. (2018) 'The mitochondrial DNA polymerase gamma degrades linear DNA fragments precluding the formation of deletions', *Nat. Commun.*, 9(1), p. 2491.

Norddahl G.L., Pronk C.J., Wahlestedt M., Sten G., Nygren J.M., Ugale A., *et al.* (2011) 'Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging', *Cell Stem Cell.* 2011/05/10, 8(5), pp. 499–510.

Nordlund P. & Reichard P. (2006) 'Ribonucleotide reductases', *Annu. Rev. Biochem.*, pp. 681–706.

Nunnari J., Marshall W.F., Straight A., Murray A., Sedat J.W., & Walter P. (1997) 'Mitochondrial transmission during mating in Saccharomyces cerevisiae is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA', *Mol. Biol. Cell*, 8(7), pp. 1233–1242.

Nunnari J. & Suomalainen A. (2012) 'Mitochondria: In sickness and in health', *Cell*, 148(6), pp. 1145–1159.

Nyhan W.L. (2005) 'Disorders of purine and pyrimidine metabolism', *Mol. Genet. Metab.*, 86(1–2), pp. 25–33.

Olson M.W. & Kaguni L.S. (1992) '3 ' + 5 ' Exonuclease in Drosophila Mitochondrial DNA Polymerase', *J. Biol. Chem.*, 267(32), pp. 23136–23142.

Orme Johnson N.R., Hansen R.E., & Beinert H. (1974) 'Electron paramagnetic resonance detectable electron acceptors in beef heart mitochondria. Ubihydroquinone cytochrome c reductase segment of the electron transfer system and complex mitochondrial fragments', *J. Biol. Chem.*, 249(6), pp. 1928–1939.

Palmer C.S., Osellame L.D., Laine D., Koutsopoulos O.S., Frazier A.E., & Ryan M.T. (2011) 'MiD49 and MiD51, new components of the mitochondrial fission machinery', *EMBO Rep.*, 12(6).

Pancsa R., Fichó E., Molnár D., Surányi É.V., Trombitás T., Füzesi D., *et al.* (2022) 'dNTPpoolDB: a manually curated database of experimentally determined dNTP pools and pool changes in biological samples', *Nucleic Acids Res.*, 50(D1), pp. D1508–D1514.

Paumard P. (2002) 'The ATP synthase is involved in generating mitochondrial cristae morphology', *EMBO J.*, 21(3), pp. 221–230.

Pearce S.F., Rebelo-Guiomar P., D'Souza A.R., Powell C.A., Van Haute L., & Minczuk M. (2017) 'Regulation of Mammalian Mitochondrial Gene Expression: Recent Advances', *Trends Biochem. Sci.*, 42(8), pp. 625–639.

Peeva V., Blei D., Trombly G., Corsi S., Szukszto M.J., Rebelo-Guiomar P., *et al.* (2018) 'Linear mitochondrial DNA is rapidly degraded by components of the replication machinery', *Nat. Commun.*, 9(1), pp. 1–11.

Pernas L., Bean C., Boothroyd J.C., & Scorrano L. (2018) 'Mitochondria Restrict Growth of the Intracellular Parasite Toxoplasma gondii by Limiting Its Uptake of Fatty Acids', *Cell Metab.*, 27(4), pp. 886-897.e4.

Pernas L. & Scorrano L. (2016) 'Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function', *Annu. Rev. Physiol.*

Pickles S., Vigié P., & Youle R.J. (2018) 'Mitophagy and Quality Control Mechanisms in Mitochondrial Maintenance', *Curr. Biol.*

Pirinen E., Auranen M., Khan N.A., Brilhante V., Urho N., Pessia A., *et al.* (2020) 'Niacin Cures Systemic NAD+ Deficiency and Improves Muscle Performance in Adult-Onset Mitochondrial Myopathy', *Cell Metab.*, 31(6), pp. 1078-1090.e5.

Pitts K.R., Yoon Y., Krueger E.W., & McNiven M.A. (1999) 'The dynamin-like protein DLP1 is essential for normal distribution and morphology of the endoplasmic reticulum and mitochondria in mammalian cells', *Mol. Biol. Cell*, 10(12), pp. 4403–4417.

