Department of Medical Biochemistry and Biophysics, 2026

Maintaining human mitochondrial DNA: Insights into Replication Stress, Pathogenic Mutations, and Related Mitochondrial Processes

Tran Vinh Hong Nguyen

UMEÅ UNIVERSITY

Dissertation for PhD
ISBN: 978-91-8070-879-1 (print)
ISBN: 978-91-8070-880-7 (pdf)
ISSN: 0346-6612
Umeå University Medical Dissertations New Series
Cover design: by Tran Vinh Hong Nguyen, generated on BioRender.com
Electronic version available at: http://umu.diva-portal.org/
Printed by: Scandinavian Print Group, Skarpnäck, 2026

“We are a way for the cosmos to know itself”
Carl Sagan

TABLE OF CONTENTS

ABSTRACT iv

ABBREVIATIONS vi

PUBLICATION LIST viii

INTRODUCTION 1

1. Mitochondria and their genome 1

1.1. Mitochondrial structure, dynamics and metabolism 1

1.2. Mitochondria as energy and cellular signalling hubs 6

1.3. The mitochondrial genome and nucleoid organization 7

2. Replication and Maintenance of mtDNA 9

2.1. Mode of mtDNA replication 9

2.2. The mtDNA replisome 11

2.3. Other proteins involved in mtDNA replication 13

2.4. Maintenance of mtDNA integrity: repair and degradation 14

3. Mitochondrial dysfunction and diseases 16

3.1. The genetics of mitochondrial disorders 16

3.2. Cellular responses to mitochondrial dysfunction 18

4. PolDIP2: a multifunctional protein involved in mitochondrial maintenance and many cellular processes 20

4.1. Structure and expression of PolDIP2 20

4.2. Mitochondrial function 23

4.3. Other possible cellular functions of PolDIP2 25

AIMS OF THE THESIS 28

RESULTS AND DISCUSSION 29

Part I - Insights into mechanisms of mtDNA instability in human cells 29

1. Pathogenic mutations in DNA polymerase gamma in mitochondrial DNA-depletion associated diseases (paper I and II) 29

2. Enhanced mitochondrial G-quadruplex formation impedes replication fork progression leading to mtDNA loss in human cells (paper III) 36

Part II - Characterization of PolDIP2 structural features and their implications in mitochondria (Manuscript IV) 40

CONCLUSION AND OUTLOOK 46

ACKNOWLEGMENT 47

REFERENCES 49

TABLE OF FIGURES

Figure 1 2
Figure 2 4
Figure 3 8
Figure 4 10
Figure 5 11
Figure 6 21
Figure 7 30
Figure 8 32
Figure 9 34
Figure 10 35
Figure 11 37
Figure 12 39
Figure 13 41
Figure 14 44

ABSTRACT

Mitochondria are essential organelles in eukaryotic cells. They contain their own genome that encodes proteins of the OXPHOS complexes essential for cellular ATP production. Failure to maintain mitochondrial DNA (mtDNA) integrity, therefore, can impair energy production and lead to mitochondrial dysfunction, which consequently results in a wide range of diseases, including rare genetic mitochondrial disorders and neurodegeneration.

In human mitochondria, the genome is replicated by a unique enzymatic machinery including the mitochondrial replicative DNA Polymerase Gamma (Polγ). Polγ is a holoenzyme consisting of a catalytic Polγ α subunit and two accessory Polγ β subunits. The catalytic subunit Polγ α has both DNA polymerase and exonuclease activities that are required for high-fidelity mtDNA replication. A precise cooperation of the two activities of Polγ during mtDNA replication is therefore critical to ensure proper mtDNA integrity. In our study, we investigated the mechanism by which the pathogenic mutation Y951N induces replication stalling and a loss of mtDNA in patient cells. Our findings showed that the Y951N mutation in the polymerase domain disrupts Polγ's ability to switch between its polymerase and exonuclease activities, leading to severe mtDNA replication stalling and eventually mtDNA depletion. Identifying Polγ residues critical for this intramolecular switching mechanism provides insights into how various pathogenic mutations affect the maintenance of the mitochondrial genome.

In response to mitochondrial dysfunction that alters the cellular energy state, particularly in the context of mitochondrial DNA depletion, AMP-activated protein kinase (AMPK), a key energy sensor and metabolic regulator can be activated to restore energy homeostasis. However, the degree of severity of mitochondrial dysfunction required to induce AMPK activation, as well as how mitochondrial biogenesis can be restored following stimulation of AMPK activity, remains to be elucidated. To address this, we used a cell model of mtDNA depletion syndromes (MDS) in which the expression of a pathogenic Polγ variant causes severe mtDNA loss, leading to progressive mitochondrial dysfunction. We observed that the activation of mitochondria-associated AMPK occurs during the early stages of advancing mitochondrial dysfunction. Moreover, our results showed that stimulation of AMPK activity using a specific agonist, A-769662, can mitigate impaired mitochondrial phenotypes and partially restore mtDNA levels. These findings contribute to our understanding of the impacts of specific activators of AMPK on mitochondrial and cellular function as well as their potential applications in mitochondrial diseases.

In addition to defects in nuclear genes involved in mtDNA replication, mutations in the mitochondrial genome that arise from errors during mtDNA replication or from repair of damaged mtDNA can also result in a loss of mitochondrial genetic integrity, e.g. due to an accumulation of large-scale mtDNA deletions. Many of these mtDNA deletion breakpoints were recently suggested to occur at sequence motifs with potential to form secondary DNA structures, G-quadruplexes (G4s). In our study, by developing a novel tool for mapping G4s in living cells, we were able to determine mtDNA G4 formation in human cells under

different cellular conditions. Our results indicated that replication stalling enhances G4 formation, which in turn blocks the replication fork progression and causes mtDNA loss, potentially leading to mitochondrial disease. The new mtG4-ChiP tool will enable future research to further investigate the factors involved in G4 formation and resolution, as well as the mechanistic roles of G4s in the generation of pathogenic mtDNA deletions.

In parallel with studying mechanisms of mtDNA deletions and depletion, we investigated how cells tolerate mitochondrial genome damage to preserve mtDNA integrity. PrimPol, a primase–polymerase that can restart stalled mtDNA replication, requires stimulation by Polymerase δ–interacting protein 2 (PolDIP2) for efficient DNA synthesis. While characterizing this interaction, we unexpectedly found that PolDIP2 forms disulfide-linked homodimers, a process that is enhanced under oxidative stress. Notably, PolDIP2 interacts with coiled-coil-helix-coiled-coil-helix domain–containing protein 2 (CHCHD2), a mitochondrial protein implicated in maintaining cristae structure and dynamics. Our findings suggest that redox-sensitive PolDIP2 dimerization may influence mitochondrial function by modulating its interaction with CHCHD2 during oxidative stress.

In summary, the findings presented here provided valuable insights into molecular mechanisms of mitochondrial genome instability caused by pathogenic mutations and cellular responses to mitochondrial dysfunctions, as well as the involvement of potential factors in mitochondrial DNA maintenance.

ABBREVIATIONS

Terms	Definitions
ATP	Adenosine triphosphate
2D-AGE	Two-dimensional agarose gel electrophoresis
ADP	Adenosine diphosphate
AMP	Adenosime monophosphate
AMPK	AMP-activated protein kinase
BER	Base excision repair
CTD	C-terminal domain
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain–containing protein 2
ddC	2,3-dideoxycytidine
dNTP	Deoxyribonucleotide triphosphate
Dox	Doxycycline
DSBs	Double-strand breaks
dsDNA	Double-stranded DNA
EMSA	Electrophoretic mobility shift assay
ETC	Electron transport chain
Exo-	Exonuclease deficient
FAD	Flavin adenine dinucleotide
FADH2	Flavin adenine dinucleotide (reduced)
G4	G-quadruplex
HSP	H-strand promoter
IMM	Inner mitochondrial membrane
IMS	Mitochondrial intermembrane space
IP	Immunoprecipitation
LSP	L-strand promoter
MDS	mtDNA depletion syndromes
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting sequence
mtSSB	Mitochondrial single-stranded binding protein
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NCR	Non-coding region
nDNA	Nuclear DNA
NTD	N-terminal domain
O_H	H-strand origin replication
O_L	L-strand origin replication
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation

PAE	Predicted aligned error
PCNA	Proliferating cell nuclear antigen
PEO	Progressive external ophthalmoplegia
PGS	G4-forming sequences
PolDIP2	Polymerase delta interacting protein 2
POLRMT	Mitochondrial RNA polymerase
Polγ	Mitochondrial DNA polymerase gamma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDM	Strand-displacement model
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
ssDNA	Single-stranded DNA
TCA	Tricarboxylic acid
TFAM	Mitochondrial transcription factor A
TLS	Translesion synthesis
TOM	Translocase of outer membrane
WT	Wild type
ΔΨ	Mitochondrial membrane potential

PUBLICATION LIST

I. The POLγ Y951N patient mutation disrupts the switch between DNA synthesis and proofreading, triggering mitochondrial DNA instability

Josefin M. E. Forslund, **Tran V. H. Nguyen**, Vimal Parkash, Andreas Berner, Steffi Goffartb, Jaakko L. O. Pohjoismäki, Paulina H. Wanrooij, Erik Johansson, and Sjoerd Wanrooij.

Proceedings of the National Academy of Sciences (2025).
DOI:10.1073/pnas.2417477122

II. Activating AMPK improves pathological phenotypes due to mtDNA depletion

Gustavo Carvalho*, **Tran V. H. Nguyen***, Bruno Repolês*, Josefin M. E. Forslund, W. M. Ruchitha Rukmal Wijethunga, Farahnaz Ranjbarian, Isabela C. Mendes, Choco Michael Gorospe, Namrata Chaudhari, Micol Falabella, Mara Doimo, Sjoerd Wanrooij, Robert D. S. Pitceathly, Anders Hofer, Paulina H. Wanrooij.

The FEBS Journal (2025). DOI: 10.1111/febs.70006

*These authors contributed equally to this work

III. Enhanced mitochondrial G-quadruplex formation impedes replication fork progression leading to mtDNA loss in human cells

Mara Doimo, Namrata Chaudhari, Sanna Abrahamsson, Valentin L'Hôte, **Tran V H Nguyen**, Andreas Berner, Mama Ndi, Alva Abrahamsson, Rabindra Nath Das, Koit Aasumets, Steffi Goffart, Jaakko L O Pohjoismäki, Marcela Dávila López, Erik Chorell, Sjoerd Wanrooij

Nucleic Acids Research (2023). DOI: 10.1093/nar/gkad535

IV. Redox-dependent dimerization of PolDIP2 and a conserved ApaG-domain motif required for CHCHD2 interaction.

Tran V H Nguyen, Andreas Berner, Kazutoshi Kasho, Anaïs Lamy, Kieran Deane-Alder, Koit Aasumets, Namrata Chaudhari, Cuncun Qiao, Louise L Fernandes, Ronnie Berntsson, Sjoerd Wanrooij

Manuscript

INTRODUCTION

1. Mitochondria and their genome

Mitochondria are double membrane-bound organelles located in the cytoplasm of eukaryotic cells. Mitochondria serve as a "powerhouse" of the cell, which is attributed to their capacity to convert uptaken nutrients into energy in the form of ATP for cellular functions. In addition to the central role in energy production, mitochondria are now recognized as a key player in diverse cellular processes, including metabolite biosynthesis, redox homeostasis, signal transduction and immunity, cellular stress response, and apoptosis[1].

A large body of evidence, including phylogenetic analyses, strongly supports the bacterial origin of mitochondria. According to the widely accepted endosymbiotic theory, mitochondria were derived from alpha-proteobacteria that became integrated into a host cell approximately 2 billion years ago, establishing an endosymbiotic relationship that formed the basis of eukaryotic cells[2,3]. Over millions of years after this acquisition of the ancestral proteobacteria, the endosymbiont underwent substantial genetic reconstruction within the host cell and evolved into mitochondria with several retained genomic and proteomic characteristics of their original ancestor[3].

It was proposed that most of the original genes from the ancestral endosymbiont were either lost or transferred to the nuclear genome of the host over the course of evolution, eventually resulting in a considerable reduction in the content and size of the genetic material present in mitochondria. Despite the diversity in overall genome sizes and gene content across a broad range of eukaryotes, most mitochondrial genomes encode only a few mitochondrial ribosomal (rRNAs), transfer RNAs (tRNAs), and a small fraction of mitochondrial proteins, typically between 12 and 20 protein-coding genes[2,3]. As a result, although mitochondria retained their own genetic information that contributes to mitochondrial biogenesis, the vast majority of mitochondrial proteins are encoded in the nucleus and subsequently imported into mitochondria for their functions.

1.1. Mitochondrial structure, dynamics and metabolism

1.1.1. The dynamic organization of mitochondria

Mitochondrial architecture

One of the features that mitochondria retained from their bacterial ancestor is the double membrane structure that encloses the organelle and separates it from the rest of the cells. The two membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), are characterized by different compositions and functions and create distinct internal compartments: mitochondrial intermembrane space (IMS) located between the outer and inner membranes, and the mitochondrial matrix (MM) surrounded by IMM.

The outer mitochondrial membrane is the organelle's outermost barrier that faces the cytosol, and is generally permeable to many ions and small molecules to pass through via voltage-dependent anion channels (VDACs)[4], which are aqueous pores embedded in the lipid bilayer and constitute up to 30% of the OMM surface[5]. In addition, the OMM hosts many proteins that are important for mitochondrial functions, as well as serves as a platform for various biochemical and signalling pathways[6]. The OMM is inhabited by the translocase of outer membrane (TOM) and the sorting and assembly machinery (SAM) complexes, which mediate the import of mitochondrial precursor proteins into the organelle. Several proteins located in the OMM also participate in establishing interfaces, known as membrane-contact sites, which connect mitochondria with other subcellular compartments (e.g. endoplasmic reticulum (ER), lysosomes, endosomes, and the plasma membrane[6]).

In contrast to the smooth OMM, the inner mitochondrial membrane forms highly dynamic structures that protrude into the mitochondrial matrix to form invaginations known as cristae. The section of IMM that does not fold inwards into the MM but runs parallel to the OMM is called the inner boundary membrane (IBM). Cristae are connected to the IBM via slit-like structures called crista junctions (CJs)[7]. The inner boundary membrane hosts various channel transporters and protein translocases that are responsible for the shuttling of ions, small metabolites, and for the transfer of mitochondrial proteins to and from the MM, respectively, whereas the cristae are enriched with protein complexes that constitute oxidative phosphorylation (OXPHOS) machinery[8,9]. Therefore, the highly folded structure of cristae, which substantially expands the total surface area of the IMM creates optimal conditions for oxidative phosphorylation to occur, thus facilitating efficient ATP production that meets cellular demand[10].

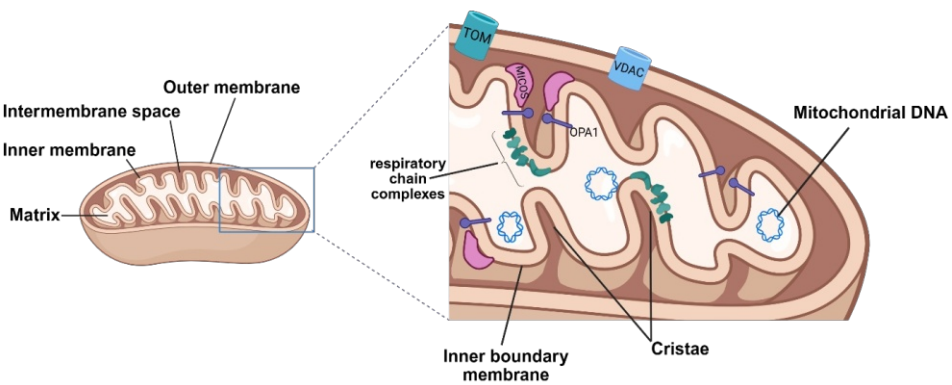

Figure 1. Schematic overview of mitochondrial structure. The close-up illustrates key components within mitochondrial subcompartments: VDAC and TOM complexes on the OMM; MICOS, OPA1, and respiratory chain complexes on the IMM; and mitochondrial nucleoids containing mtDNA in the matrix.

Mitochondrial cristae exhibit diversity in shape and number depending on cell type, which can be rapidly adjusted to cellular energy requirements[11]. The maintenance of the cristae structures in physiological conditions, as well as their dynamic reorganization in response to internal and external stimuli are orchestrated by numerous key regulators distributed across the IMM, including mitochondrial contact site and cristae organizing system (MICOS) complex[12–14], ATP synthase dimers[15,16], and dynamin-related protein optic atrophy protein 1 (OPA1)[17,18].

Mitochondria are dynamic organelles that continuously undergo fusion and fission. These two opposing processes are tightly coordinated to regulate mitochondrial morphology and adaptation to cellular metabolic needs[19]. Both mitochondrial fission and fusion are highly active processes mediated by specialized proteins that act on the OMM and IMM[19]. Mitochondrial fusion results in tubular or elongated mitochondria, forming interconnected networks that enable the exchange of intramitochondrial material within the mitochondrial population for optimal functioning, especially under metabolic stress[20]. On the other hand, mitochondrial fission occurs when a single mitochondrion divides into two or more separate structures. This process allows the formation of new mitochondria during cell division and the redistribution of mitochondria to the energy-demanding regions within the cell. Additionally, mitochondrial fission contributes to mitochondrial quality control by segregating damaged segments of mitochondria[20].

Import of mitochondrial proteins

As mentioned earlier, in modern organisms, the mitochondrial genome encodes only a small portion of the organelle's proteome (e.g. 13 mitochondria-encoded proteins in mammalian cells). Most mitochondrial proteins are nuclear encoded and must be transported from the cytosol across the double-membrane system of mitochondria and subsequently sorted into their destination within the mitochondria. These precursor proteins possess targeting signals that are recognized sequentially by translocase complexes located in both OMM and IMM. The targeting signals can be either canonical mitochondria-targeting sequences or so-called pre-sequences, which typically consist of 18-20 amino acids and are proteolytically removed upon entry into mitochondria, or internal targeting sequences that are found within the primary structure and retained in the mature protein[21,22].

The entry of mitochondrial precursor proteins from the cytosol is initiated when their targeting signals are recognized by the translocase of the outer membrane (TOM) complex for the transport across the OMM. After passage through the TOM complex, the precursor proteins can use different pathways to reach their final destinations in mitochondrial sub-compartments. Many proteins destined for the IMS possess internal targeting signals and characteristic cysteine (Cys) motifs that are recognized and sorted by the TOM complex and the mitochondrial intermembrane space assembly (MIA) machinery[23]. Other precursors that contain hydrophobic sorting signals are transferred through the intermembrane space to either the TIM22 complex that sorts and incorporates them into the IMM[24,25], or to the sorting and assembly machinery (SAM) complex of the OMM for their insertions into this

membrane[25–27]. Those proteins targeted to the mitochondrial matrix commonly contain cleavable presequences and are transported across the IMM by the presequence translocase of the inner membrane (TIM23) complex and the presequence translocase-associated motor (PAM). Once these preproteins are translocated to the matrix, the mitochondrial processing peptidase (MPP) carries out the cleavage of their presequences, which in some cases is followed by additional cleavage events by other peptidases, to give rise to mature proteins[22,28].

1.1.2. Oxidative phosphorylation and Mitochondrial membrane potential

Oxidative phosphorylation

Nutrients taken up by cells, including carbohydrates, fats, and proteins are metabolized in several cellular processes to generate molecules that are subsequently used in the tricarboxylic acid (TCA) cycle in the matrix of mitochondria. NADH and $FADH_2$, electron donors generated by TCA cycle are then utilized to produce ATP in oxidative phosphorylation (OXPHOS), a process that involves the coupling of electron transport with the synthesis of ATP[30,31]. This series of redox reactions is catalyzed by several protein complexes of the electron transport chain (ETC) embedded in the cristae of the inner mitochondrial membrane.

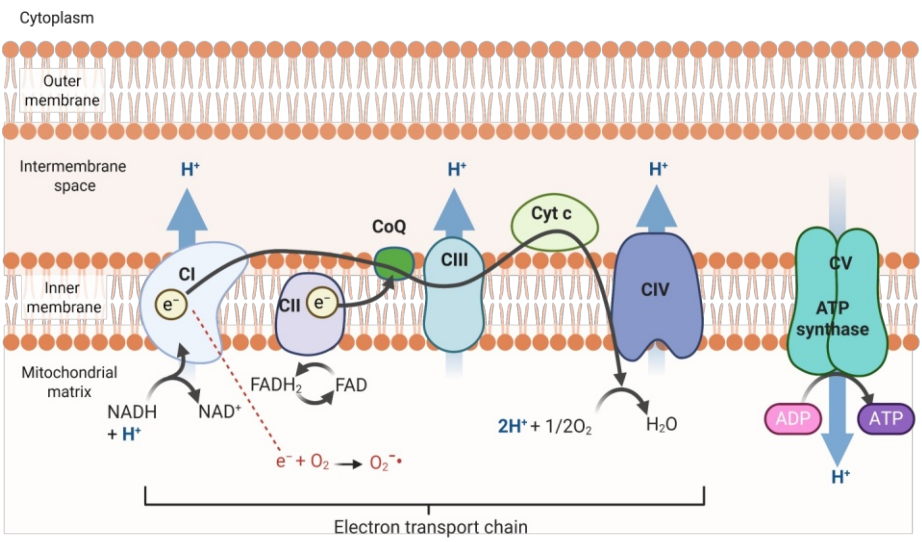

Figure 2. Overview of mitochondrial oxidative phosphorylation. Electrons provided by NADH and $FADH_2$ are transferred through complex I-III of the electron transport chain via electron carriers, ubiquinone (CoQ) and cyrtochrome c (Cyt c), ultimately reaching complex IV where they are donated to oxygen to produce water. The electron flow through the ETC generates energy to pump protons (H^+) from the mitochondrial matrix to the mitochondrial intermembrane space, creating an electrochemical potential gradient across the inner membrane. This proton-motive force is then used by complex V (ATP synthase) to catalyse the conversion of ADP to ATP. During OXPHOS, a fraction of electrons can prematurely leak out of the ETC and react with O_2, generating primarily superoxide anion O_2^-•.

The ETC is composed of four multi-subunit OXPHOS complexes (I-IV) and the electron carriers, ubiquinone (CoQ) and cytochrome c (Cyt c). The flow of electrons through the ETC generates energy to pump protons (H^+) from the MM to the IMS, resulting in electrochemical potential gradient across the inner membrane. The energy stored in this proton gradient is ultimately used by the last OXPHOS complex, F_0F_1ATP synthase, to catalyse the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) for cellular use[30].

Mitochondrial membrane potential

During oxidative phosphorylation, energy from the electron flux along the ETC is used to transport protons from the mitochondrial matrix to the intermembrane space, generating a proton gradient. This proton gradient creates an electrochemical potential difference across the inner mitochondrial membrane, which is known as mitochondrial membrane potential (MMP, often denoted as $\Delta\Psi$). The MMP is essential in driving ATP synthesis as when protons move back to the matrix to dissipate the gradient through the transmembrane component F_0 of ATP synthase, the catalytic component F_1 uses the stored energy from proton transfer to convert ADP to ATP[30].

Mitochondrial reactive oxygen species (ROS)

Reactive oxygen species (ROS) are highly reactive chemicals that can cause irreversible damage to biological macromolecules such as nucleic acids, proteins, and lipids[32]. An important source of cellular ROS is mitochondria, with the premature leakage of electrons from the ETC generating the majority of mitochondrial ROS as a by-product of respiration[33]. Several studies have estimated that up to 2% of the electrons during oxidative phosphorylation can prematurely leak out of the ETC and react with molecular oxygen to generate primarily superoxide anion (O_2^{-}•)[28,34]. O_2^{-}• can then be converted either spontaneously or by mitochondrial superoxide dismutases to hydrogen peroxide (H_2O_2), which can be removed by mitochondrial antioxidant systems such as catalase, peroxiredoxins (Prx), and associated reductases. If not eliminated by the peroxide scavengers, H_2O_2 may be reduced to hydroxyl radicals (OH•) in Fenton reactions catalysed by transition metals. In addition, O_2^{-}• may directly react with nitric oxide (NO•) to form peroxynitrite, one of the reactive nitrogen species (RNS). Although O_2^{-}• and H_2O_2 are not strong oxidants, OH• and peroxynitrite formed from these precursors are extremely reactive and can cause significant damage to essential cellular components[35], subsequently leading to cellular oxidative stress and cell death.

Recent studies, however, have indicated that ROS are not merely harmful byproducts that cause oxidative damage and diseases. Mitochondrial ROS, especially H_2O_2, which has a relatively long half-life and can diffuse across membranes within the cell, also act as signalling molecules that regulate several cellular processes such as cell proliferation and differentiation, redox balance and immune responses[36]. ROS levels are therefore highly regulated by the balance between ROS production and cellular antioxidant defence systems

to enable rapid adaptive responses to changing environmental conditions without causing widespread oxidative damage[37].

1.2. Mitochondria as energy and cellular signalling hubs

Mitochondria as an energy hub

Mitochondria are well known not only as the primary producer of ATP but also for their central roles in coordinating cellular bioenergetics, allowing cells to effectively adapt to changes in nutrient and oxygen availability, as well as to fluctuations in energy demand.

Cells first break down glucose into pyruvate via glycolysis in the cytoplasm, releasing energy in the form of ATP. However, this process extracts only a small fraction of the total energy stored within a glucose molecule to produce two ATP molecules. Pyruvate is then transported to mitochondria where it is oxidized to form acetyl Coenzyme A (CoA) and further catabolized through the TCA cycle in the mitochondrial matrix. Electrons harvested throughout the cycle are carried by electron carriers NADH and $FADH_2$, which in turn fuel the ETC to produce significantly more ATP via oxidative phosphorylation[30,38]. Under anaerobic conditions, however, mitochondrial respiration is repressed, pyruvate is instead fermented into lactate in the cytosol. This pathway regenerates NAD+ to sustain glycolysis, thus ensuring sufficient ATP production for cellular activities even when oxygen availability is limited[38]. Therefore, by integrating differentially compartmentalized processes, pyruvate synthesis in the cytoplasm versus the TCA cycle and oxidative phosphorylation within mitochondria, cells can adjust their metabolic pathways to efficiently adapt to environmental changes and altered cellular energy states.

Mitochondria as a source of metabolites

In addition to ATP production, mitochondria also take part in the biosynthesis of numerous metabolites needed for building macromolecules (fatty acids, cholesterol, nucleotides, amino acids, and heme) and for other cellular functions. These biosynthetic pathways are tightly regulated to maintain cellular homeostasis and are finely coordinated in response to stresses or changes in cell state[38].

Mitochondria control calcium homeostasis

While the outer mitochondrial membrane is permeable to Ca^{2+} ions[1], the transport of this essential ion across the IMM to reach the matrix is mediated by the mitochondrial calcium uniporter (MCU) channel in $\Delta\Psi$-dependent manner[39]. Ca^{2+} can be accumulated within mitochondria at considerably high concentrations, up to 20-fold higher than those in the cytosol[40]. This allows mitochondria to function as a buffering system for cytosolic Ca^{2+} levels, thereby controlling intracellular Ca^{2+} homeostasis[40]. Accumulation of Ca^{2+} within the mitochondria also modulates the activity of calcium-sensitive enzymes of the TCA cycle, consequently regulating ATP production[41–43].

Mitochondria as a key regulator in immunity and inflammation

Extensive studies over the past decades have revealed the central role of mitochondria in immune response through various signalling pathways and metabolic processes[44].

Given the prokaryotic origin of mitochondria, mitochondrial DNA shares high similarities with the bacterial genome, including its content of hypomethylated CpG motifs. As a result, released mtDNA in the cytosol can be recognized as a damage-associated molecular pattern (DAMP) by pattern recognition receptors (PRRs) of the innate immune system, thereby potently triggering inflammation[45,46]. Cytosolic mtDNA can activate three major innate immune signalling pathways: (1) activation of NF-kB signalling pathway[46] and (2) activation of Nod-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome, which consequently induces the production of multiple pro-inflammatory cytokines[46,47], and (3) activation of STING (stimulator of interferon gene) pathway via cGAS (cyclic GMAP-AMPs), which ultimately stimulates type I interferon (IFN) production[48].

In addition, mitochondria produce metabolites that are not only used as building blocks and signalling molecules for intracellular communication but also for modulating immune cell differentiation and activation[49]. For instance, intermediates generated in the TCA cycle can be released into the cytosol during bacterial infection to trigger inflammatory responses[50]. Additionally, mitochondrial ROS act as critical second messengers in both innate and adaptive immune responses, and a balanced level of mtROS is critical for maintaining proper immune function[46,51].

1.3. The mitochondrial genome and nucleoid organization

Despite large variations in size and organization of mitochondrial genomes across eukaryotes, their key characteristics and basic functions are largely similar. With a few exceptions, nearly all mitochondrial genomes exist as a circular molecule of double-stranded DNA that is distinct from the nuclear counterpart. A single cell can contain multiple mitochondria, and since each mitochondrion can have several copies of mitochondrial DNA genome, the number of mtDNA molecules generally ranges from hundreds to several thousand copies per cell depending on cell type[52–54]. A unique characteristic of mtDNA that arises from its multicopy nature is heteroplasmy, a state in which several different types of mtDNA variants, including the wild type mtDNA and its mutated forms can co-exist within a cell[55]. These mtDNA variants can derive from maternal inheritance or from somatic mutations that occur and accumulate during an individual's development[56]. The proportion of different mtDNA variants, which is referred to as the level of heteroplasmy, can vary between different cells and tissues within an individual, as well as between individuals[57]. This level of heteroplasmy is of importance for determining the severity of mitochondrial dysfunction and related pathological symptoms in mitochondrial diseases[58].

The nucleotide sequence of human mitochondrial DNA was the first portion of human genome to be sequenced in 1981[59]. Human mitochondrial genome consists of 16569 base pairs and encodes for 37 different genes, of which 13 protein-coding genes encode for

subunits of four of the OXPHOS complexes and the other 24 genes encode tRNA and rRNAs for their own translational apparatus[59–61]. Contrary to nuclear DNA, the gene organization of mitochondrial DNA in mammals is highly compact, as it lacks introns and has minimal intergenic regions between individual genes, except for a single large noncoding region present within the genome[62]. In humans, this major noncoding region (NCR), also known as the control region, is approximately 1100 bp long and located between $tRNA^{Pro}$ and $tRNA^{Phe}$ genes[59] (**Fig. 3**).

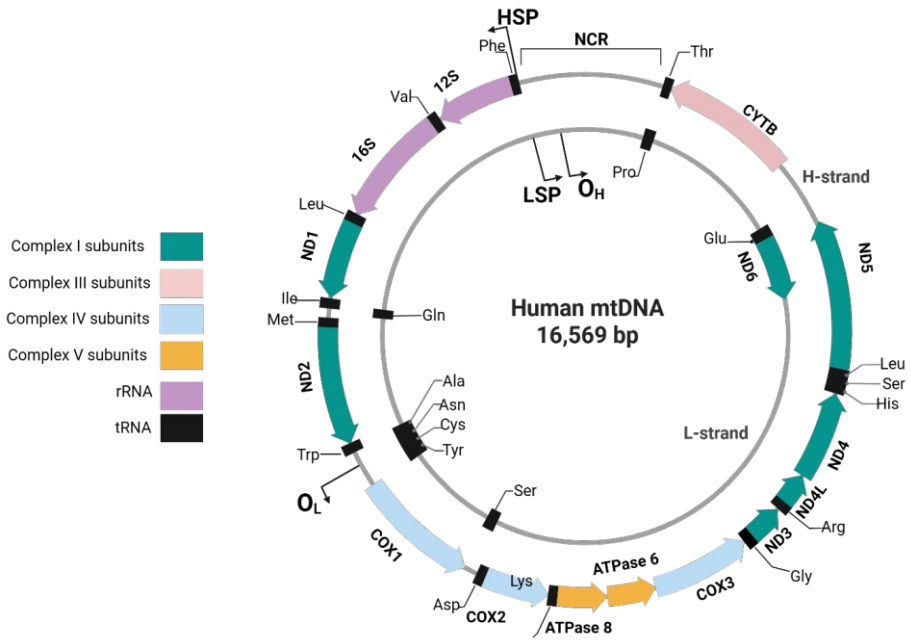

Figure 3. Overview of the human mitochondrial genome. The outer and inner circles represent the heavy- (H) and light- (L) strands, respectively. The noncoding region (NCR) contains both the H- and L- strand promoters (HSP, LSP), as well as the H-strand origin of replication (O_H).

The two strands of mitochondrial DNA differ in their G+T base composition, with the heavy (H) strand being rich in guanine and the light (L) strand with low guanine content, resulting in different buoyancies in denaturing caesium chloride gradients[63]. The guanine enrichment in the H-strand increases its propensity to form non-canonical DNA secondary structures known as G-quadruplexes (G4s). A collection of evidence has shown that multiple sequence motifs with G4-forming potential on the H-strand are associated with mtDNA deletion breakpoints, suggesting that G4 formation might interfere with mtDNA replication machinery[64–67].

mtDNA is organized into discrete nucleoprotein complexes that are known as mitochondrial nucleoids. When visualized with confocal fluorescence microscopy, human mitochondrial nucleoids appear as discrete punctate of approximately 100 nm in diameter distributed

throughout the mitochondrial network[68]. The distribution of mitochondrial nucleoids in the mitochondrial matrix, however, is not random but found in proximity to the IMM.

Each mitochondrial nucleoid typically consists of one or two mtDNA molecules and numerous nucleoid-associated proteins[54,68,69]. Although the exact compositions of mitochondrial nucleoids remain to be further investigated, a multilayer model was suggested to describe the organization of mitochondrial nucleoids[70]. According to the model, mtDNA and key mtDNA-associated proteins involved in replication and transcription are located in the inner core of the nucleoids, whereas factors that are temporarily recruited to or transiently associated with the nucleoid to exert their specific functions reside in the peripheral area of the nucleoid. One of the components in the central core essential for mtDNA transcription is mitochondrial transcription factor A (TFAM). This abundant mitochondrial protein is also a crucial factor for nucleoid packaging as it binds to mtDNA and coordinates the compaction of DNA molecules to form spheroid-like structures[71]. Similar to histone compaction of nuclear DNA which negatively regulates DNA replication and transcription, in fully compacted mitochondrial nucleoids, DNA-bound TFAM can block the progression of mitochondrial replication and transcription, and variations in the ratios of TFAM to DNA can differently impact mtDNA compaction and ultimately mtDNA transactions[72].

2. Replication and Maintenance of mtDNA

Replication of mtDNA is generally regulated by the cell's energy status rather than by the cell cycle. The mitochondrial genome is replicated by a unique replisome of nuclear-encoded proteins including mtDNA polymerase gamma (Polγ), mtDNA helicase Twinkle, mitochondrial single-stranded DNA binding protein (mtSSB), and mitochondrial RNA polymerase (POLRMT). These proteins of mtDNA replication machinery are distinct from those of nuclear DNA replisome but many of them are reminiscent of replication factors found in certain bacteriophages[73].

2.1. Mode of mtDNA replication

Extensive research has been conducted to investigate mechanisms of mitochondrial DNA replication in mammalian cells, from which several models have been proposed. Among them, the most widely accepted one is the strand-displacement model (SDM) that was first proposed in 1972, describing an asymmetric mechanism for mtDNA replication[74]. The SDM model has been strongly supported by later studies that used various experimental methods such as electron microscopy, pulse and pulse-chase labelling studies, and 5′-end characterization[75–78].