Pohjoismäki J.L. & Goffart S. (2017) 'The role of mitochondria in cardiac development and protection', *Free Radic. Biol. Med.*

Pohjoismäki J.L.O., Holmes J.B., Wood S.R., Yang M.Y., Yasukawa T., Reyes A., *et al.* (2010) 'Mammalian mitochondrial DNA replication intermediates are essentially duplex but contain extensive tracts of RNA/DNA hybrid', *J. Mol. Biol.*, 397(5), pp. 1144–1155.

Pohjoismäki J.L.O., Williams S.L., Boettger T., Goffart S., Kim J., Suomalainen A., *et al.* (2013a) 'Overexpression of Twinkle-helicase protects cardiomyocytes from genotoxic stress caused by reactive oxygen species', *Proc. Natl. Acad. Sci. U. S. A.*, 110(48).

Pohjoismäki J.L.O., Krüger M., Al-Furoukh N., Lagerstedt A., Karhunen P.J., & Braun T. (2013b) 'Postnatal cardiomyocyte growth and mitochondrial reorganization cause multiple changes in the proteome of human cardiomyocytes', *Mol. Biosyst.*, 9(6), pp. 1210–1219.

Purhonen J., Rajendran J., Tegelberg S., Smolander O.P., Pirinen E., Kallijärvi J., *et al.* (2018) 'NAD+ repletion produces no therapeutic effect in mice with respiratory chain complex III deficiency and chronic energy deprivation', *FASEB J.*, 32(11), pp. 5913–5926.

Raimondi V., Ciccarese F., & Ciminale V. (2020) 'Oncogenic pathways and the electron transport chain: a dangeROS liaison', *Br. J. Cancer*, 122(2), pp. 168–181.

Rajala N., Gerhold J.M., Martinsson P., Klymov A., & Spelbrink J.N. (2014) 'Replication factors transiently associate with mtDNA at the mitochondrial inner membrane to facilitate replication', *Nucleic Acids Res.*, 42(2), pp. 952–967.

Ramón J., Vila-Julià F., Molina-Granada D., Molina-Berenguer M., Melià M.J., García-Arumí E., *et al.* (2021) 'Therapy prospects for mitochondrial dna maintenance disorders', *Int. J. Mol. Sci.*, 22(12), p. 6447.

Rantamäki M., Krahe R., Paetau A., Cormand B., Mononen I., & Udd B. (2001) 'Adult-onset autosomal recessive ataxia with thalamic lesions in a Finnish family', *Neurology*, 57(6), pp. 1043–1049.

Rath S., Sharma R., Gupta R., Ast T., Chan C., Durham T.J., *et al.* (2021) 'MitoCarta3.0: An updated mitochondrial proteome now with sub-organelle localization and pathway annotations', *Nucleic Acids Res.*, 49(D1), pp. D1541–D1547.

Raudvere U., Kolberg L., Kuzmin I., Arak T., Adler P., Peterson H., *et al.* (2019) 'G:Profiler: A web server for functional enrichment analysis and conversions of gene lists (2019 update)', *Nucleic Acids Res.*, 47(W1), pp. W191–W198.

Reichard P. (1988) 'Interactions between deoxyribonucleotide and DNA synthesis', *Annu. Rev. Biochem.*, 57, pp. 349–374.

Reis A. & Spickett C.M. (2012) 'Chemistry of phospholipid oxidation', *Biochim. Biophys. Acta - Biomembr.*, 1818(10), pp. 2374–2387.

Rey T., Zaganelli S., Cuillery E., Vartholomaiou E., Croisier M., Martinou J.C., *et al.* (2020) 'Mitochondrial RNA granules are fluid condensates positioned by membrane dynamics', *Nat. Cell Biol.*, 22(10), pp. 1180–1186.

Riley J.S. & Tait S.W. (2020) ' Mitochondrial DNA in inflammation and immunity ', *EMBO Rep.*, 21(4), p. e49799.

Robberson D.L., Kasamatsu H., & Vinograd J. (1972) 'Replication of mitochondrial DNA. Circular replicative intermediates in mouse L cells.', *Proc. Natl. Acad. Sci. U. S. A.*, 69(3), pp. 737–741.