In the SDM model (**Fig. 4**), both mtDNA strands are synthesized continuously but in an asymmetric manner and no Okazaki fragments are formed during the process[79]. The replication of mitochondrial DNA is first initiated at replication origin of the H-strand (oriH) located on the L-strand using RNA primers generated by POLRMT. During this initial phase, Twinkle helicase is required to unwind the double helix to provide single-

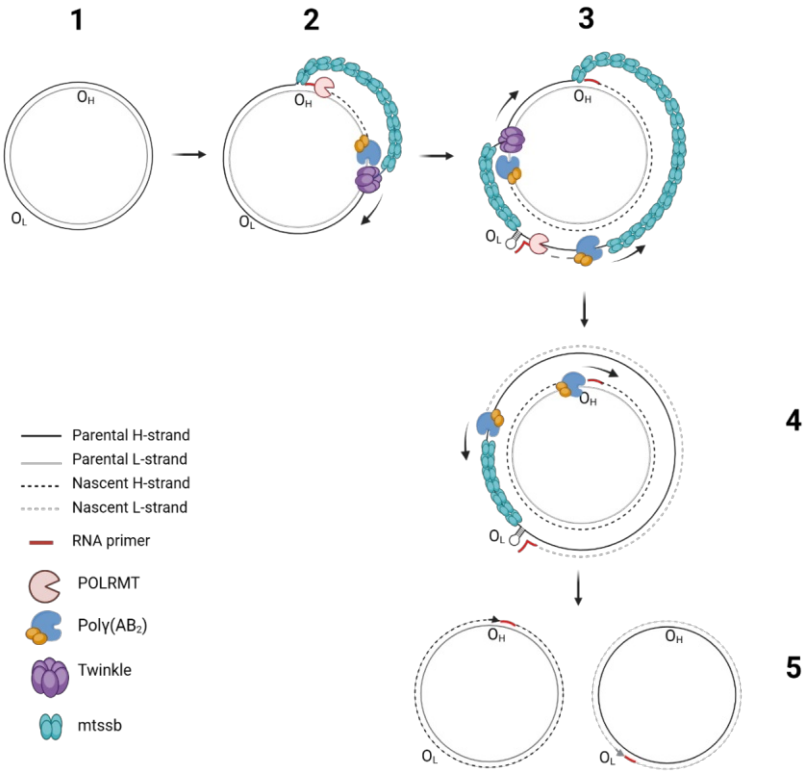

Figure 4. Model of mitochondrial DNA replication. (1) Mitochondria DNA contains two origins of replication, the origin of H-strand synthesis (O_H), located within the control region, and the origin of L-strand synthesis (O_L), a stem-loop forming sequence located on the H-strand. (2) mtDNA replication initiates at O_H, where POLRMT generates an RNA primer that is used by Polγ to begin nascent H-strand synthesis, resulting in the displacement of the parental H-strand. (3) As replication progresses and the displaced H-strand exposes O_L, the O_L stem-loop structure forms and allows POLRMT to synthesize an RNA primer that initiates the synthesis of the nascent L-strand. (4) Synthesis of the H- and L-strand proceeds in opposite directions around the genome until both strands are fully replicated (5). Upon completion of mtDNA synthesis, replication terminates near the respective origins, O_H or O_L, producing two daughter DNA molecules.

stranded template for Polγ to use for DNA synthesis. The replication machinery proceeds in one direction to synthesize new H-strand, consequently resulting in the displacement of the single-stranded, parental H-strand which is now covered by mtSSB. The binding of mtSSB to the displaced H-strand not only stabilizes the exposed single-strand DNA at the replication fork but also prevents random priming by POLRMT on this strand[77,80]. When the replication machinery has completed about two-thirds of the circular mtDNA, it reaches the second origin of replication, oriL, on the parental H-strand. At this stage, the oriL region becomes single stranded and adopts a stem-loop structure that prevents mtSSB binding, which makes it accessible for POLRMT to synthesize a primer to initiate replication of the L-strand[77,81]. From this point, the synthesis of two strands continues in opposite directions

until they complete the full circle. Once both strands are fully replicated, the replication terminates near the respective origins, O_H or O_L, giving rise to two daughter DNA molecules.

2.2. The mtDNA replisome

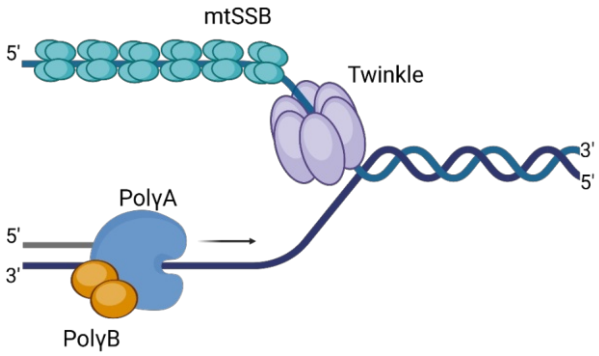

Figure 5. Core components of the mtDNA replication machinery. The hexameric Twinkle helicase translocases in a 5′ to 3′ direction along the DNA template to unwind the double-stranded DNA and generates a fork-like structure. The mitochondrial single-stranded DNA binding protein (mtSSB), which functions as a homotetramer, binds and stabilizes the displaced single-stranded DNA. The heterotrimeric Polγ(AB_2) synthesizes the nascent DNA strand in the 5′-3′ direction.

DNA Polymerase Gamma

DNA polymerase gamma (Polγ) is the only known replicative polymerase found in mammalian mitochondria and is essential for replication and repair of mtDNA. The structural composition of Polγ varies among eukaryotes, with some having a single catalytic subunit such as MIP1 in yeast (*Saccharomyces cerevisiae*) while others possessing both catalytic and accessory subunits in different oligomerization forms, such as a heterodimer in Drosophila or a heterotrimer in mammals[82].

In human cells, Polγ is a holoenzyme consisting of one PolγA catalytic subunit and two Polγ B accessory subunits[82–84].The catalytic subunit PolγA of 140 kDa is encoded by the *POLG* gene in the nuclear genome and belongs to the family A of DNA polymerases that also includes polymerase I of *Escherichia coli* and T7 DNA polymerase of bacteriophage[83]. PolγA possesses both 5′-3′ DNA polymerase and 3′-5′exonuclease activities. The exonuclease proofreading activity of PolγA is remarkably efficient with a frequency of nucleotide misincorporation less than 1 x 10^{-6} per base, making Polγ among the most accurate DNA polymerases in cells[85]. Several recent studies have demonstrated that, upon recognizing mismatches, Polγ can transfer the 3′-end of the nascent strand from the polymerase site to the exonuclease site and shift back after mismatch removal through a series of delicate protein conformational changes[86,87]. This intramolecular transfer enables Polγ to rapidly switch between polymerase and exonuclease modes without dissociating from the DNA template[86,87], likely making this process faster than intermolecular transfer in which the polymerase dissociates and rebinds to the DNA template[88]. In addition, PolγA also has 5′-

deoxyribose phosphate (dRP) lyase activity that could potentially be involved in base excision repair of mtDNA[89,90].

The accessory subunit PolγB in mammalian cells is encoded by *POLG2* gene and has a molecular mass of 55 kDa. PolγB enhances DNA binding and nucleotide binding affinity of PolγA, thereby stimulating both catalytic activity and processivity of PolγA[91]. In solution, PolγB exists as a dimer that binds to monomeric PolγA to form a functional heterotrimer[82]. Each monomer of PolγB in the holoenzyme has a distinct role in stimulation of Polγ processivity: the subunit proximal to PolγA stabilizes interactions with the DNA template whereas the distal monomer works in concert with PolγA to accelerate the polymerization rate[92].

Mitochondrial DNA helicase Twinkle

Twinkle, a nuclear-encoded helicase, has a molecular weight of 72 kDa and is located in the core region of mitochondrial nucleoids[70]. Twinkle helicase is a member of the superfamily 4 (SF4) helicases and highly homologous to the bacteriophage T7 gene protein 4 (gp4)[93].

To assist Polγ that is able to use only single-stranded DNA (ssDNA) as template for DNA synthesis, Twinkle helicase is required for unwinding the double-stranded DNA helix. During replication, Twinkle forms a ring-shaped, hexameric structure that travels along the DNA template and catalyses unwinding of double-stranded DNA in the 5′-3′ direction[94,95]. Each monomer of Twinkle hexamer consists of a N-terminal domain that shares sequence similarity with the primase domain of T7 gp4, a C-terminal domain that contains the helicase motifs, and a flexible linker helix connecting the two functional domains[93,96]. However, unlike the bifunctional T7 gp4 primase-helicase, Twinkle has lost its primase activity due to mutations in key primase motifs in the N-terminal domain during evolution[97,98].

Similar to Polγ, Twinkle helicase is also essential for mitochondrial DNA maintenance as conditional knockout of Twinkle led to severely impaired progression of mtDNA replication and subsequent mtDNA depletion in mice[99]. Several mutations in the *PEO1* gene, which encodes Twinkle, have also been reported in patients with deficient respiratory chain and neuromuscular symptoms caused by mtDNA deletions or depletions[99,100].

Mitochondrial Single-Stranded DNA Binding Protein

Mitochondrial single-stranded DNA binding protein (mtSSB) is another core component of mitochondrial replication machinery and shares high similarities in primary sequence as well as homotetrameric structure with *E.coli* SSB[101]. Four identical mtSSB monomers of 16kDa form a tetramer to bind and coat ssDNA[101,102]. While mtSSB alone can considerably stimulate Twinkle helicase activity on short stretches of DNA[95], its action in concert with Polγ at the replication fork can further assist Twinkle in unwinding longer dsDNA stretches, thus significantly improving mtDNA synthesis[94]. In addition to stimulating Twinkle activity, mtSSB is also involved in directing RNA primer formation during mtDNA

replication, a role that was well characterized for the *E.coli* SSB protein. By preventing nonspecific priming at multiple sites across the genome, mtSSB ensures specific primer synthesis at the proper origins of replication[103].

2.3. Other proteins involved in mtDNA replication

Mitochondrial RNA polymerase (POLRMT)

In addition to its role in mitochondrial transcription, POLRMT synthesizes RNA primers for the initiation of mtDNA replication at both oriH and oriL. Since the L-strand promoter is located upstream of oriH site, transcription by POLRMT from LSP not only generates full-length transcript for L-strand gene expression, but also creates shorter primers to initiate replication at oriH[80,104]. In contrast, the primer formation at oriL initiates when POLRMT uses a short ssDNA stretch of a DNA stem-loop structure at oriL as a template[81]. This loop is formed only when oriL is exposed as single-stranded DNA during replication.

Mitochondrial Topoisomerases

At least two members of type IA and type IB family of topoisomerases, TOP3A and TOP1mt, respectively, have been shown to be localized and active in human mitochondria. Mitochondrial TOP3A is produced by utilizing an alternative transcription initiation site in *TOPIIIα* gene, resulting in an isoform with additional mitochondrial targeting signal[105]. TOP3A is required to resolve hemicatenane structures formed near the ori-H during termination of mtDNA replication, thus ensuring proper mtDNA segregation[106]. Depletion of TOP3A in cells leads to the accumulation of catenated mtDNA structures, and various mutations in *TOPIIIα* gene have been reported in many mitochondrial disorders[107]. The second topoisomerase in mitochondria, TOP1mt, is also highly homologous to the nuclear topoisomerase I isoform, apart from its N-terminus that contains a mitochondrial targeting signal instead of nuclear localization sequence[108]. TOP1mt removes the positive supercoils that arise during replication and transcription, and knockout of TOP1mt in mice leads to increased levels of mtDNA supercoiling and consequently dysfunctional mitochondrial respiration[109,110]. Both TOP3A and TOP1mt are therefore important for proper replication and expression of mtDNA.

Mitochondrial nucleases

After mtDNA replication has been completed, the RNA primers synthesized by POLRMT to initiate DNA synthesis must be removed. The removal of RNA primers involves several factors that differ between oriH and oriL. RNase H1 processes the primers at oriL, leaving 1-3 ribonucleotides at the 5′end of the nascent DNA strand[111]. These remaining ribonucleotides are then removed by the second nuclease to enable the ligation by mitochondrial DNA ligase 3 (LIG3). The identity of this second nuclease, however, is still under debate. Results from *in vitro* experiments have suggested mitochondrial exonuclease G for this role, but aside from its clear mitochondrial localization, *in vivo* evidence supporting this function is still lacking[111,112]. Another enzyme that was suggested in this process is the mitochondrial variant of the flap-structure specific endonuclease 1 (FEN1).

FEN1 has dual mitochondrial and nuclear localization and the mitochondrial isoform of this protein, called FENMIT, is generated by using alternative translation initiation site[113]. Although FENMIT has a binding preference for short RNA or DNA flaps[113], different biochemical assays to examine its 5′-endonuclease activity have shown conflicting results, leaving the proposal for FENMIT's role in mitochondria to be further investigated[114].

Primer processing at OriH requires even more complex cooperations of different nucleases, which remains poorly understood. Nevertheless, a collection of strong evidence has demonstrated the actions of two main nucleases, RNase H1 and the mitochondrial genome maintenance exonuclease (MGME1) in RNA primer removal at oriH. RNase H1 cleaves a major part of the RNA primer, and the remaining ribonucleotides are most likely removed by MGME1[117], a member of the RecB family that preferentially binds to ssDNA and exhibits both 5′ and 3′ exonuclease activity[115–117].

Mitochondrial ligase 3

After RNA primers are removed, the two ends of nascent mtDNA strands are ligated by mitochondrial DNA ligase 3 (mtLIG3). mtLIG3 is encoded by human DNA ligase III gene that also gives rise to the nuclear isoform by using different in-frame starting codon[118]. The longer mitochondrial LIG3 variant is exclusively localized to mitochondrial matrix where its ligase activity plays an essential role in mtDNA replication and repair[118,119].

2.4. Maintenance of mtDNA integrity: repair and degradation

Like nuclear DNA, the mitochondrial genome is continuously exposed to both external and internal DNA-damaging sources. However, mtDNA is more vulnerable and estimated to have higher mutation rates than the nuclear genome[120], mainly due to close proximity of mtDNA to the ROS-generating ETC, which can give rise to various oxidative DNA adducts and lesions[121]. As mitochondrial DNA encodes for essential components of mitochondrial respiratory complexes, defects of which result in impaired oxidative phosphorylation and cellular ATP production, it is not surprising that mtDNA damage can lead to mitochondrial dysfunction and mitochondrial diseases[122]. Integrity of mitochondrial genome is therefore critical for mitochondrial functions and cellular health.

For a long time, mitochondria were thought to lack DNA repair pathways, since early research initially did not observe the repair of pyrimidine dimers produced by ultraviolet (UV) irradiation[123] and some types of alkylation damage[124] in mitochondria. This view has changed overtime as there is increasing evidence for the existence of several DNA repair enzymes and DNA repair activity in mitochondria, and it is now known that certain types of mitochondrial DNA damage can be repaired.

The most well-characterized DNA repair pathway in mitochondria is **base excision repair (BER),** which is responsible for repairing small non-bulky base damage. BER is of particular importance in preserving mtDNA integrity as most common oxidative DNA lesions caused by mtROS are efficiently repaired by this pathway[125]. The first step in the BER pathway is recognition of a modified base by DNA glycosylase, which then cleaves

the N-glycosylic bond and leaves an apurinic (AP) site. The AP site generated upon the removal of the damaged base is subsequently incised by an AP endonuclease, creating a single strand break with 5′-abasic deoxyribose phosphate (dRP) and a free 3′-OH. APE1 is the main AP endonuclease in human mitochondria[126]. The resulting dRP group, which is not ligatable and has to removed prior to the insertion of a new correct base, is excised by dRP lyase activity of mitochondrial Polγ, leaving a gap with 5′-phosphate and 3′-OH. The gap is ultimately filled with a new nucleotide by polymerase activity of Polγ and ligation activity of mitochondrial DNA ligase III[121].

While BER has been well understood and widely accepted as the dominant DNA repair pathway in mammalian mitochondria, much less is known about the existence of other DNA repair pathways for different types of mitochondrial DNA damage.

Mismatch repair (MMR) pathway corrects misincorporation of bases or short insertions/deletions (indels) that are introduced by DNA polymerases during replication. While MMR has been reported in yeast mitochondria[127,128], there is limited evidence that mammalian mitochondria also possess similar MMR mechanisms. A mismatch binding protein, YB-1, has been shown to localize to human mitochondria and largely account for the MMR activity observed in the mitochondrial extract of Hela cells[129]. Additionally, cells depleted of YB-1 exhibited increased mutagenesis, suggesting that YB-1 may play a role in mitochondrial MMR.

Double-strand breaks (DSBs) are one of the most deleterious lesions to a cell, with high probability of causing genomic instability, large-scale alterations, and cell death. DBSs generated in nuclear DNA can be repaired via non-homologous end joining (NHEJ) or homologous recombination (HR). The ability to repair DSBs via HR has been characterized in yeast mitochondria[130,131] and more recently in plant mitochondria[132,133]. However, there is still little evidence of DSBs repair in mammalian mitochondria.

Double-strand breaks in the circular mitochondrial DNA generate linear DNA fragments, but whether these fragments are repaired at all and, if so, by which mechanism, remains unknown. Given the multicopy nature of the mitochondrial genome, it has been proposed that when severely damaged DNA, such as that containing DSBs, cannot be repaired, mitochondria may simply eliminate the damaged molecule without detrimental effects, with replication of the remaining intact genomes compensating for the loss. Consistent with this idea, rapid loss of damaged mtDNA molecules and substantial reduction in overall mtDNA content have been observed following the accumulation of DSBs caused either by oxidative stress[134,135] or by the persistent expression of mitochondrially targeting exonucleases[136,137]. Additionally, recent evidence has indicated that rapid degradation of linearized mtDNA was performed by a concerted action of 5′-3′ exonuclease MGME1 and 3′-5′ exonuclease activity of Polγ[138,139].

3. Mitochondrial dysfunction and diseases

Since mitochondrial proteins are encoded by genes on both nuclear and mitochondrial DNA, mutations in either genome can impair oxidative phosphorylation and mitochondrial biogenesis, leading to mitochondrial dysfunction. Given the essential roles of mitochondria in regulating multiple cellular processes, it is not surprising that mitochondrial dysfunction is associated with a broad spectrum of human diseases, particularly affecting tissues and organs with high energy demands, such as the heart, skeletal muscles, and brain[122].

3.1. The genetics of mitochondrial disorders

3.1.1. Mutations in nuclear DNA

Most of mitochondrial proteins are encoded by nuclear genes and mutations in these genes can therefore disrupt all aspects of mitochondrial homeostasis. In particular, impaired maintenance and expression of mtDNA may arise from pathogenic variants in nuclear genes required for mtDNA replication, nucleotide transport, and dNTP synthesis and salvage pathways[122]. For instance, pathogenic mutations in *POLG* and *POLG2* genes, which encode the catalytic and accessory subunits of the mitochondrial replicative polymerase Polγ, respectively, are well-established causes of mtDNA instability. These mutations can lead to large-scale mtDNA deletions, as observed in patients with chronic progressive external ophthalmoplegia (PEO) and Kearns-Sayre syndrome (KSS)[140,141]. In many cases, multiple mtDNA deletions of variable sizes are accumulated particularly in skeletal muscle, a hallmark that correlates with more severe clinical symptoms in patients with autosomal PEO (adPEO)[100,142]. Defects in the *POLG* gene can also cause mtDNA depletion, which has been described to associate with severe, early-onset clinical phenotypes[143]. Additionally, mutations in nuclear genes involved in multiple mitochondrial processes, including respiration, mitochondrial protein synthesis, and mitochondrial fusion and fission, have also been implicated in a variety of mitochondrial disorders. An example is the *OPA1* gene encoding a dynamin-related GTPase, mutations of which disrupt its function in maintaining the IMM architecture, eventually resulting in multiple DNA deletions associated with clinical features including progressive vision failure, hearing loss, and ataxia[144].

3.1.2. Mutations in mitochondrial DNA

Altered integrity of mitochondrial genome due to point mutations or rearrangements in mtDNA can directly impact the OXPHOS system, leading to a cellular energy deficit.

As previously stated, due to multicopy nature of mitochondrial DNA, both wild-type and mutated mtDNA molecules can coexist within a cell, a state known as heteroplasmy. During cell division, mitochondria carrying mutated mtDNA are randomly distributed to daughter cells. As a result, the proportions of mutant to wild-type mtDNA can shift over time, eventually leading to diverse levels of heteroplasmy both between cells within a tissue and across different tissues[145]. The presence of a mixed population of mtDNA variants allows cells to tolerate a certain proportion of mutant genomes. However, once the mutation load exceeds a threshold at which the wildtype mtDNA molecules can no longer compensate,

mitochondrial functions such as ATP production become compromised, leading to cellular dysfunction. This threshold depends on the specific mutations, cell types, and tissue, and usually ranges between 60-90%, with lower thresholds observed in tissue with high energy needs[122,145].

mtDNA point mutations

Mitochondrial DNA point mutations can arise from errors during mtDNA replication or from ROS-induced DNA damage, which may not be efficiently corrected by the mtDNA repair system[145]. mtDNA point mutations are usually maternally inherited and represent one of the most common causes of mtDNA diseases. Since the mitochondrial genome encodes 13 structural peptide subunits of the OXPHOS system, as well as rRNAs and tRNAs, pathogenic mutations in these genes can directly impair specific OXPHOS complexes or disrupt overall mitochondrial protein synthesis, respectively[146]. Ultimately, ATP production is reduced because of impaired oxidative phosphorylation, leading to dysfunctions in tissues with high energy demands. More than 300 pathogenic point mutations have been reported in mitochondrial diseases, and most of them occur in a heteroplasmic state[145]. The first mitochondrial disease identified to be associated with mtDNA point mutations was Leber's hereditary optic neuropathy (LHON)[147]. LHON-associated point mutations are found across the mitochondrial genome, but most occur in *MT-ND1*, *MT-ND4*, and *MT-ND6* genes, which all encode for subunits of Complex I[145]. LHON predominantly affects males and is characterized by acute or subacute, bilateral vision loss[148]. Another common disease-causing mtDNA point mutation is found in the $tRNA^{Leu(UUR)}$ gene, leading to tRNA dysfunction and eventually defective mitochondrial translation. The mutation m.3243A>D in this gene is associated with two different clinical presentations: a low mutation load causes maternally inherited diabetes and deafness (MIDD) characterized by isolated diabetes, hearing loss, and migraines[149], whereas a high heteroplasmy level results in a more severe disease, mitochondrial encephalopathy lactic acidosis stroke-like episodes (MELAS)[150].

Large-scale mtDNA deletions

Unlike point mutations, large-scale mtDNA deletions are usually sporadic and often arise during embryonic development or normal aging[151–154]. These deletions differ in size, ranging from 1.8-8 kb and are thought to result from replication slippage or erroneous repair of damaged mtDNA, although the precise molecular mechanisms underlying their formation remain unclear[145]. Nevertheless, certain regions of mitochondrial genome are more prone to deletion events, many of which are flanked by tandem repeat sequences[155,156]. Additionally, several studies have provided evidence of an association between mtDNA deletion breakpoints and potential G4-forming sequences[64,66,67]. Large-scale mtDNA deletions have been implicated in aging, various types of cancer and mitochondrial disorders including three main deletion-associated clinical phenotypes: chronic progressive external ophthalmoplegia (PEO), Kearns-Sayre syndrome (KSS), and Pearson's syndrome[157].

A large proportion of pathogenic mtDNA deletions occurs within the major arc that spans the region between oriH and oriL. The most frequently reported deletion is the loss of 4977 bp within this region (Δ4977), often referred to as the "common deletion". This deletion, whose breakpoints are flanked by 13-bp direct repeats, results in the loss of 7 OXPHOS genes, thereby severely hampering mitochondrial respiration and cellular energy production[155]. The common deletion has been identified as the causative mutation for all three syndromes: chronic PEO, Kearns-Sayre syndrome (KSS), and Pearson's syndrome[157].

3.2. Cellular responses to mitochondrial dysfunction

Dysfunctional mitochondria can trigger various cellular responses, including the activation of the mitochondrial unfolded protein response (UPRmt) to increase mitochondrial biogenesis, activation of retrograde signalling pathways (e.g. upregulating AMPK pathway) to restore cellular energy homeostasis, and initiation of mitophagy to remove damaged mitochondria. These processes aim to help the cell adapt to mitochondrial stress and maintain the cellular energy balance; however, if the dysfunction is severe or prolonged, it can cause widespread inflammation and apoptosis, eventually leading to tissue damage and disease progression. The following section will mainly discuss the activation of AMPK pathway in the context of mitochondrial health and energy stress.

3.2.1. AMPK activation and metabolic reprogramming

AMP-activated protein kinase (AMPK) is a heterotrimeric complex consisting of one catalytic α-subunit and two regulatory subunits, β and γ. In human, each subunit has multiple isoforms that are encoded by different genes, potentially giving rise to 12 distinct combinations of AMPK complexes[158]. The difference in subunit compositions of AMPK complex has been proposed to influence both its activity and subcellular localization, allowing the protein kinase to respond to various types of stimuli. AMPK has been reported in different subcellular compartments, including nucleus, lysosomes, mitochondria, and endoplasmic reticulum, where it is suggested to target different substrates to generate specific responses[159].

AMPK is a key energy sensor and metabolic regulator in the maintenance of cellular energy homeostasis. AMPK senses changes in ratios of ATP-to-AMP/ADP caused by imbalance of ATP consumption and ATP production. Upon energy stress signalled by a decrease in ATP levels, AMPK becomes activated and phosphorylates a variety of downstream targets involved in multiple metabolic pathways to restore the cellular energy balance. This restoration is achieved by the promotion of catabolic pathways (including glucose and lipid breakdown, autophagy and mitophagy, and mitochondrial biogenesis) to stimulate ATP production, and the inhibition of anabolic processes (such as protein and lipid synthesis) to minimize ATP consumption[159].

3.2.2. AMPK signalling in mitochondrial functions

Extensive research over the past decades has revealed regulatory roles of AMPK in various aspects of mitochondrial homeostasis, including mitochondrial biogenesis, mitochondrial dynamics, and mitophagy.

AMPK has been established as a central regulator of mitochondrial biogenesis, which occurs through the growth of the existing mitochondrial network to enhance ATP production in response to increased energy expenditure. Several studies have described that AMPK activation induces the expression of the peroxisome proliferator-activated receptor-γ-co-activator 1α (PGC1-α), a master regulator required for transcriptional regulation of several genes involved in mitochondrial metabolism[160,161], thereby promoting mitochondrial biogenesis.

Mitochondrial dynamics involves two opposing processes, fission and fusion, the balance of which is critical to maintain mitochondrial health and function. Elongated mitochondria resulted from mitochondrial hyperfusion have been proposed to sustain ATP production during starvation, whereas in response to mitochondrial stress, mitochondrial fission leads to segregation of damaged mitochondria, facilitating the removal of dysfunctional parts from the mitochondrial network by mitophagy[159]. A collection of evidence has suggested a role for AMPK in regulating mitochondrial dynamics. Activated AMPK was shown *in vivo* to directly phosphorylate mitochondrial fission factor (MFF) in the OMM, a core component of the mitochondrial fission pathway, thereby potentially contributing to the modulation of the mitochondrial network during energy stress[162,163].

Additionally, AMPK has been shown to be directly involved in mitophagy, a process that selectively removes dysfunctional mitochondria, by activating key proteins involved in the autophagy pathway. AMPK activation was shown to induce the phosphorylation of Unc-51 like autophagy activating kinase 1 (ULK1), a kinase essential for autophagy induction, which subsequently promotes the formation of the autophagosome to clear the damaged mitochondrion during various stresses[164,165]. In addition to AMPK-ULK1 axis, mitophagy can also be mediated via the phosphatase and tension homolog-induced putative kinase 1 (PINK1) – the E3 ubiquitin ligase (Parkin) pathway, which is activated upon mitochondrial depolarization. It was shown that dissipation of the mitochondrial membrane potential (ΔΨ) by a proton ionophore carbonylcyanide m-chlorophenylhydrazone (CCCP) inhibits mitochondrial ATP synthesis, thereby strongly activating AMPK. The loss of ΔΨ also results in accumulation of PINK in the OMM[162]. The activated AMPK then phosphorylates Ser495 in PINK1[163], subsequently triggering the recruitment of Parkin to the OMM to mark the depolarized mitochondria for degradation by the mitophagy machinery[168].

In summary, by coordinating mitochondrial biogenesis, dynamics, and mitophagy, AMPK plays a central role in maintaining mitochondrial health under physiological conditions as well as in response to mitochondrial dysfunctions, helping cells adapt to energetic stress, indicating the potentials of AMPK modulation for therapeutic implications in many diseases including mitochondrial disorders.

4. PolDIP2: a multifunctional protein involved in mitochondrial maintenance and many cellular processes

As described above, mitochondrial DNA is replicated by a unique machinery that includes the main replicative DNA polymerase, Polγ. However, under stress conditions, such as in the presence of DNA damage, Polγ is unable to bypass DNA damage sites, causing replication stalling which, if not repaired, can lead to mtDNA depletion and deletions. Several specialized enzymes have been found to be recruited to stalled replication sites to ensure the continuation of DNA synthesis by Polγ. One of them is PrimPol, a DNA primase-polymerase, and its putative processivity factor, Polymerase-delta interacting protein 2 or PolDIP2, which have been suggested to work together to efficiently restart DNA synthesis downstream of DNA lesions or stalled replication[169–171].

PolDIP2, also known as PDIP38 and Mitogenin-1, was initially identified through yeast two-hybrid screening as an interacting partner of p50 subunit of DNA polymerase delta (Polδ) and Proliferating Cell Nuclear Antigen (PCNA)[172]. Subsequent studies suggested additional functional interactions of PolDIP2 with several DNA translesion synthesis (TLS) polymerases including Y-family Pol η[173], X-family Pol λ[174], and primase-polymerase PrimPol[171], suggesting PolDIP2 involvement in DNA damage tolerance pathway. However, a growing body of evidence over the past decade has suggested that PolDIP2 may also play roles beyond DNA maintenance, as it associates with diverse binding partners involved in various cellular processes such as cell-cycle regulation[175], cell migration and cellular adhesion[176,177], oxidative signalling[176], and mitochondrial metabolism[178].

While the mechanisms by which PolDIP2 regulates these processes are not well understood, the wide range of PolDIP2 binding partners and its potential functions in a variety of cellular pathways appear to be attributed to the protein abundance, its multiple subcellular localizations, and its distinct structure, which allows highly dynamic conformational changes[179,180]. However, it remains unclear whether PolDIP2 directly participates in all of these proposed processes, as some reported effects may reflect indirect cellular responses or *in vitro* artefacts that do not necessarily translate to the *in vivo* context. Further mechanistic studies will be needed to delineate the physiological roles of PolDIP2.

4.1. Structure and expression of PolDIP2

4.1.1. Overall structure and domain organization

Phylogenetic analysis suggested that PolDIP2 first emerged within Holozoa as there were no PolDIP2 orthologues found in prokaryotes, plants and fungi[179,180]. Within Holozoan clades, PolDIP2 is present and highly conserved across the Metazoa[180].

The human *PolDIP2* gene is located at 17q11.2 and encodes for the full-length protein of 368 amino acids. Human PolDIP2 (hPolDIP2) consists of an N-terminal mitochondrial targeting sequence (MTS) and two main functional domains: a YccV-like domain at the N-terminus and a C-terminal ApaG/DUF525-like domain[172,175]. The two domains are connected by a short α/β linker region[181]. Interestingly, although YccV and ApaG domains

are individually present in various prokaryotic proteins, PolDIP2 is found only within the Holozoa, suggesting that PolDIP2 likely resulted from the fusion of these two domains during eukaryotic evolution, which probably occurred in the very beginning of the Holozoan establishment[179,180].

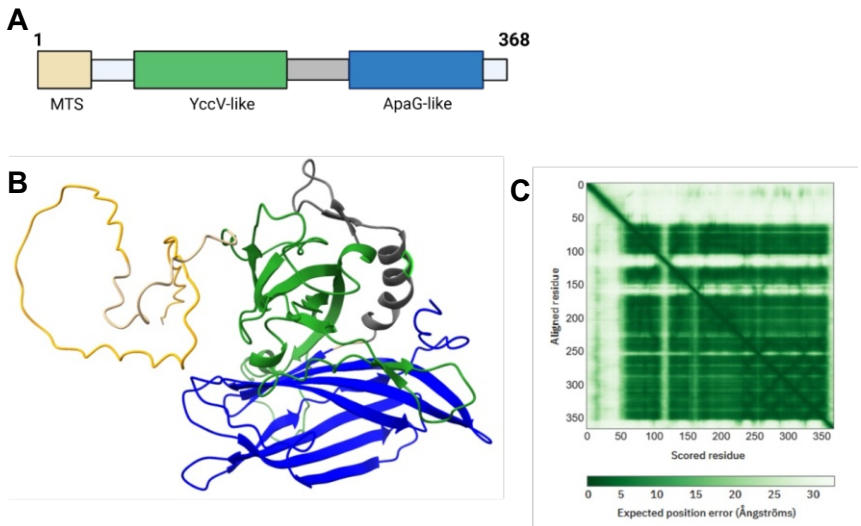

Figure 6. Overview of human PolDIP2 structure. (A) Schematic representation of the domain architecture of human PolDIP2. (B) Predicted full-length PolDIP2 structure generated by Alphafold3 (Database ID: AF-Q9Y2S7-F1-v6). Domain colouring: mitochondrial targeting sequence in yellow, YccV-like domain in green, linker (extended YccV) in gray, and ApaG-like domain in blue. (C) Predicted Aligned Error (PAE) plot corresponding to the structure shown in panel B.

The YccV-like domain of PolDIP2 shares structural similarity with domains found in bacterial heat-shock protein Q (HspQ) and the ClpF adaptor protein of plant Clp protease system, both of which participate in the regulation of proteolysis in bacteria and chloroplasts, respectively[182–184]. In *E.coli*, the YccV domain of HspQ not only functions in protein quality control during heat shock stress, but also binds hemimethylated DNA to regulate gene expression[185]. Although the YccV-like domain of human PolDIP2 shares 34% sequence homology with *E.coli* YccV, early studies reported no detectable DNA binding activity under standard conditions used for electrophoretic mobility shift assay (EMSA)[174]. However, later studies have demonstrated a relatively low DNA binding affinity of PolDIP2 when present in excess relative to primer/template DNA[186]. In addition, PolDIP2 YccV-like domain harbours three putative PCNA-interacting protein (PIP) motifs that are suggested to contribute to its functional association with PCNA[172,179].

The ApaG/DUF525-like domain is also present in several bacterial ApaG proteins and in some eukaryotic F-box family proteins, where it is suggested to play a role in protein-protein interactions, substrate recognition within ubiquitin-dependent protein degradation pathways, and cation efflux[187,188]. In human, PolDIP2 and F-Box only 3 (FBxo3) are the

only known proteins that contain an ApaG-like domain. Although the ApaG-like domain in human PolDIP2 shows low primary sequence similarity to bacterial ApaG proteins (i.e. *Xanthomonas axonopodis* ApaG*)* and F-Box proteins (i.e. human FBxo3), their tertiary structures are highly conserved, suggesting potential functional similarities[172,181]. A conserved glycine-rich motif (GxGxxG, a.a. 298-303 in human PolDIP2) is present within this ApaG domain[172]. While this motif commonly mediates pyrophosphate or nucleotide binding in many ATP-, NAD-, and FAD- binding proteins[172,189], such activity has not been detected in ApaG homologues from *X.axonopodis* or human FBxo3[190,191]. Furthermore, PolDIP2 also contains a unique positively-charged arginine cluster that partly overlaps with the glycine-rich motif and has been shown to stimulate PrimPol-dependent DNA synthesis *in vitro*[186]. This Arg cluster appears to form a binding channel that facilitates optimal dNTP positioning during PolDIP2-PrimPol association, thereby enhancing PrimPol polymerase activity[186].

4.1.2. Tissue expression and subcellular localization

Tissue expression

PolDIP2 is ubiquitously expressed across a wide range of human tissues and cell types. However, its expression levels vary considerably between tissues, with highest PolDIP2 expression in metabolically active organs such as the liver, kidney, and muscle tissues, whereas markedly lower expression levels are detected in myeloid cells[176,180,192]. In addition, altered PolDIP2 expression has been reported in several cancer types, including human breast cancer cells and lung adenocarcinoma cells [193,194].

Subcellular localization

PolDIP2 contains a canonical N-terminal mitochondrial targeting sequence (MTS), which targets the protein to mitochondria where the MTS is cleaved, giving rise to a mature functional mitochondrial isoform (mito-PolDIP2). Consistent with this, early studies confirmed mitochondrial localization of human PolDIP2[195], and a subsequent study led by Cheng et al reported that PolDIP2 was exclusively localized within the mitochondrial matrix of HeLa cells and associated with mitochondria nucleoid components[178]. More recent studies, however, have described additional subcellular localizations patterns, raising questions whether PolDIP2 is strictly mitochondrial or possesses a broader context-dependent distribution. In these studies, PolDIP2 has been implicated in several cellular processes beyond mitochondrial function, including cell-cycle regulation[196], nuclear DNA maintenance[171,174,197], and oxidative signalling[176,198].