Rocha A.G., Franco A., Krezel A.M., Rumsey J.M., Alberti J.M., Knight W.C., *et al.* (2018) 'MFN2 agonists reverse mitochondrial defects in preclinical models of Charcot-Marie-Tooth disease type 2A', *Science (80-.).*, 360(6386), pp. 336–341.

Rouault T.A. & Tong W.H. (2005) 'Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis', *Nat. Rev. Mol. Cell Biol.*, 6(4), pp. 345–351.

Rovio A.T., Marchington D.R., Donat S., Schuppe H.C., Abel J., Fritsche E., *et al.* (2001) 'Mutations at the mitochondrial DNA polymerase (POLG) locus associated with male infertility', *Nat. Genet.*, 29(3), pp. 261–262.

Rubio-Cosials A., Sidow J.F., Jiménez-Menéndez N., Fernández-Millán P., Montoya J., Jacobs H.T., *et al.* (2011) 'Human mitochondrial transcription factor A induces a U-turn structure in the light strand promoter', *Nat. Struct. Mol. Biol.*, 18(11), pp. 1281–1289.

Russell O.M., Gorman G.S., Lightowlers R.N., & Turnbull D.M. (2020) 'Mitochondrial Diseases: Hope for the Future', *Cell*, 181(1), pp. 168–188.

Saada A., Shaag A., Mandel H., Nevo Y., Eriksson S., & Elpeleg O. (2001) 'Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy', *Nat. Genet.*, 29(3), pp. 342–344.

Sagan L. (1967) 'On the origin of mitosing cells', *J. Theor. Biol.* 1967/03/01, 14(3), pp. 255–274.

Santel A. & Fuller M.T. (2001) 'Control of mitochondrial morphology by a human mitofusin', *J. Cell Sci.*, 114(5), pp. 867–874.

Santra S., Gilkerson R.W., Davidson M., & Schon E.A. (2004) 'Ketogenic treatment reduces deleted mitochondrial DNAs in cultured human cells', *Ann. Neurol.*, 56(5), pp. 662–669.

Sarzi E., Seveno M., Piro-Mégy C., Elzière L., Quilès M., Péquignot M., *et al.* (2018) 'OPA1 gene therapy prevents retinal ganglion cell loss in a Dominant Optic Atrophy mouse model', *Sci. Rep.*, 8(1), pp. 1–6.

Schägger H. & Pfeiffer K. (2000) 'Supercomplexes in the respiratory chains of yeast and mammalian mitochondria', *EMBO J.*, 19(8), pp. 1777–1783.

Schriner S.E., Linford N.J., Martin G.M., Treuting P., Ogburn C.E., Emond M., *et al.* (2005) 'Medecine: Extension of murine life span by overexpression of catalase targeted to mitochondria', *Science (80-.).*, 308(5730), pp. 1909–1911.

Schumacher B. & Vijg J. (2019) 'Age is in the nucleus', *Nat. Metab.*

Selcuklu S.D., Donoghue M.T.A., Rehmet K., De Gomes M.S., Fort A., Kovvuru P., *et al.* (2012) 'MicroRNA-9 inhibition of cell proliferation and identification of novel miR-9 targets by transcriptome profiling in breast cancer cells', *J. Biol. Chem.*, 287(35), pp. 29516–29528.

Shaibani A., Shchelochkov O.A., Zhang S., Katsonis P., Lichtarge O., Wong L.J., *et al.* (2009) 'Mitochondrial neurogastrointestinal encephalopathy due to mutations in RRM2B', *Arch. Neurol.*, 66(8), pp. 1028–1032.

Sharma S., Koolmeister C., Tran P., Nilsson A.K., Larsson N.G., & Chabes A. (2020) 'Proofreading deficiency in mitochondrial DNA polymerase does not affect total dNTP pools in mouse embryos', *Nat. Metab.* Edited by Guthrie, c and Fink, G., 2(8), pp. 673–675.

Shaw G.C., Cope J.J., Li L., Corson K., Hersey C., Ackermann G.E., *et al.* (2006) 'Mitoferrin is essential for erythroid iron assimilation', *Nature*, 440(7080), pp. 96–100.