Evidence also indicates that PolDIP2 may undergo dynamic intracellular relocalization. Specifically, PolDIP2 has been shown to shuttle between the cytoplasm and nucleus in a cell-cycle dependent manner, a process regulated by the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Perturbation of CEACAM1 with antibodies caused PolDIP2 to relocate to the plasma membrane, while in quiescent cells, the same treatment led to recruitment of PolDIP2 to the nucleus[199]. Cell-cycle dependent localization was also observed in mouse myoblast C2C12 cells, where the mouse PolDIP2 homolog was detected

in either mitochondria or the nucleus depending on proliferative status[200]. Furthermore, in rat vascular smooth muscle cells (VSMC), PolDIP2 colocalized with NADPH oxidase 4 (Nox4) in both the nucleus and cytoplasm, specifically at focal adhesions and along stress fibers, where the two proteins were suggested to cooperatively mediate ROS generation and subsequent VSMC migration[176].

Collectively, the reported differences in subcellular localization across cell types imply that PolDIP2 distribution may vary according to cellular context, interacting partners, or physiological conditions.

4.2. Mitochondrial function

Although PolDIP2 was initially described to bind nuclear polymerases Polδ and PCNA, many subsequent studies have provided strong evidence of PolDIP2 localization to mitochondria where the protein has been described as an important factor in multiple mitochondrial processes and pathways.

4.2.1. Role in mitochondrial morphology

In mouse myoblast C2C12 cells, overexpression of the mouse PolDIP2 induced elongation of mitochondria, whereas shRNA silencing of this gene increased fragmented mitochondria, thereby affecting the mitochondrial morphology[200]. Similar observations were made in human umbilical endothelial cells (HUVECs) and central nervous system (CNS) catecholaminergic cells, further supporting the potential role of PolDIP2 in mitochondrial fusion, although the underlying molecular mechanism remains unresolved [200].

4.2.2. Role in mtDNA maintenance

As PrimPol's processivity factor

PrimPol belongs to the archaeo-eukaryotic primase (AEP) superfamily and possesses both primase and polymerase activity[169,201]. PrimPol has the capacity to re-prime downstream of DNA lesion or stalled replication fork *in vitro*, thus ensuring the continuation of DNA replication progression, indicating an important role for PrimPol in DNA translesion synthesis (TLS)[170,202,203]. In addition, PrimPol can restart DNA synthesis downstream of G-quadruplex structures that impede replication progression[204]. As is typical for TLS DNA polymerases, PrimPol exhibits low processivity and a relatively high error rate during DNA synthesis[205]. PrimPol localizes to both nucleus and mitochondria and was suggested to play an important role in DNA damage tolerance in both genomes in mammalian cells[169]. In particular, while wild-type cells were able to resume mtDNA replication following drug-induced inhibition of the replicative DNA polymerase gamma, PrimPol KO cells failed to do so and rapidly lost all detectable mtDNA replication intermediates, as shown by two-dimensional DNA gel electrophoresis[169].

Using pull-down assay in cultured human cells, Guilliam and colleagues identified PolDIP2 as a binding partner of PrimPol, in addition to replication protein A (RPA), the nuclear and mitochondrial single-stranded DNA binding protein (SSB and mtSSB, respectively)[205]. Follow-up studies confirmed the interaction between PolDIP2 and PrimPol in human cells

using chemical cross-linking combined with mass spectrometry analysis[171]. While mtSSB was reported to suppress PrimPol activities, likely to limit unscheduled or error-prone DNA synthesis[205], PolDIP2 was shown to enhance PrimPol polymerase activity but not its primase activity[117].

PrimPol is a non-processive DNA polymerase with a relatively low affinity for DNA and limited DNA synthesis activity *in vitro*[186]. Interacting with PolDIP2 enhanced DNA primer-template and dNTP binding affinity of PrimPol, thereby increasing PrimPol's processivity and polymerase activity[171,186]. The interaction between PolDIP2 and PrimPol was shown to involve multiple binding sites on both proteins[171,186]. The N-terminal region of PolDIP2, including the MTS, exhibits considerable conformation flexibility, which facilitates its interaction with the catalytic domain of PrimPol[171,179]. This interaction appears functionally important, as only the full-length (FL) PolDIP2, and not the mitochondrial variant, was able to enhance DNA binding and fully stimulate processivity of PrimPol[171,186]. In addition to the N-terminus, the ApaG-like domain of PolDIP2 interacts with a flexible loop in PrimPol (aa 201-260 in human PrimPol), thereby improving PrimPol affinity for the DNA template[171,186]. Importantly, a unique arginine cluster adjacent to the glycine-rich motif in the ApaG-like domain was found to be essential for PrimPol stimulation by increasing its affinity for the incoming dNTPs[171,186].

While both PolDIP2 and PrimPol have been reported to co-localize in mitochondria, their functional interaction has so far only been observed *in vitro*, and only when using full-length PolDIP2 but not the MTS-free mitochondrial variant[171,186]. This raises questions about the physiological relevance of PolDIP2-mediated stimulation of PrimPol activity within mitochondria. Nevertheless, it has been speculated that additional proteins or post-translational modifications present in mitochondria may mediate the interaction between PrimPol and mito-PolDIP2.

4.2.3. Role in mitochondrial homeostasis

In humans, mitochondrial protein homeostasis is maintained by at least five different AAA+ (ATPases associated with a variety of cellular activities) proteases, two of which are matrix-localized, LONP1 and CLPXP. Human CLPXP protease is composed of a hexameric unfoldase CLPX component and a heptameric CLPP peptidase component[206–208]. The unfoldase CLPX recognizes and unfolds substrates before translocating them to the proteolytic CLPP component in an ATP-dependent manner[209]. For many AAA+ proteases, the recognition of different substrates sometimes requires adaptor proteins (also referred to as specialized cofactors)[210–212]. Many substrate adaptors have been described for the bacterial homolog of CLPX[213], however, no such adaptor proteins had been identified in mammalian mitochondria until recently.

Using a cross-linking approach combined with immunoprecipitation, Cheng and colleagues performed the earliest systematic protein-interaction screens for human PolDIP2 and identified several potential binding partners[174]. In addition to single-stranded DNA binding proteins and constituents of mitochondrial nucleoid, two mitochondrial proteases, LONP1

and CLPX, as well as mitochondrial chaperons were found to associate with endogenous human PolDIP2 after crosslinking[178]. In line with this, a subsequent study identified PolDIP2 as a putative CLPX-interacting protein in a pull-down assay using recombinant human PolDIP2 to capture interacting partners from isolated mouse mitochondria[214]. Follow-up work from the same group later confirmed PolDIP2-CLPX interaction within the mitochondria matrix and further characterized PolDIP2 as a novel adaptor protein for the CLPXP protease[181]. PolDIP2 was shown to directly bind to CLPX, which specifically inhibits CLPX turnover by LONP1[181]. The reduced protein levels of CLPX observed in PolDIP2-deficient cells indicated that PolDIP2 plays a role in maintaining the steady-state levels of CLPX, ultimately regulating the cellular activity of CLPXP system[181]. Moreover, PolDIP2 has also been proposed to function as an adaptor protein for mitochondrial CLPXP[181]. In this model, the C-terminal ApaG-like domain of PolDIP2 is suggested to recognize and accommodate specific, yet unidentified, protein substrates within its conserved binding pocket. These substrates would then be delivered to the CLPXP protease for degradation via docking of the PolDIP2 NTD to a defined loop within the Zinc-finger binding domain of CLPX, thereby modulating the substrate specificity of CLPXP system.

In agreement with this proposed role of PolDIP2 in CLPX-mediated degradation of protein substrates in mitochondrial matrix, PolDIP2 has recently been shown to bind to ALA synthase (ALAS), the first and rate-limiting enzyme in heme biosynthesis, in the presence of heme and promote the recognition of ALAS by CLPX for subsequent degradation[215].

4.3. Other possible cellular functions of PolDIP2

4.3.1. DNA replication and repair

As previously stated, PolDIP2 was initially described as an interacting partner of the p50 subunit of Pol δ and proliferating cell nuclear antigen (PCNA) in a yeast two-hybrid screen[172]. The ability of PolDIP2 to bind p50 and PCNA was further supported by GST pull-down assay and coimmunoprecipitation experiments using human cell extracts, providing some evidence that these interactions may occur within the Pol δ complex under physiological conditions. In addition, the authors identified three putative PCNA-binding motifs within the PolDIP2 sequence, similar to those found in established PCNA-binding proteins[172]. This suggests that PolDIP2 could potentially regulate the Pol δ-PCNA complex activity, which plays an important role in the nuclear DNA replication and damage repair.

Subsequent studies demonstrated that PolDIP2 interacted with several translesion DNA synthesis (TLS) polymerases, including DNA polymerase eta (Pol η), Rev7 subunit of DNA polymerase zeta (Pol ζ), and Rev1 (dCMP transferase)[173]. PolDIP2 directly interacts with the ubiquitin-binding zinc (UBZ) finger domain of Pol η and was suggested to regulate the focal localization of Pol η. siRNA-mediated depletion of PolDIP2 in human MRC5 cells increased the proportion of cells habouring Pol η foci under basal conditions and reduced cell viability following UV radiation. These observations, in combination with PolDIP2's reported interactions with several TLS polymerases, raising the possibility that PolDIP2 is

involved in the switching between replicative and specialized DNA polymerases at damaged DNA sites[173].

The proposed role of PolDIP2 as a mediator protein in translesion DNA synthesis is further supported by evidence that PolDIP2 physically interacted with and assisted TLS polymerase lamda (Pol λ) in efficiently bypassing the 8-oxoG DNA lesions *in vitro*[174]. PolDIP2 was shown to enhance the processivity of both Pol η and Pol λ on non-damaged DNA templates, as well as increase the efficiency of accurate 8-oxoG bypass. Similar stimulatory effects of PolDIP2 on Pol η and Pol λ TLS activity were also observed with other two common DNA lesions, abasic (AP) site and cyclobutene thymine dimers (T-T)[174]. Furthermore, mouse embryonic fibroblast (MEF) cells depleted of PolDIP2 exhibited increased sensitivity to oxidative stress, a phenotype similar to that observed in Pol $\lambda^{-/-}$ cells, suggesting that both Pol λ and PolDIP2 are important for cellular tolerance to oxidative damage[174].

Despite evidence of PolDIP2 interacting with these nuclear DNA polymerases both *in vitro* and in cells, it remains unclear how PolDIP2 is able to bind such diverse protein partners and by which mechanism PolDIP2 exerts its function in coordination with them during DNA damage tolerance.

4.3.2. Cell cycle regulation and proliferation

Previous findings have suggested that the expression and subcellular distribution of PolDIP2 in mammalian cells appear to be not only tissue and cell-type specific but also cell-cycle-status dependent[200]. PolDIP2 was shown to be exclusively localized to mitochondria of mouse myoblast cells throughout most of the cell cycle, but was detected in both mitochondrial and nuclear compartments from the G2/M phase through the late G1 phase[200], suggesting a potential role in cell cycle regulation. However, the mechanism by which PolDIP2 is shuttled between these compartments in cell-cycle dependent manner remained unclear.

A subsequent study further investigated a role of PolDIP2 in cell division using mice homozygous for a PolDIP2 deletion[175]. PolDIP2$^{-/-}$ mice exhibited severely impaired embryonic development and perinatal lethality. Cell growth of PolDIP2$^{-/-}$ mouse embryonic fibroblasts (MEFs) was significantly reduced, potentially in part due to increased autophagy in knockout cells. Additionally, flow cytometry revealed an accumulation of PolDIP2$^{-/-}$ MEFs cells in the G1 and G2/M phases compared to wild-type cells, suggesting that loss of PolDIP2 altered expression of key cell cycle regulators[175]. While phosphorylation of p53 and expressions of its downstream transcriptional targets were increased in early-passage PolDIP2$^{-/-}$ MEFs, E2F/Rb-dependent expressions of PCNA, Cdk1, and Cyclin A2 were found to be downregulated at late passages. Notably, the SV40 immortalization restored both growth rate and cell cycle profile of PolDIP2$^{-/-}$ MEFs cells to wild-type levels[175]. Together, these findings suggest that PolDIP2 plays an important role in regulating cell cycle progression.

4.3.3. Oxidative stress signalling and cell migration

A yeast two-hybrid screen using rat vascular smooth muscle cells (VSMC) proteins revealed an interaction between PolDIP2 and the $p22^{phox}$ subunit of NADPH oxidase (Nox) 4 enzyme, which was further confirmed by pull-down and coimmunoprecipitation assays[176,177]. PolDIP2 and $p22^{phox}$ /Nox4 were additionally found to colocalize at focal adhesions, stress fiber and the nucleus of VSMCs. Overexpression of PolDIP2 stimulated Nox4-mediated ROS production, whereas PolDIP2 knockdown decreased basal ROS levels in VSCMs and resulted in morphological changes similar to those observed in Nox4 depleted cells[119]. Moreover, siRNA silencing PolDIP2 or Nox4 resulted in the loss of focal adhesion-like structures, while overexpressing PolDIP2 increased focal adhesion and stress fiber formation. ROS can activate RhoA, a master regulator of the actin cytoskeleton that plays a crucial role in cell migration[176,177]. Based on these findings, it was proposed that PolDIP2 regulates ROS production through Nox4, which in turn modulates RhoA activity required for cell migration[176,177]. Supporting this model, the loss of stress fibers and focal adhesions observed in PolDIP2-depleted VSMCs could be rescued by expression of constitutively active RhoA[119].

AIMS OF THE THESIS

The present study comprises four interconnected projects that aim at advancing our understanding of human mitochondrial DNA maintenance and its implication in mitochondrial dysfunction. The first part of the thesis focuses on the molecular mechanisms underlying mitochondrial genome instability, either through intrinsic replication errors caused by pathogenic mutations in DNA polymerase gamma or through the formation of secondary DNA structures that impedes mtDNA replication. In addition, we investigate how cells respond to mitochondrial dysfunctions caused by mtDNA instability. In the second part of the thesis, we employ biochemical and cell biological approaches to characterize redox-dependent dimerization of mitochondrial PolDIP2 and to explore its potential roles in mitochondrial homeostasis.

The specific aims of this thesis were to:

1. Define the molecular basis by which the pathogenic Y951N variant in mitochondrial DNA polymerase gamma disrupts mtDNA replication and contributes to pathological phenotypes (**paper I**)
2. Characterize cellular activation of the energy-sensing kinase AMPK in response to mtDNA depletion and evaluate the therapeutic potential of the AMPK activator A-769662 in mitigating mitochondrial dysfunction (**paper II**)
3. Develop and validate a novel method for mapping G-quadruplexes (G4s) specifically within mitochondria and use this tool to examine how G4 formation influences mtDNA stability under various cellular conditions (**paper III**)
4. Investigate redox-regulated dimerization of PolDIP2 and examine its physiological relevance and potential functions within human mitochondria. (**Manuscript IV)**

Achieving these objectives provides new insights into the mechanisms that safeguard mitochondrial genome integrity and regulate mitochondrial homeostasis. Furthermore, the work presented in projects II and III highlights potential therapeutic directions for mitochondrial diseases and offers valuable tools for future research in mitochondrial biology.

RESULTS AND DISCUSSION

Part I - Insights into mechanisms of mtDNA instability in human cells

1. Pathogenic mutations in DNA polymerase gamma in mitochondrial DNA-depletion associated diseases (paper I and II)

1.1. The Polγ Y951N patient mutation disrupts the switch between DNA synthesis and proofreading, triggering mitochondrial DNA instability (paper I)

As previously mentioned, DNA polymerase gamma is the only replicative polymerase in human mitochondria. Human Polγ is a holoenzyme consisting of a catalytic PolγA subunit and two accessory PolγB subunits[82,84]. The catalytic subunit PolγA has 5′-3′ DNA polymerase activity responsible for DNA synthesis, and 3′- 5′ exonuclease activity that functions as a proofreading mechanism to ensure the correct nucleotide incorporation. A precise cooperation between the two activities of PolγA during mtDNA replication is therefore critical to ensure proper mitochondrial genome integrity. Like other DNA polymerases with proofreading activity, PolγA continuously shuttles the 3′-end of the nascent DNA strand between the polymerase and the exonuclease sites during replication via either intermolecular or intramolecular switching mechanisms[88]. The intermolecular transfer occurs when the polymerase dissociates and rebinds to the DNA template in a different domain[88]. In contrast, the intramolecular mechanism involves a series of delicate conformational changes within each domain of PolγA, enabling the enzyme to rapidly switch between polymerase and exonuclease modes without dissociating from the DNA template, making the process faster than the intermolecular transfer[86,87].

To date, more than 300 pathogenic mutations in Polγ have been implicated in various mitochondrial disorders[216]. Most patient mutations in PolγA occur either in its polymerase domain that primarily impairs DNA synthesis or in the exonuclease domain which mostly compromises its proofreading activity, thereby affecting the fidelity of mtDNA replication and eventually leading to mtDNA deletions or depletion[217]. However, much less is known about how different disease-causing mutations may influence the intramolecular switch of Polγ.

PolγA Y951N expression leads to mtDNA replication stalling and depletion in human cells

In this study, we were interested in the tyrosine-to-asparagine substitution of residue 951 (Y951N) in PolγA found in patients with mitochondrial disorder phenotypes such as bilateral congenital cataracts, marked muscle weakness, and atrophy[218]. The Y951 residue, located in the polymerase domain of PolγA, is highly conserved among A-family DNA polymerases and important for nucleotide selection (**Fig. 7**).