Shen Q., Yamano K., Head B.P., Kawajiri S., Cheung J.T.M., Wang C., *et al.* (2014) 'Mutations in Fis1 disrupt orderly disposal of defective mitochondria', *Mol. Biol. Cell*, 25(1).

Shepard K.A. & Yaffe M.P. (1999) 'The yeast dynamin-like protein, Mgm1p, functions on the mitochondrial outer membrane to mediate mitochondrial inheritance', *J. Cell Biol.*, 144(4), pp. 711–719.

Shewach D.S. (1992) 'Quantitation of deoxyribonucleoside 5'-triphosphates bya sequential boronate and anion-exchange high-pressure liquid chromatographic procedure', *Anal. Biochem.*, 206(1), pp. 178–182.

Shutt T.E. & Gray M.W. (2006) 'Bacteriophage origins of mitochondrial replication and transcription proteins', *Trends Genet.*, 22(2), pp. 90–95.

Siegmund S.E., Yang H., Sharma R., Javors M., Skinner O., Mootha V., *et al.* (2017) 'Low-dose rapamycin extends lifespan in a mouse model of mtDNA depletion syndrome', *Hum. Mol. Genet.*, 26(23), pp. 4588–4605.

Silva-Pinheiro P., Nash P.A., Van Haute L., Mutti C.D., Turner K., & Minczuk M. (2022) 'In vivo mitochondrial base editing via adeno-associated viral delivery to mouse post-mitotic tissue', *Nat. Commun.*, 13(1), p. 750.

Smirnova E., Griparic L., Shurland D.L., & Van der Bliek A.M. (2001) 'Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells', *Mol. Biol. Cell*, 12(8), pp. 2245–2256.

Song Z., Ghochani M., McCaffery J.M., Frey T.G., & Chan D.C. (2009) 'Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion', *Mol. Biol. Cell*, 20(15).

Soonpaa M.H., Kim K.K., Pajak L., Franklin M., & Field L.J. (1996) 'Cardiomyocyte DNA synthesis and binucleation during murine development', *Am. J. Physiol. - Hear. Circ. Physiol.*, 271(5 40-5).

Soubannier V., McLelland G.L., Zunino R., Braschi E., Rippstein P., Fon E.A., *et al.* (2012) 'A vesicular transport pathway shuttles cargo from mitochondria to lysosomes', *Curr. Biol.*, 22(2), pp. 135–141.

Spang A., Saw J.H., Jørgensen S.L., Zaremba-Niedzwiedzka K., Martijn J., Lind A.E., *et al.* (2015) 'Complex archaea that bridge the gap between prokaryotes and eukaryotes', *Nature*, 521(7551), pp. 173–179.

Spelbrink J.N., Li F.Y., Tiranti V., Nikali K., Yuan Q.P., Tariq M., *et al.* (2001) 'Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria', *Nat. Genet.*, 28(3), pp. 223–231.

Spinazzola A., Viscomi C., Fernandez-Vizarra E., Carrara F., D'Adamo P., Calvo S., *et al.* (2006) 'MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion', *Nat. Genet.* 2006/04/04, 38(5), pp. 570–576.

Stanley W.C., Recchia F.A., & Lopaschuk G.D. (2005) 'Myocardial substrate metabolism in the normal and failing heart', *Physiol. Rev.*

Stehling O. & Lill R. (2013) 'The role of mitochondria in cellular iron-sulfur protein biogenesis: Mechanisms, connected processes, and diseases', *Cold Spring Harb. Perspect. Biol.*, 5(8), p. a011312.

Stewart J.D., Horvath R., Baruffini E., Ferrero I., Bulst S., Watkins P.B., *et al.* (2010) 'Polymerase γGene POLG determines the risk of sodium valproate-induced liver toxicity', *Hepatology*, 52(5), pp. 1791–1796.