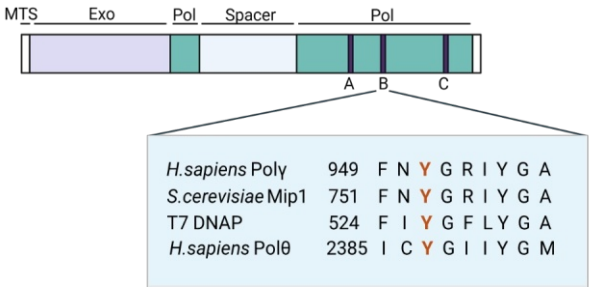

Figure 7. Domain organization of human PolγA and sequence alignment of the B motif in PolγA with other A-family DNA polymerases. The schematic (top) depicts the major domains of human PolγA, including the mitochondrial targeting sequence (MTS), exonuclease (Exo) domain, and polymerase (Pol) domain. The pathogenic residue tyrosine 951 (Y951, in bold orange) is located within the conserved B motif of the polymerase domain. The sequence alignment (bottom) shows the B motif of human PolγA compared with representative A-Family DNA polymerases from multiple species, illustrating the strong evolutionary conservation of the tyrosine at this position.

To investigate the mechanism by which the pathogenic mutation Y951N causes mtDNA-associated disorders, we first examined the impact of this mutation on mtDNA replication and cell growth in human cells. To do this, we established stable inducible Flp-In T-Rex HEK293 cell lines expressing either wildtype (WT) Polγ-Flag or Y951N mutant upon doxycycline (Dox) induction and monitored the expression of these exogenous proteins over a 10-day period. By performing multiplex qPCR analysis, we observed a progressive decline in mtDNA content in cells expressing the Y951N variant. The relative mtDNA copy number in Y951N-induced cells was rapidly reduced to below 20% of that in the WT-expressing cells already on day 3 of Dox induction. The mtDNA depletion coincided with an accumulation of mtDNA replication intermediates in the mutant-induced cells, indicating marked replication stalling. Furthermore, the pronounced effects of the Y951N variant expression on mitochondrial genomic integrity were followed by a significant decrease in cell proliferation and an increase in cell death. These results indicate that the Y951N mutation acts in a dominant-negative manner and causes mtDNA replication stalling, consequently leading to mtDNA depletion and impaired cell growth.

The Y951N substitution does not affect DNA binding but causes a moderate reduction in DNA synthesis activity and dNTP binding affinity

To elucidate the mechanism underlying mtDNA replication stalling caused by the Y951N mutation, we first assessed DNA binding using electrophoretic mobility shift assay (EMSA). We found that the Y951N variant bound the DNA template comparably to the WT protein, both in the absence and presence of the processivity subunit PolγB.

We then examined DNA synthesis activity by performing primer extension assays on different DNA substrates. Interestingly, when a 25-70 nt primer/template DNA substrate was used, the Y951N variant was able to synthesize full-length DNA products despite a moderate reduction in DNA synthesis activity, whereas its DNA synthesis efficiency was

considerably affected on a longer DNA template (a 3-kb single-stranded plasmid). The altered processivity of the Y951N variant as well as its retained capacity to extend DNA products to full-length were also observed in single-hit conditions, in which heparin was added to prevent any rebinding events of Polγ that could potentially mask the actual defects in Y951N's synthesis activity.

Furthermore, we reconstituted a minimal mtDNA replisome consisting of Polγ, the mtDNA helicase Twinkle, and mitochondrial single-stranded DNA binding protein (mtSSB) and performed a rolling-circle replication assay to assess DNA synthesis by the WT and Y951N Polγ variants on a primed single-stranded mini-circle substrate. The results showed that replication by the Y951N variant was substantially impaired in comparison with the WT protein even in the presence of Twinkle stimulation. In addition, when the combination of WT and Y951N Polγ at a 1:1 ratio to mimic the heterogeneous state was used, the WT protein could partially compensate for the Y951N mutant. Taken together, these findings show that the Y951 mutant exhibits reduced processivity, especially over longer DNA sequences, indicating polymerase dysfunction.

Since the Y951 residue has been suggested to play a key role in binding and incorporating incoming nucleotides during replication, we questioned whether the Y951N substitution hampers dNTP binding by PolγA, thereby causing the reduction in DNA synthesis. Our results showed that the Y951N mutant exhibited decreased dNTP binding affinity; however, increasing dNTP levels in primer extension reactions did not restore the polymerase activity of the Y951N protein, suggesting that the reduced dNTP binding was not the major cause of the impaired DNA synthesis. Interestingly, we observed a decreased capacity of Y951N variant to degrade the DNA substrate in the absence of dNTPs, which prompted us to hypothesize that the Y951N mutation, despite being in the polymerase active site, could unexpectedly influence the enzyme's exonuclease activity.

The Y951N mutation disrupts intramolecular switching between polymerase and exonuclease activities.

To test the hypothesis that the Y951N mutation might affect DNA degradation by PolγA, we performed exonuclease assays on different substrates in the absence of dNTPs. While the WT protein was able to efficiently degrade both single-stranded DNA and partially double-stranded DNA primer/template substrates, DNA degradation by the Y951N variant was only observed on ssDNA but was not efficient on partially dsDNA substrate. It is worth noting that, upon binding to ssDNA, PolγA directly engages in exonuclease mode to degrade DNA substrate whereas on partially dsDNA, the enzyme first binds in polymerase mode and then switches to exonuclease mode for DNA degradation. Therefore, our results indicated that Y951N's failing to degrade partially dsDNA was not caused by impaired exonuclease activity but instead resulted from a defect in transitioning from polymerase to exonuclease mode.

Moreover, using primer/template DNA substrates with mismatches incorporated at the 3′-terminus of the primer, we demonstrated that the WT Polγ was efficient in removing the

mismatches using its exonuclease activity and transitioning to polymerase mode to continue to extend the primer. In contrast, the Y951N mutant could excise the mismatches but failed to switch back to polymerase mode after mismatch removal, thus impeding the extension of the mismatched substrates. The reduction in extending these substrates by the Y951N mutant became more significant when intermolecular switching was restricted in the presence of heparin. Taken together, these results showed that the Y951N mutant was also defective in switching from exonuclease to polymerase activity.

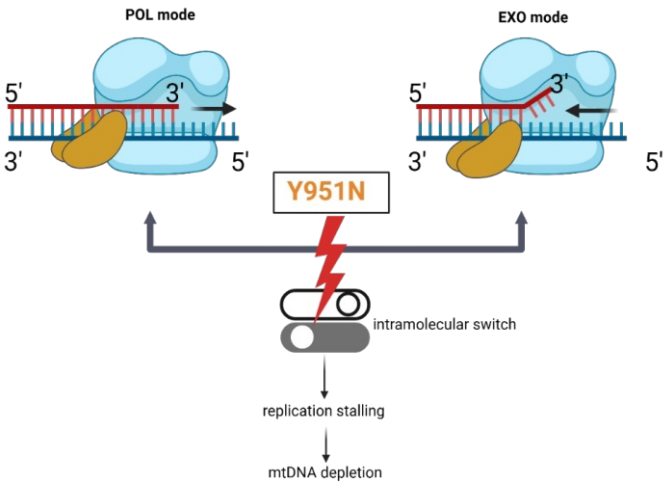

Figure 8. Schematic illustrating the defective intramolecular switch in Y951N PolγA. The pathogenic mutation Y951N disrupts the switching between Polγ's polymerase (POL) and exonuclease (EXO) activities, leading to severe mtDNA replication stalling and subsequent mtDNA loss in cells.

Conclusions

In summary, we have demonstrated that the patient mutation, Y951N, located in the polymerase domain of mitochondrial PolγA, severely impairs the enzyme's ability to switch between polymerase and exonuclease activities, leading to replication stalling and mtDNA instability in human cells **(Fig. 8)**. Thus, our study provides further insights into the distinct mechanism underlying how different mutations in PolγA can cause mitochondrial genome instability associated with various mitochondrial disorders.

1.2. Activating AMPK improves pathological phenotypes due to mtDNA depletion (paper II)

As described earlier (see Introduction 3.2), in response to mitochondrial dysfunction that alters cellular energy state, AMP-activated protein kinase (AMPK) becomes activated and promotes metabolic reprogramming to restore the energy balance. Given AMPK's central role in maintaining energy homeostasis, pharmacological strategies utilizing small-molecule activators to stimulate AMPK activity have shown positive effects in several models of mitochondrial dysfunction[219]. However, the degree of mitochondrial dysfunction required to induce AMPK activation, as well as how defects in mitochondrial functions can

be alleviated following AMPK stimulation by activators, remain unclear. Our study therefore aimed to address these questions.

Cells expressing the D1135A Polγ variant display a progressive mtDNA depletion that is followed by impaired OXPHOS and cell proliferation.

In this study, we used the previously established Flp-In T-Rex HEK293 cell line that expresses the polymerase deficient D1135A Polγ mutant upon doxycycline (Dox) induction. We specifically chose the D1135A mutant as it acts in a dominant-negative manner and previous work has shown that inducible overexpression of D1135A resulted in pronounced mtDNA replication stalling and subsequent rapid loss of mtDNA in cultured human cells[220,221], making this an excellent model to study progression of mtDNA depletion syndromes (MDS).

To monitor the mitochondrial and cellular effects of progressive mtDNA depletion caused by D1135A Polγ overexpression, we collected cells at different time points after doxycycline addition over a 9-day period. Consistent with previous studies, cells expressing the D1135A mutant showed a substantial decrease in mitochondrial DNA content, with mtDNA copy number falling below 15% of starting levels already on day 3 post-induction. Western blot analysis also showed that the protein levels of all respiratory chain (RC) subunits were affected in mutant-induced cells, with a severe loss of most RC complexes and supercomplexes by day 6 of induction, when mtDNA copy number had reached critically low levels. Moreover, measurements of mitochondrial membrane potential ($\Delta\Psi$) and cell proliferation revealed substantial decreases by day 6 post-induction in cells expressing D1135A Polγ compared with the wildtype counterpart.

We also observed an increase in cellular ADP and AMP levels in D1135A-expressing cells after 6 days of Dox induction, resulting in a decreased ratio of ATP to (AMP+ADP) compared to WT and uninduced cells. In addition, flow cytometry analysis revealed alterations in the cell cycle profile of mutant-induced cells, with an increased number of cells arrested in the S and G2 phases.

Mitochondria-associated AMPK is activated early during the course of mitochondrial dysfunction and stimulation of AMPK activity restores MMP in cells expressing D1135A Polγ

Since D1135A Polγ-expressing cells displayed altered cellular ADP and AMP levels following mtDNA depletion, we next examined the status of AMPK, a key metabolic regulator that senses changes in the ratios of ATP to AMP/ADP and becomes activated, subsequently triggering multiple metabolic pathways to restore cellular energy balance. We initially performed cellular fractionation assays to evaluate AMPK phosphorylation in different subcellular compartments. The results revealed that AMPK activation in the crude mitochondrial fraction was initiated already on day 3 post-induction, whereas the phosphorylation state of cytosolic AMPK remained unchanged throughout the time course. Further separation of the crude mitochondrial fraction into pure lysosomal and

mitochondrial subfractions using density gradient centrifugation confirmed that mitochondria-associated AMPK became activated following D1135A expression. Importantly, we did not observe a change in partitioning of AMPK between subfractions, suggesting that the energy stress caused by mtDNA depletion in the mutant-induced cells primarily activates AMPK that is already associated with mitochondria.

Several aspects of the advancing mitochondrial dysfunction following the inducible expression of D1135A Polγ in our experimental time course can be summarized in **Fig. 9**.

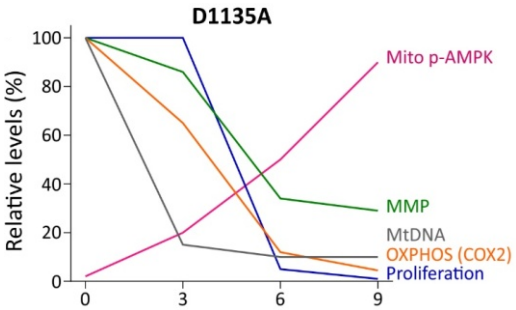

Figure 9. Schematic summary of the progressive cellular phenotypes associated with mtDNA depletion. Following inducible expression of the D1135A Polγ, cells exhibited a rapid loss of mtDNA by day 3, coinciding with the first detectable reduction in respiratory chain protein levels and the onset of energy stress that primarily activates mitochondria-associated AMPK. By day 6 of induction, most respiratory chain proteins were diminished, accompanied by a significant decrease in mitochondrial membrane potential (MMP) and severely impaired cell proliferation.

Based on the timeline in Figure 9, we conducted further analysis with Dox induction for 4 days, because this was the time point when we observed mild mitochondrial dysfunction in cells, which could help us estimate the situation in mild to moderate mitochondrial disorders. Given the central role of AMPK in maintaining cellular energy homeostasis, we questioned whether pharmacological modulation of AMPK activity can mitigate some of the phenotypes associated with mitochondrial dysfunction in our cell model. To address this, we treated uninduced and D1135A-induced cells with the specific AMPK activator A-769662 and evaluated its effects on cellular phenotypes. Interestingly, we found that A-769662 treatment increased ΔΨ in both uninduced and D1135A-induced cells, albeit with a more pronounced impact in the induced cells. Specifically, AMPK activation by A-769662 was sufficient to restore the reduced ΔΨ in the mutant-induced cells. Moreover, the impact of A-769662 on ΔΨ was diminished when AMPK was depleted by siRNA knockdown, confirming that the stimulatory effect of A-769662 on mitochondrial membrane potential is AMPK dependent. Of note, the substantial decline in ΔΨ in uninduced cells upon siRNA silencing indicated an essential role of AMPK in maintaining ΔΨ under normal cellular conditions.

AMPK activation by A-769662 results in a modest increase in mtDNA copy number and restores the integrated stress response in D1135A-expressing cells

Although A-769662 treatment could rescue the decreased ΔΨ in the mutant-induced cells, it was not sufficient to improve impaired cell proliferation. Nevertheless, we observed positive effects of A-769662-mediated AMPK activation on some phenotypes of D1135A-induced cells, including a small increase in mtDNA copy number and protein levels of OXPHOS complexes. Treatment with A-769662 did not change mtDNA content in uninduced cells despite the observed increase in ΔΨ, suggesting that the effect of the AMPK activator on ΔΨ might be not mediated by mtDNA increases.

To further investigate the molecular mechanism by which A-769662 restored the mitochondrial membrane potential, we performed RNA-seq analysis and compared the transcriptional profiles of uninduced and D1135A-induced cells in the absence or presence of A-769662. We identified several genes involved in the integrated stress response (ISR) whose expression was upregulated in mutant-induced cells compared to the uninduced cells. Treatment with A-769662 did not cause any significant changes in gene expression in the uninduced cells, while the expression levels of 88 genes were restored in mutant-induced cells upon treatment with the activator. These results indicated that the elevated ΔΨ induced by A-769662 was not achieved through major transcriptional changes.

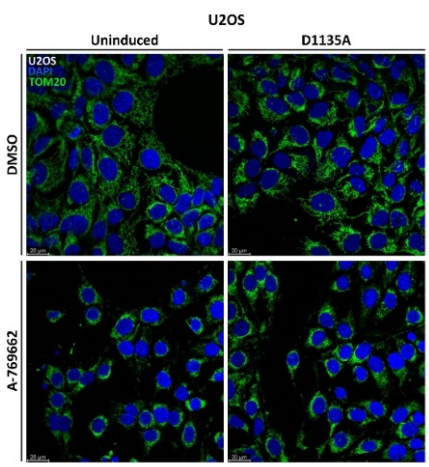

Figure 10. Representative confocal microscopy images of uninduced and D1135A-induced Flp-In T-REx U2OS cells (6 days) either untreated or treated with A-769662 for 48h. Following fixation, cells were immunostained with DAPI to label nuclei (blue) and TOM20 to visualize mitochondria (green), scale bar 20 μm.

We next examined mitochondrial dynamics and morphology which are reported to respond to energy-stress-induced AMPK activation and may impact mitochondrial membrane potential. Confocal microscopy revealed an altered mitochondrial network in D1135A-induced cells, with shorter and fewer branches compared to uninduced cells, indicating

mitochondrial fragmentation. Interestingly, we observed that, in both uninduced and mutant-induced cells, AMPK activation by A-769662 resulted in a considerable decline in number of branches and summed length of branches in the mitochondrial network as well as a clustering of mitochondria in the perinuclear region of the cell (**Fig. 10**).

Given the modest but promising effects of AMPK stimulation by A-769662 in D1135A-induced cells that exhibit severe mtDNA loss and mitochondrial dysfunction, we next applied this activator to cells with a milder and more clinically relevant level of mtDNA depletion. The patient-derived fibroblasts were homozygous for either V1106A Polγ or Y955C Polγ mutation and displayed a ~ 40 % reduction in mtDNA copy number compared to the control cells. A-769662 treatment caused a slight increase, although not statistical significance, in the mtDNA content of the patient cells but not in the control lines. Similar to observations in uninduced and D1135A-induced cells, treatment with A-769662 elevated mitochondrial membrane potential both in control and patient cell lines, suggesting a beneficial effect of pharmacological stimulation of AMPK by this specific agonist on mitochondrial function.

Conclusions

Taken together, our findings show that the activation of mitochondria-associated AMPK occurs during the early stage of advancing mitochondrial dysfunction, which is in line with its established role in maintaining energy homeostasis. Moreover, we demonstrated that stimulation of AMPK activity using the specific activator A-769662 can increase mitochondrial membrane potential both in our cell model with severe mtDNA depletion and in myopathy-associated patient fibroblasts. Our results suggest that the positive effect of AMPK activation on mitochondrial membrane potential was not mediated through mtDNA increases or major transcriptional changes. Instead, perinuclear mitochondrial clustering observed in cells treated with A-769662 prompts us to speculate that this AMPK-mediated redistribution of mitochondrial network could be part of the retrograde signalling pathway triggered by mitochondrial stress, resulting in a compensatory increase in $\Delta\Psi$. Further investigation, however, will be needed to address this hypothesis. Nevertheless, this study broadens our understanding of the beneficial impacts of specific AMPK agonists on mitochondrial and cellular functions, thus contributing to the development of therapeutic strategies for mitochondrial disorders.

2. Enhanced mitochondrial G-quadruplex formation impedes replication fork progression leading to mtDNA loss in human cells (paper III)

Loss of mitochondrial genome integrity results not only from defects in mtDNA replisome proteins (e.g. Polγ) but also from errors during mtDNA replication or repair of damaged mtDNA that can lead to the formation of point mutations and/or deletions in mtDNA. Many large-scale mtDNA deletion breakpoints implicated in mitochondrial disorders have been suggested to occur at mtDNA sequences with potential to form G-quadruplexes (G4s)[64,66]. In support of this, biochemical studies have shown that these G4 structures may pose an

obstacle to the progression of the mtDNA replication machinery[66,222]. However, the role of G4s in the formation of mtDNA deletions has remained unclear. Studying the dynamics of mitochondrial DNA G4s has been challenging, primarily due to the lack of tools to specifically detect these structures within mitochondria. Therefore, in this study, we developed a novel method that allows for the detection and mapping of mtDNA G4s in human cells under different cellular conditions.

Establishing a cell line expressing BG4 that is specifically targeted to mitochondria (mitoBG4)

One of the approaches to detect G4s in cultured cells is based on the use of synthetic antibodies that specifically recognize and bind to G4 structures, such as BG4[223–225]. This single chain antibody fragment was previously used to analyse nuclear G4s. To study G4s in mtDNA, we generated a construct consisting of BG4 sequence fused with the mitochondrial targeting signal (MTS) of TFAM to drive the protein to the mitochondrial matrix, and a C-terminal Flag tag to monitor BG4 expression in cells (**Fig. 11**). We then established a Flp-In T-REx HEK293 cell line expressing the MTS-BG4-Flag protein upon induction with doxycycline.

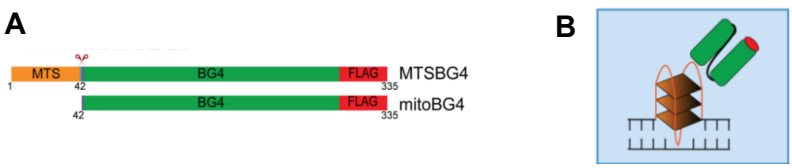

Figure 11. A. Schematic representation of the BG4 construct used to establish the mitoBG4 cell model. The BG4 sequence is fused with the N-terminal mitochondrial targeting sequence (MTS) of TFAM and carries a C-terminal Flag tag. Upon import into the mitochondrial matrix, the MTS is recognized and cleaved by specific proteases to produce mitoBG4. **B.** Properly folded mitoBG4 binds specifically to G4 structures within the mtDNA.

The expression of mitoBG4 after Dox addition was detected by Western blotting using an anti-Flag antibody. We next performed immunofluorescence (IF) and cellular fractionation assays and confirmed the correct mitochondrial localization of mitoBG4. Additionally, using a Proteinase K accessibility (PKA) assay we further demonstrated that mitoBG4 was localized to the mitochondrial matrix, where mtDNA resides. Importantly, the analysis of non-reducing SDS-PAGE confirmed the proper folding of mitoBG4, which is achieved through disulfide bonds when expressed in cells.

MitoBG4 colocalizes with mitochondrial nucleoids and does not disrupt mtDNA replication

After confirming that mitoBG4 localized to the mitochondrial matrix, we next asked whether it interacts with the components constituting mitochondrial DNA nucleoid. By performing immunofluorescence in mitoBG4-induced cells, we showed that mitoBG4 co-localized with TFAM and mtSSB, two essential mtDNA nucleoid proteins. Our immunoprecipitation (IP) assay further supported the IF results by demonstrating that several mtDNA-associated proteins (including Polγ and Twinkle) were co-precipitated with

mitoBG4, while no enrichment of cytosolic and nuclear proteins was detected. Collectively, these findings indicate that mitoBG4 directly associates with mitochondrial nucleoids.

Furthermore, we observed that long-term expression of mitoBG4 had no effect on mtDNA maintenance, as evidenced by the unchanged mtDNA copy number and patterns of mtDNA replication intermediates comparable to the control cells. Taken together, we show that our cell model can be used to detect mtDNA G4s in human cells without disturbing mtDNA metabolism.

MtG4-ChIP can specifically enrich G4-containing regions on mtDNA

We next set up a Chromatin Immunoprecipitation protocol to map mtDNA G4s (mtG4-ChIP) in the mitoBG4 cells. Briefly, we performed a cross-linking step on intact cells and shearing of total genomic DNA prior to mitoBG4-pulldown with the anti-Flag antibody. The subsequent analysis revealed the enrichment of several mtDNA regions with predicted putative G4-forming sequences (PGS), many of which were indeed shown to fold into G4 structures and be specifically recognized by the recombinant BG4 *in vitro*. Additionally, we did not observe an enrichment for nDNA G4s in cells expressing mitoBG4, or detection of mtDNA sequence reads from mitoBG4 p^0 cells (i.e. cells devoid of mtDNA), confirming that the signal detected in the mitoBG4 cells by mtG4-ChIP-seq is specific for mitochondrial DNA. Our data also indicated that the level of mtDNA G4s is relatively low in human cells under normal growth conditions.

mtDNA G4 stabilization blocks the progression of mtDNA replication fork, which in turn enhances mtDNA G4 formation

Next, we applied mtG4-ChIP-seq to investigate the consequences of G4 structures on mtDNA replication progression. Upon treatment of mitoBG4 cells with RHPS4, a known G4 stabilizer that has been shown to localize to mitochondria, we found a considerable increase in mtDNA sequence reads harbouring PGS. The elevated mtG4-ChIP-seq signal coincided with increased replication stalling and reduced mtDNA copy number in cells treated with RHPS4, indicating that the enhanced stabilization of mtDNA G4s by RHPS4 impedes the mtDNA replication machinery. These results thus validated the feasibility of our method in detecting and mapping mtDNA G4s formation in human cell lines.

Due to the asynchronous replication mode of mtDNA, the GC-rich heavy strand (H-strand) is particularly susceptible to the formation of G4 structures during mtDNA replication, where the H-strand is exposed as a partially single-stranded intermediate for an extended period. Therefore, to investigate whether impaired mtDNA replication could lead to G4 formation in mtDNA, we took advantage of ddC (2,3-dideoxycytidine), a chain-terminating nucleoside analogue that is selectively incorporated by Polγ, thereby resulting in chain termination and subsequent mtDNA replication stalling. The analysis of mtG4-ChIP-seq in mitoBG4-expressing cells upon ddC treatment showed an increase in the enrichment of multiple G4 regions on mtDNA but not on nuclear DNA. In particular, the elevated mtDNA G4 signal was more pronounced on the major arc, likely because this region of the parental H-strand can be exposed in single-stranded form for even longer when the mtDNA

replication progresses more slowly in the presence of ddC. As most mtDNA deletion breakpoints in patients have been mapped within the major arc, these findings suggest an interplay between mtDNA G4 accumulation during the replication process and the formation of mtDNA deletions.

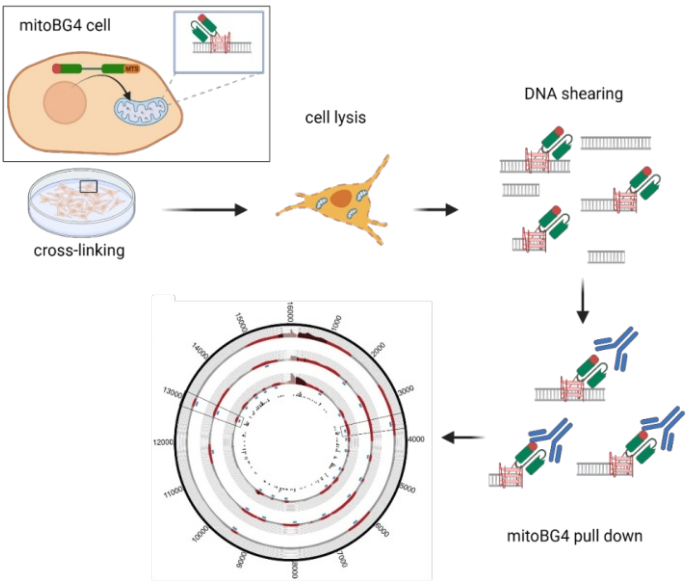

Figure 12. Schematic representation of a cell model and mtG4-ChIP-seq method that we developed in the study. Inducible cells expressing mitoBG4-Flag were subjected to cross-linking prior to cell lysis and DNA shearing. The mitoBG4-Flag was enriched with an anti-Flag antibody, and the pulldown extract was subsequently analysed by next generation sequencing, allowing the detection and mapping of G4s specifically in mtDNA.

Conclusions

In this study, we established a cell model expressing mitochondrially targeted G4-binding antibody BG4 under doxycycline induction and developed a mtG4-ChIP approach coupled with sequencing, enabling us to detect and map G4s specifically in mitochondrial DNA of human cells (**Fig. 12**). Our results show that mitoBG4 was correctly localized and folded within the mitochondrial matrix and its inducible expression did not perturb mtDNA maintenance or the dynamics of G4 structures. Using mtG4-ChIP to specifically enrich mtDNA G4 sequences, we demonstrated that G4 structures are present in mitochondrial DNA at a low level under normal cell culture conditions, and that G4 stabilizer RHPS4 enhanced mtDNA G4 formation, leading to increased mtDNA replication blockage and subsequent mtDNA depletion in cultured cells. On the other hand, the stalling of mtDNA replication fork enhanced G4 formation in mtDNA, particularly on the major arc region. Overall, the mtG4-ChIP approach provides a useful tool for future research for studying factors involved in the formation of mtDNA G4 structures as well as their mechanistic roles in mtDNA instability.

Part II - Characterization of PolDIP2 structural features and their implications in mitochondria (Manuscript IV)

In addition to studying mechanisms underlying mtDNA deletions and depletion, we also investigated how cells tolerate mtDNA damage to preserve mitochondrial genome integrity. We focused on the primase–polymerase PrimPol that can restart stalled mtDNA replication, thereby playing a role in mtDNA maintenance[170]. Previous evidence, including our own work, has demonstrated that PrimPol-dependent DNA synthesis requires stimulation by Polymerase δ–interacting protein 2 (PolDIP2)[171,186]. While characterizing this functional interaction, we unexpectedly found that PolDIP2 forms dimers, which is enhanced under oxidative conditions. This observation broadened our research focus and motivated us to explore PolDIP2 oligomerization and its potential cellular relevance, ultimately leading to **Manuscript IV** (**Redox-dependent dimerization of PolDIP2 and a conserved ApaG-domain motif required for CHCHD2 interaction**)

PolDIP2 can form dimers in vitro and in cultured human cells

While investigating the biochemical properties of PolDIP2 and its potential physiological functions, we detected higher-molecular-weight forms of PolDIP2. By analysing endogenous PolDIP2 in HEK293T cells on non-reducing SDS-PAGE followed by immunoblotting with an anti-PolDIP2 antibody, we observed the expected ~37 kDa band corresponding to the mitochondrial mature form and an additional slower-migrating species at ~85 kDa (**Fig. 13A**). This higher-molecular-weight band was absent when β-mercaptoethanol was added, suggesting it represents a disulfide-linked oligomer (**Fig. 13B**). Importantly, despite having a lower abundance than the monomer, the oligomer was similarly depleted upon PolDIP2 silencing, indicating that both species are specific forms of PolDIP2 (**Fig. 13A**). Furthermore, in doxycycline-inducible Flp-In T-Rex HEK293 cells expressing Flag-tagged PolDIP2, both the monomeric and oligomeric species were detected using anti-Flag and anti-PolDIP2 antibodies.

Consistent with PolDIP2 oligomerization observed in cultured cells, the recombinant PolDIP2 protein also eluted as monomers and oligomers from a size exclusion chromatography (SEC) column. Mass photometry analysis confirmed that the higher-molecular weight species corresponds to PolDIP2 dimer. Together, these results demonstrate that PolDIP2 can form dimers both *in vitro* and in cells.

Cysteine 143 residue is important for PolDIP2 dimer formation via a disulfide bridge

To gain insight into the molecular basis of PolDIP2 dimerization, we solved the crystal structure of recombinant human PolDIP2 dimers. The structural analysis revealed an intermolecular disulfide linkage formed between two PolDIP2 monomers via cysteine 143 residues (Cys143). Sequence alignment of PolDIP2 from various vertebrate species identified two highly conserved cysteines, Cys143 in the YccV-like domain and Cys266 in the ApaG-like. Therefore, to evaluate the contribution of both Cys residues to dimer formation in cells, we generated doxycycline-inducible cell lines expressing PolDIP2

variants carrying cysteine-to-alanine substitutions. The results showed that while the dimerization of PolDIP2 C266A mutant was comparable to that of the wild-type, dimer formation was not detected for C143A-expressing cells (**Fig. 13B**). Consistent with observations in cells, both the recombinant C143A and Δ143–170 mutants failed to form dimers *in vitro*. Altogether, these results indicate that Cysteine 143 is required for disulfide bond-mediated dimerization of PolDIP2 both in cells and *in vitro*.

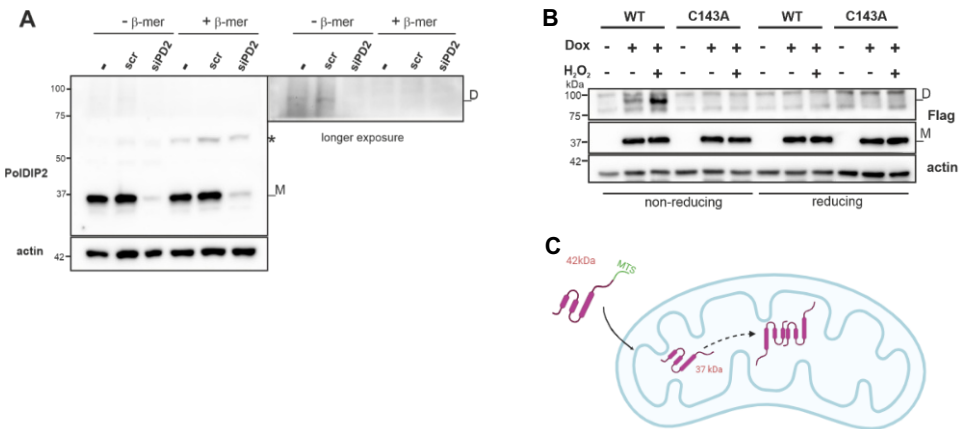

Figure 13. (A) Western Blot analysis of PolDIP2 in HEK293T cells transfected with siRNA against PolDIP2 or scramble (scr) siRNA (control) for 72 hours. Both short (left) and long (right) exposures are shown. Both the monomeric and dimeric species are lost upon PolDIP2 knockdown (**B**) Western blot analysis of Flp-In™ HEK293 T-REx cells expressing Flag-tagged PolDIP2 variants. While wildtype (WT) PolDIP2 forms both monomeric (M) and dimeric (D) species, the C143A PolDIP2 mutation abolishes dimer formation. (**C**) Schematic overview of PolDIP2 dimerization in mitochondria. Following mitochondrial import, the precursor PolDIP2 is processed to the mature 37kDa form, which can undergo Cys143-dependent dimerization within the organelle.

As disulfide bonds can contribute significantly to the stabilization of protein structures, we questioned whether mutating Cys143 had an effect on PolDIP2 protein stability. By treating PolDIP2 WT- and C143A-induced cells with protein synthesis inhibitor Cycloheximide (CHX), we found that C143A mutant exhibited a turnover rate comparable to the WT in the CHX-chase assay, suggesting that Cys143-mediated disulfide linkage does not contribute to the steady-state stability of PolDIP2 in cultured cells.

PolDIP2 dimerization occurs strictly within mitochondria and can be enhanced by ROS-induced oxidative stress

Given relatively low levels of endogenous PolDIP2 dimers in HEK293T cells under basal conditions, we next investigated whether its dimer formation could be induced by certain stimuli. As PolDIP2 dimerizes via disulfide bond formation between two cysteine residues, and dimerization of the recombinant PolDIP2 can be triggered by oxidizing agents (potassium ferricyanide $K_3Fe(CN)_6$ or hydrogen peroxide (H_2O_2)), we examined PolDIP2 oligomerization in cells in response to oxidative stress. The results showed that H_2O_2

treatment increased dimer formation of both endogenous and exogenous PolDIP2 WT. Importantly, PolDIP2 C143A mutant did not respond to H_2O_2 treatment, indicating the importance of Cys143 for oxidation-dependent PolDIP2 dimerization. Furthermore, cell exposure to rotenone, an inhibitor of mitochondrial complex I that enhances intracellular reactive oxygen species (ROS), also caused a modest increase in the dimerization of PolDIP2 WT. Altogether, these findings indicate that PolDIP2 dimerization is selectively promoted in response to ROS-mediated oxidative stress.

Next, to investigate the significance of PolDIP2 dimerization in cells, we examined the subcellular localization of PolDIP2 dimers using a cell fractionation assay. While the monomeric form of PolDIP2 was detected in both the cytoplasmic and mitochondrial fractions, dimeric form was exclusively distributed to mitochondria. Moreover, by transiently expressing different PolDIP2 constructs in HEK293T cells, we demonstrated that only the PolDIP2 variant that was properly imported into mitochondria and processed to the mature form could form dimers. Taken together, these results indicate that PolDIP2 dimerization occurs strictly within mitochondria, suggesting that dimer formation may be linked to a mitochondrial function (**Fig. 13C**).

PolDIP2 dimerization does not alter PrimPol stimulation or mtDNA replication in cells

As previously discussed, PolDIP2 has been demonstrated as a processivity factor of PrimPol by stimulating PrimPol's processivity and polymerase activity *in vitro*[171,186]. We therefore examined whether PolDIP2 dimerization influences PrimPol stimulation. The results showed that the recombinant PolDIP2 dimer exhibited stimulatory effects on PrimPol activity comparable to the monomer, indicating that disulfide-bond-dependent dimerization does not affect PolDIP2-mediated PrimPol stimulation *in vitro*.

Since PolDIP2 has also been suggested to be involved in mtDNA-associated processes[178,226], we next asked whether its dimerization affects mtDNA replication in cells. By employing two-dimensional agarose gel electrophoresis (2D-AGE), we detected a modest increase in the abundance of replication intermediates in WT-expressing cells compared to uninduced control counterparts, implying a mildly slower progression of mtDNA replication forks. A similar pattern, however, was observed when expressing the dimer-deficient C143A variant, suggesting that while PolDIP2 overexpression may modestly influence mtDNA replication dynamics, this effect is independent of Cys143-mediated dimerization.