Stipanuk M.H., Dominy J.E., Lee J.I., & Coloso R.M. (2006) 'Mammalian cysteine metabolism: New insights into regulation of cysteine metabolism', in *J. Nutr.*

Stojkovic G., Makarova A. V., Wanrooij P.H., Forslund J., Burgers P.M., & Wanrooij S. (2016) 'Oxidative DNA damage stalls the human mitochondrial replisome', *Sci. Rep.*, 6(1), pp. 1–13.

Sugiura A., McLelland G., Fon E.A., & McBride H.M. (2014) 'A new pathway for mitochondrial quality control: mitochondrial-derived vesicles', *EMBO J.*, 33(19), pp. 2142–2156.

Suomalainen A., Majander A., Haltia M., Somer H., Lönnqvist J., Savontaus M.L., *et al.* (1992) 'Multiple deletions of mitochondrial DNA in several tissues of a patient with severe retarded depression and familial progressive external ophthalmoplegia', *J. Clin. Invest.*, 90(1), pp. 61–66.

Suomalainen A., Kaukonen J., Amati P., Timonen R., Haltia M., Weissenbach J., *et al.* (1995) 'An autosomal locus predisposing to deletions of mitochondrial DNA', *Nat. Genet.*, 9(2), pp. 146–151.

Suomalainen A. & Battersby B.J. (2018) *Mitochondrial diseases: The contribution of organelle stress responses to pathology*, *Nat. Rev. Mol. Cell Biol.* Nature Publishing Group.

Suomalainen A. & Syvänen A.C. (1996) 'Analysis of nucleotide sequence variations by solid-phase minisequencing.', *Methods Mol. Biol.*, 65(1064-3745 (Print)), pp. 73–79.

Suomalainen A. & Syvänen A.C. (2003) 'Analysis of nucleotide sequence variations by solid-phase minisequencing.', *Methods Mol. Biol.*, 226, pp. 361–366.

Szczepanowska K. & Trifunovic A. (2015) 'Different faces of mitochondrial DNA mutators', *Biochim. Biophys. Acta - Bioenerg.*, pp. 1362–1372.

Szent-Györgyi A. (1928) 'Observations on the function of peroxidase systems and the chemistry of the adrenal cortex', *Biochem. J.*, 22(6), pp. 1387–1409.

Szklarczyk D., Gable A.L., Lyon D., Junge A., Wyder S., Huerta-Cepas J., *et al.* (2019) 'STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets', *Nucleic Acids Res.*, 47(D1), pp. D607–D613.

Tadokoro T., Ikeda M., Ide T., Deguchi H., Ikeda S., Okabe K., *et al.* (2020) 'Mitochondria-dependent ferroptosis plays a pivotal role in doxorubicin cardiotoxicity', *JCI Insight*, 5(9).

Tanaka A., Ide T., Fujino T., Onitsuka K., Ikeda M., Takehara T., *et al.* (2013) 'The Overexpression of Twinkle Helicase Ameliorates the Progression of Cardiac Fibrosis and Heart Failure in Pressure Overload Model in Mice', *PLoS One*, 8(6).

Thelander L. & Reichard P. (1979) 'Reduction of ribonucleotides.', *Annu. Rev. Biochem.*, 48, pp. 133–158.

Thompson K., Majd H., Dallabona C., Reinson K., King M.S., Alston C.L., *et al.* (2016) 'Recurrent De Novo Dominant Mutations in SLC25A4 Cause Severe Early-Onset Mitochondrial Disease and Loss of Mitochondrial DNA Copy Number', *Am. J. Hum. Genet.*, 99(4), pp. 860–876.

Tinker R.J., Lim A.Z., Stefanetti R.J., & McFarland R. (2021) 'Current and Emerging Clinical Treatment in Mitochondrial Disease', *Mol. Diagnosis Ther.*, 25(2), pp. 181–206.

Tiranti V., Rossi E., Ruiz-Carrillo A., Rossi G., Rocchi M., Didonato S., *et al.* (1995) 'Chromosomal localization of mitochondrial transcription factor A (TCF6), single-stranded DNA-binding protein (SSBP), and

Endonuclease G (ENDOG), three human housekeeping genes involved in mitochondrial biogenesis', *Genomics*, 25(2), pp. 559–564.

Torregrosa-Muñumer R., Forslund J.M.E., Goffart S., Pfeiffer A., Stojkovič G., Carvalho G., *et al.* (2017) 'PrimPol is required for replication reinitiation after mtDNA damage', *Proc. Natl. Acad. Sci. U. S. A.*, 114(43), pp. 11398–11403.

Torregrosa-Muñumer R., Hangas A., Goffart S., Blei D., Zsurka G., Griffith J., *et al.* (2019) 'Replication fork rescue in mammalian mitochondria', *Sci. Rep.*, 9(1).

Torres-Torronteras J., Viscomi C., Cabrera-Pérez R., Cámara Y., Di Meo I., Barquinero J., *et al.* (2014) 'Gene therapy using a liver-targeted AAV vector restores nucleoside and nucleotide homeostasis in a murine model of MNGIE', *Mol. Ther.*, 22(5), pp. 901–907.

Tovar J., León-Avila G., Sánchez L.B., Sutak R., Tachezy J., Van Der Giezen M., *et al.* (2003) 'Mitochondrial remnant organelles of Giardia function in iron-sulphur protein maturation', *Nature*, 426(6963), pp. 172–176.

Traut T.W. (1994) 'Physiological concentrations of purines and pyrimidines', *Mol. Cell. Biochem.*, pp. 1–22.

Trifunovic A., Wredenberg A., Falkenberg M., Spelbrink J.N., Rovio A.T., Bruder C.E., *et al.* (2004) 'Premature ageing in mice expressing defective mitochondrial DNA polymerase', *Nature*, 429(6990), pp. 417–423.

Trifunovic A., Hansson A., Wredenberg A., Rovio A.T., Dufour E., Khvorostov I., *et al.* (2005) 'Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production', *Proc. Natl. Acad. Sci. U. S. A.*, 102(50), pp. 17993–17998.

Tyynismaa H., Sembongi H., Bokori-Brown M., Granycome C., Ashley N., Poulton J., *et al.* (2004) 'Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number', *Hum. Mol. Genet.* 2004/10/29, 13(24), pp. 3219–3227.

Tyynismaa H., Ylikallio E., Patel M., Molnar M.J., Haller R.G., & Suomalainen A. (2009) 'A Heterozygous Truncating Mutation in RRM2B Causes Autosomal-Dominant Progressive External Ophthalmoplegia with Multiple mtDNA Deletions', *Am. J. Hum. Genet.*, 85(2), pp. 290–295.

Tyynismaa H., Carroll C.J., Raimundo N., Ahola-erkkilä S., Wenz T., Ruhanen H., *et al.* (2010) 'Mitochondrial myopathy induces a starvation-like response', *Hum. Mol. Genet.*, 19(20), pp. 3948–3958.

Tyynismaa H., Sun R., Ahola-Erkkilä S., Almusa H., Pöyhönen R., Korpela M., *et al.* (2012) 'Thymidine kinase 2 mutations in autosomal recessive progressive external ophthalmoplegia with multiple mitochondrial DNA deletions', *Hum. Mol. Genet.*, 21(1), pp. 66–75.

Tyynismaa H. & Suomalainen A. (2009) 'Mouse models of mitochondrial DNA defects and their relevance for human disease', *EMBO Rep.* 2009/01/17, pp. 137–143.

Tyynismaa H. & Suomalainen A. (2010) 'Mouse models of mtDNA replication diseases', *Methods*. 2010/04/14, pp. 405–410.

Uygur A. & Lee R.T. (2016) 'Mechanisms of Cardiac Regeneration', *Dev. Cell*.

Vasilescu C., Ojala T.H., Brilhante V., Ojanen S., Hinterding H.M., Palin E., *et al.* (2018) 'Genetic Basis of Severe Childhood-Onset Cardiomyopathies', *J. Am. Coll. Cardiol.*, 72(19), pp. 2324–2338.

Veech R.L. (2004) 'The therapeutic implications of ketone bodies: The effects of ketone bodies in pathological conditions: Ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism', *Prostaglandins Leukot. Essent. Fat. Acids*, 70(3), pp. 309–319.

Vermulst M., Bielas J.H., Kujoth G.C., Ladiges W.C., Rabinovitch P.S., Prolla T.A., *et al.* (2007) 'Mitochondrial point mutations do not limit the natural lifespan of mice', *Nat. Genet.*, 39(4), pp. 540–543.

Vijg J. & Suh Y. (2013) 'Genome instability and aging', *Annu. Rev. Physiol.*

Viscomi C. & Zeviani M. (2017) 'MtDNA-maintenance defects: syndromes and genes', *J. Inherit. Metab. Dis.*, 40(4), pp. 587–599.

De Vos K.J., Allan V.J., Grierson A.J., & Sheetz M.P. (2005) 'Mitochondrial function and actin regulate dynamin-related protein 1-dependent mitochondrial fission', *Curr. Biol.*, 15(7).

Wahlestedt M., Ameur A., Moraghebi R., Norddahl G.L., Sten G., Woods N.B., *et al.* (2014) 'Somatic cells with a heavy mitochondrial DNA mutational load render induced pluripotent stem cells with distinct differentiation defects', *Stem Cells*, 32(5), pp. 1173–1182.

Wai T. & Langer T. (2016) *Mitochondrial Dynamics and Metabolic Regulation*, *Trends Endocrinol. Metab.* Elsevier Current Trends.

Wan X., Pei H., Zhao M.J., Yang S., Hu W.K., He H., *et al.* (2016) 'Efficacy and Safety of rAAV2-ND4 Treatment for Leberâ (tm) s Hereditary Optic Neuropathy', *Sci. Rep.*, 6(1), pp. 1–10.

Wang A., Mouser J., Pitt J., Promislow D., & Kaeberlein M. (2016) 'Rapamycin enhances survival in a Drosophila model of mitochondrial disease', *Oncotarget*, 7(49), pp. 80131–80139.

Wang H., Liu C., Zhao Y., & Gao G. (2020) 'Mitochondria regulation in ferroptosis', *Eur. J. Cell Biol.*

Wang L. (2010) 'Deoxynucleoside salvage enzymes and tissue specific mitochondrial DNA depletion', *Nucleosides, Nucleotides and Nucleic Acids*, pp. 370–381.

Wang Y., Yen F.S., Zhu X.G., Timson R.C., Weber R., Xing C., *et al.* (2021) 'SLC25A39 is necessary for mitochondrial glutathione import in mammalian cells', *Nature*, 599(7883), pp. 136–140.

Wanrooij S., Fusté J.M., Farge G., Shi Y., Gustafsson C.M., & Falkenberg M. (2008) 'Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro', *Proc. Natl. Acad. Sci. U. S. A.*, 105(32), pp. 11122–11127.

Wernette C.M. & Kaguni L.S. (1986) 'A mitochondrial DNA polymerase from embryos of Drosophila melanogaster. Purification, subunit structure, and partial characterization', *J. Biol. Chem.*, 261(31), pp. 14764–14770.

West A.P., Khoury-Hanold W., Staron M., Tal M.C., Pineda C.M., Lang S.M., *et al.* (2015) 'Mitochondrial DNA stress primes the antiviral innate immune response', *Nature*, 520(7548), pp. 553–557.

Wheaton K., Campuzano D., Ma W., Sheinis M., Ho B., Brown G.W., *et al.* (2017) 'Progerin-Induced Replication Stress Facilitates Premature Senescence in Hutchinson-Gilford Progeria Syndrome', *Mol. Cell. Biol.*, 37(14).

Wheeler L.J. & Mathews C.K. (2011) 'Nucleoside triphosphate pool asymmetry in mammalian mitochondria', *J. Biol. Chem.*, 286(19), pp. 16992–16996.

Wheeler L.J., Rajagopal I., & Mathews C.K. (2005) 'Stimulation of mutagenesis by proportional deoxyribonucleoside triphosphate accumulation in Escherichia coli', in *DNA Repair (Amst).*, pp. 1450–1456.

Williams S.L., Huang J., Edwards Y.J.K., Ulloa R.H., Dillon L.M., Prolla T.A., *et al.* (2010) 'The mtDNA mutation spectrum of the progeroid polg mutator mouse includes abundant control region multimers', *Cell Metab.*, 12(6).

Winterthun S., Ferrari G., He L., Taylor R.W., Zeviani M., Turnbull D.M., *et al.* (2005) 'Autosomal recessive mitochondrial ataxic syndrome due to mitochondrial polymerase γ mutations', *Neurology*, 64(7), pp. 1204–1208.

Wong E.D., Wagner J.A., Gorsich S.W., McCaffery J.M., Shaw J.M., & Nunnari J. (2000) 'The dynamin-related GTPase, Mgm1p, is intermembrane space protein required for maintenance of fusion competent mitochondria', *J. Cell Biol.*, 151(2), pp. 341–352.

Wong Y.C., Ysselstein D., & Krainc D. (2018) 'Mitochondria-lysosome contacts regulate mitochondrial fission via RAB7 GTP hydrolysis', *Nature*, 554(7692), pp. 382–386.

Xu B. & Clayton D.A. (1996) 'RNA-DNA hybrid formation at the human mitochondrial heavy-strand origin ceases at replication start sites: An implication for RNA-DNA hybrids serving as primers', *EMBO J.*, 15(12), pp. 3135–3143.

Yang W., Li J., & Hekimi S. (2007) 'A measurable increase in oxidative damage due to reduction in superoxide detoxification fails to shorten the life span of long-lived mitochondrial mutants of Caenorhabditis elegans', *Genetics*, 177(4), pp. 2063–2074.

Yasukawa T., Reyes A., Cluett T.J., Yang M.Y., Bowmaker M., Jacobs H.T., *et al.* (2006) 'Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand', *EMBO J.*, 25(22), pp. 5358–5371.

Yatsuga S. & Suomalainen A. (2012) 'Effect of bezafibrate treatment on late-onset mitochondrial myopathy in mice', *Hum. Mol. Genet.*, 21(3), pp. 526–535.

Ylikallio E., Tyynismaa H., Tsutsui H., Ide T., & Suomalainen A. (2010) 'High mitochondrial DNA copy number has detrimental effects in mice', *Hum. Mol. Genet.* 2010/04/24, 19(13), pp. 2695–2705.

Ylikallio E. & Suomalainen A. (2012) 'Mechanisms of mitochondrial diseases', *Ann. Med.*, 44(1), pp. 41–59.

Youle R.J. & Narendra D.P. (2011) 'Mechanisms of mitophagy', *Nat. Rev. Mol. Cell Biol.*, 12(1), pp. 9–14.

Zaremba-Niedzwiedzka K., Caceres E.F., Saw J.H., Bäckström Di., Juzokaite L., Vancaester E., *et al.* (2017) 'Asgard archaea illuminate the origin of eukaryotic cellular complexity', *Nature*, 541(7637), pp. 353–358.

Zekonyte U., Bacman S.R., Smith J., Shoop W., Pereira C. V., Tomberlin G., *et al.* (2021) 'Mitochondrial targeted meganuclease as a platform to eliminate mutant mtDNA in vivo', *Nat. Commun.*, 12(1).

Zeviani M., Servidei S., Gellera C., Bertini E., DiMauro S., & DiDonato S. (1989) 'An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region', *Nature*, 339(6222), pp. 309–311.

Zhou N. & Bao J. (2020) 'FerrDb: A manually curated resource for regulators and markers of ferroptosis and ferroptosis-disease associations', *Database*, 2020.

Ziebarth T.D., Farr C.L., & Kaguni L.S. (2007) 'Modular Architecture of the Hexameric Human Mitochondrial DNA Helicase', *J. Mol. Biol.*, 367(5), pp. 1382–1391.

Ziegler D.M. & Doeg K.A. (1962) 'Studies on the electron transport system. XLIII. The isolation of a succinic-coenzyme Q reductase from beef heart mitochondria', *Arch. Biochem. Biophys.*, 97(1), pp. 41–50.

ISBN 978-951-51-8096-4 (*PRINT*)
ISBN 978-951-51-8097-1 (*ONLINE*)
ISSN 2342-3161 (*PRINT*)
ISSN 2342-317X (*ONLINE*)

http://ethesis.helsinki.fi

HELSINKI 2022