PolDIP2 interacts with CHCHD2 and a conserved glycine-rich motif in the ApaG-like domain of PolDIP2 is required for this association

Given the redox sensitivity of PolDIP2 specifically within mitochondria, we next investigated its interactome in this organelle, focusing on PolDIP2 associations with distinct mitochondrial protein partners that might be involved in or influenced by its redox-regulated dimerization. To this end, we performed immunoprecipitation (IP) of PolDIP2-Flag followed by mass spectrometry (MS) analysis in Flp-In HEK293 cells. In addition to previously reported protein associations with PolDIP2 in mitochondria, including the two

components of CLPXP protease, CLPX and CLPP, as well as ALAS1[181,215], we also identified three mitochondrial proteins that have not been previously confirmed as PolDIP2 interacting partners: coiled-coil-helix-coiled-coil-helix domain–containing protein 2 (CHCHD2), HSP60 (60-kDa heat-shock protein), and LONP1. These mitochondrial associations were then validated by co-immunoprecipitation (co-IP) in cells expressing PolDIP2 WT.

Among the newly validated interacting proteins, we primarily focused on CHCHD2 for further interaction characterization, as its function as a regulatory protein involved in redox-sensitive stress signaling[227] may provide a potential mechanistic link between CHCHD2 and the redox-regulated dimerization and mitochondrial function of PolDIP2.

We first generated AlphaFold3 complex predictions for CHCHD2-PolDIP2 association. Based on the predicted model, the interaction interface between the two proteins localized to a surface on the C-terminal ApaG-like domain of PolDIP2, adjacent to a conserved glycine-rich motif (GxGxxG; residues 298–303 in human PolDIP2) (**Fig. 14A-B**). Notably, the deletion of the glycine motif in PolDIP2 led to a substantial reduction in predicted complex confidence (ipTM: 0.23 compared to ipTM: 0.57 for the WT) without a notable change in the overall model confidence, suggesting that this region contributes to the interaction. Interestingly, the glycine-rich motif is positioned at the base of a hydrophobic groove that is formed within PolDIP2 ApaG-like domain and reminiscent of the substrate-binding pocket of human Fbxo3. This prompted us to investigate whether this motif contributes to shaping the local surface through which PolDIP2 can engage CHCHD2.

To address this, we generated two variants that disrupt the GxGxxG sequence: a triple glycine-to-aspartate substitution (3G>D; G298D/G300D/G303D) and a deletion of the motif (Δ3G; Δ298–303). The co-IP analysis showed that, while interaction profiles of 3G>D and Δ3G mutants were largely comparable to those of the wildtype for most tested mitochondrial interactors, the binding of these mutants to CHCHD2 was completely lost (**Fig. 14C**). Taken together, these findings indicate that the conserved glycine-rich motif in PolDIP2 is specifically required for its association with CHCHD2.

Disruption of the glycine-rich motif enhances PolDIP2 dimerization

Given CHCHD2's characterized roles in oxidative-stress responses and the loss of CHCHD2 association observed in the glycine-rich motif mutants, we next questioned whether disruption of the motif also affects the redox-sensitive dimerization of PolDIP2. Western Blot analysis revealed that, despite having a substantially lower total protein abundance compared to the PolDIP2 WT, possibly due to effects on protein stability or expression caused by motif alteration, both 3G>D and Δ3G variants exhibited considerably increased relative proportion of dimer to monomer. Moreover, H_2O_2 treatment further promoted dimerization of the motif mutants whereas introduction of the C143A substitution into the 3G>D or Δ3G backgrounds abolished dimer formation, indicating that dimerization of the motif mutants is also responsive to oxidative stress and via the same Cys143-mediated disulfide linkage as in wildtype PolDIP2. Altogether, these results indicate that the

conserved glycine-rich motif not only contributes to PolDIP2 interaction with CHCHD2 but also influences its redox-dependent dimerization.

The observation of the increased dimer formation and the loss of CHCHD2 binding in PolDIP2 motif variants suggested a potential link between the oligomeric state of PolDIP2 and its interaction with CHCHD2. Supporting this model, CHCHD2 was consistently enriched in co-Ips when dimer-deficient C143A mutant was used, indicating that CHCHD2 interacts preferentially with the monomeric form of PolDIP2 (**Fig. 14C**).

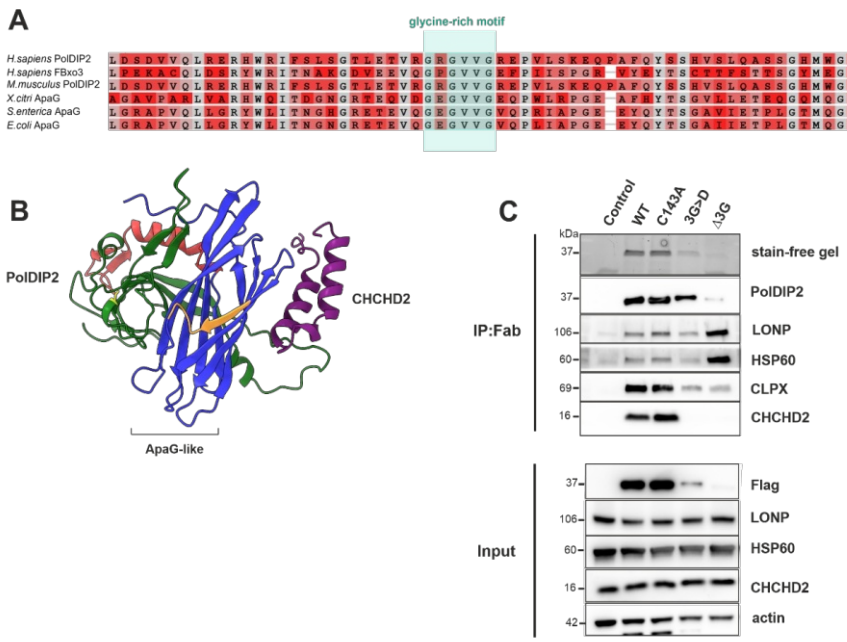

Figure 14. PolDIP2 interacts with CHCHD2, and a conserved glycine-rich motif is important for this interaction (**A**) Sequence alignment of the C-terminal domain of human PolDIP2 with other ApaG containing proteins reveals a highly conserved glycine-rich motif (boxed in green), corresponding to, $G^{298}x^{G300}xxG^{303}$ in human PolDIP2. (**B**). Alphafold3-predicted model of the PolDIP2-CHCHD2 complex. The glycine-rich motif of PolDIP2 is highlighted in yellow. The unstructured, low-confidence regions of both proteins are omitted for clarity. (**C**) Western blot analysis of input and pull-down (IP:Flag) fractions from Fab-Trap immunoprecipitation in parental Flp-In T-Rex (control) or cells expressing either wild-type or mutants.

Conclusions

In summary, our study investigated the biochemical and cellular properties of human PolDIP2. In particular, we show that PolDIP2 forms dimers via intermolecular disulfide bonds between the conserved Cys143, and that dimer formation is restricted to mitochondria and promoted by oxidative stress. Furthermore, using proteomic and biochemical approaches, we identified CHCHD2 as a specific mitochondrial binding partner of PolDIP2

and demonstrated that a conserved glycine-rich motif in the C-terminal ApaG-like domain of PolDIP2 is required for this association. Interestingly, disruption of this motif specifically abolished CHCHD2 binding while substantially increasing PolDIP2 dimer formation, suggesting that PolDIP2's oligomeric state directly influences its interaction with CHCHD2. Given CHCHD2's role in mitochondrial morphology and stress signaling and the implication of pathogenic CHCHD2 mutations in Parkinson's disease[228–230], it is much of interest to further investigate the functional consequences of PolDIP2-CHCHD2 interaction. Nevertheless, our findings provide new insight into structural features of PolDIP2 that regulate its mitochondrial interaction network as well as into its potential role in mitochondrial redox homeostasis.

CONCLUSION AND OUTLOOK

Mitochondrial DNA integrity is crucial for various key mitochondrial processes, including oxidative phosphorylation, mitochondrial biogenesis and homeostasis. Consequently, failure to maintain mitochondrial genome directly affects cellular energy production and metabolism, eventually leading to mitochondrial dysfunction implicated in a wide range of human diseases. Therefore, understanding the molecular mechanisms underlying mitochondrial dysfunction is essential for developing diagnostic methods and therapeutic interventions.

In paper I and II, our findings reinforce the crucial role of DNA polymerase gamma (Polγ) in maintaining mitochondrial genome stability and the clinical relevance of Polγ pathogenic mutations in mitochondrial dysfunction. We revealed a distinct mechanism by which the patient mutation in Polγ, Y951N, leads to mtDNA loss in human cells, providing additional insights into the origin of mitochondrial disorders (paper I). We further demonstrated that, following the energy deficit caused by mtDNA depletion, mitochondria-associated AMPK is activated early to restore cellular energy balance, and that the specific pharmacological AMPK agonist A-769662 has beneficial impacts on mitochondrial phenotypes (paper II). In addition, given the potential implications of G4 structures for mtDNA instability, in project III we developed a new method that enables the detection and mapping of G4 formation in mtDNA under different cellular conditions. This provides a valuable tool for further research to identify factors affecting G4 dynamics in mtDNA and decipher G4's roles in mtDNA maintenance and expression. Altogether, these findings not only improve our understanding of factors contributing to mtDNA instability and mitochondrial dysfunction but also provide valuable insights into potential applications of therapeutic strategies.

Lastly, we delved into the biochemical and cellular properties of human PolDIP2, a mitochondrial protein that has been suggested to play a role in several mitochondrial processes, including mtDNA maintenance and mitochondrial proteolysis. Our study describes for the first time the redox-dependent dimerization of human PolDIP2 and identifies the structural determinants regulating its interaction with CHCHD2, whose mutations are linked to Parkinson's disease in some patients. The findings suggest that the redox-sensitive conformational changes of PolDIP2 might introduce additional complexity to its interactions within the mitochondrial networks, particularly with CHCHD2, potentially influencing related mitochondrial processes. However, further experiments are needed to verify the association between PolDIP2 and CHCHD2 *in vitro* and investigate the functional cellular consequences of this interaction.

In conclusion, the work presented in this thesis explores several aspects of human mitochondrial genome maintenance and related mitochondrial processes, highlighting their implication for both mitochondrial and cellular health. The findings not only advance fundamental research on mitochondrial DNA but also support potential applications for targeted therapies in treating mitochondrial-related diseases.

ACKNOWLEGMENT

I may not be good with words, but I want to take this chance to express my gratitude to everyone who has added so much to my PhD chapter. Writing this brought back all the memories in the past five years, as a movie replayed in my head. And I have to say, it was quite a journey!

First of all, I would like to thank Sjoerd for everything! Thank you for giving me the opportunity to come to Umeå for my PhD. Thank you for motivating me to follow my curiosity in research while keeping me on the ground. Although there were unexpected turns during my PhD, thank you for being so supportive and optimistic. I am truly grateful for that.

I also want to thank all the current and former members of SW's group. To Gorazd, you were like a burst of "vitamin joy" for the lab, it was so fun having you around. To Mara, thank you for patiently guiding me in the beginning when I first started the "fancy" fiber analysis, and I truly appreciate all your support, both professionally and personally. To Josefin, thank you so much for being so helpful since the very beginning of my PhD, and for all your physical and mental support during my rainy days :). To Nam, thank you for sharing all the laughter, joy, and emotional rollercoasters when experiments ended up unexpectedly, for the delicious comfort food and cakes that always helped lift my mood, and for everything you have taught me in and outside the lab, I am genuinely thankful! Our dear Sonja, I cannot imagine the lab (and the entire department) without you. Your endless support and enthusiasm, especially for newcomers, always amaze me. And thank you for the fun (and spontaneous) 101 Swedish lessons and many small talks on various topics, you truly are the Wikipedia of our group :). I hope you enjoy your retirement to the fullest! And thank you, Koit, Andi, and Val for all your helpful suggestions and good company in the lab.

My gratitude also extends to the members of PW's lab. I am especially thankful to Paulina and Gustavo for all their support and encouragement. Thank you for trusting me and giving me the chance to contribute to your project. It really means a lot to me!

I would like to thank my co-supervisors, Nasim Sabouri and Anders Hofer, and my examiner, Stefan Björklund. Thank you for all the input and advice throughout the years. I also want to thank Francesca Aguilo's support for being on my Midterm committee earlier and now for my thesis defence.

I also thank Jeanette, Anna Åberg, Anna Lena, Farah, and Elin for lots of fun and unique experiences in teaching, I really enjoyed helping you in the lab courses! A big thank you to Anette and Erlinda as well! Thank you for being so helpful and responsive over the years, and for the enjoyable and sugar-boosting fika. I will miss your delicious treats a lot :).

Thank you, everyone, for creating such a friendly and supportive environment at Medchem.

I would also like to thank my “travel buddies”: Nam, Noopur, Gustavo, and Abdulla. You guys have added so much colour to my journey in Umeå. Thank you all, not only for taking the initiative for our gatherings, road trips, or simply just a fika that kept me sane and helped me enjoy things outside the lab, but also for always being so helpful and considerate.

A thank you also goes to the badminton club’s fellows: Ana Campos, Koit, Manik, and Ruchitha, for so much fun on the court. I think you have seen me laugh more than play in the match, but that shows how much I enjoyed playing with you guys!

I would like to especially thank my dear friends and people whom I was fortunate to meet in Oulu. To Thomas, thank you so much for your kindness and support over the years. To Alex, thank you for everything! You were the coordinator for my Master’s programme in Oulu and later became my reference person for my PhD in Umeå, you are always so supportive and encouraging! I cannot express my gratitude enough. To Ganga, Saara, and Umair, it has been almost a decade since we met and became friends. I am so happy to have you guys in my life, so a thank you may be just not enough!

A special thank you I want to send to my friends back home. Mấy đứa nhóm mình Pit, Mít, Kít, Hằng, Tịt, và chị Vịt ơi, mới đó mà chơi với nhau ngót nghét gần hai thập kỉ rồi đó. Cảm ơn cả nhà mình đã luôn ủng hộ và có mặt mỗi khi Rít có dịp về nước, nhiều khi cả mấy năm không gặp nhưng mỗi lần tụ họp là đều mới như hôm qua. Rít yêu cả nhà mình nhiều nè! Mắm bò Ngân nữa, nếu mai mốt tao có đưa cuốn luận văn này cho mày đọc thì biết là tao cảm ơn vì có đứa bạn là mày nha!

And finally, I want to express my deepest gratitude to my family, without whom I would not be where I am today. Mommy, I am so grateful for your unwavering love, support, and encouragement. Mẹ ơi, con cảm ơn mẹ vì đã để con theo đuổi ước mơ của mình, dù nhiều lúc con cũng hoài nghi chính mình và làm mẹ lo lắng. Con cảm ơn mẹ đã cho con có được ngày hôm nay. Con yêu mẹ nhiều!

Sincerely, thank you so much, everyone!

REFERENCES

1. Anderson, A. J., Jackson, T. D., Stroud, D. A. & Stojanovski, D. Mitochondria—hubs for regulating cellular biochemistry: emerging concepts and networks. *Open Biol.* **9**, 190126 (2019).

2. Gray, M. W., Burger, G. & Lang, B. F. Mitochondrial Evolution. *Science* **283**, 1476–1481 (1999).

3. Andersson, G. E., Karlberg, O., Canbäck, B. & Kurland, C. G. On the origin of mitochondria: a genomics perspective. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **358**, 165–179 (2003).

4. Colombini, M. Voltage gating in the mitochondrial channel, VDAC. *J. Membr. Biol.* **111**, 103–111 (1989).

5. Zeth, K. & Thein, M. Porins in prokaryotes and eukaryotes: common themes and variations. *Biochem. J.* **431**, 13–22 (2010).

6. Moura, J. P., Oliveira, P. J. & Urbano, A. M. Mitochondria: An overview of their origin, genome, architecture, and dynamics. *Biochim. Biophys. Acta BBA - Mol. Basis Dis.* **1871**, 167803 (2025).

7. Protasoni, M. & Zeviani, M. Mitochondrial Structure and Bioenergetics in Normal and Disease Conditions. *Int. J. Mol. Sci.* **22**, 586 (2021).

8. Scorrano, L. *et al.* A Distinct Pathway Remodels Mitochondrial Cristae and Mobilizes Cytochrome c during Apoptosis. *Dev. Cell* **2**, 55–67 (2002).

9. Gilkerson, R. W., Selker, J. M. L. & Capaldi, R. A. The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. *FEBS Lett.* **546**, 355–358 (2003).

10. Mannella, C. A. Consequences of Folding the Mitochondrial Inner Membrane. *Front. Physiol.* **11**, (2020).

11. Pánek, T., Eliáš, M., Vancová, M., Lukeš, J. & Hashimi, H. Returning to the Fold for Lessons in Mitochondrial Crista Diversity and Evolution. *Curr. Biol.* **30**, R575–R588 (2020).

12. Harner, M. *et al.* The mitochondrial contact site complex, a determinant of mitochondrial architecture. *EMBO J.* **30**, 4356–4370 (2011).

13. Hoppins, S. *et al.* A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J. Cell Biol.* **195**, 323–340 (2011).

14. Huynen, M. A., Mühlmeister, M., Gotthardt, K., Guerrero-Castillo, S. & Brandt, U. Evolution and structural organization of the mitochondrial contact site (MICOS) complex and the mitochondrial intermembrane space bridging (MIB) complex. *Biochim. Biophys. Acta* **1863**, 91–101 (2016).

15. Strauss, M., Hofhaus, G., Schröder, R. R. & Kühlbrandt, W. Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. *EMBO J.* **27**, 1154–1160 (2008).

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

17. Frezza, C. *et al.* OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* **126**, 177–189 (2006).

18. Griparic, L., van der Wel, N. N., Orozco, I. J., Peters, P. J. & van der Bliek, A. M. Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. *J. Biol. Chem.* **279**, 18792–18798 (2004).

19. Scott, I. & Youle, R. J. Mitochondrial fission and fusion. *Essays Biochem.* **47**, 85–98 (2010).

20. Al Ojaimi, M., Salah, A. & El-Hattab, A. Mitochondrial Fission and Fusion: Molecular Mechanisms, Biological Functions, and Related Disorders. *Membranes* **12**, 893 (2022).

21. Dolezal, P., Likic, V., Tachezy, J. & Lithgow, T. Evolution of the molecular machines for protein import into mitochondria. *Science* **313**, 314–318 (2006).

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

23. Chacinska, A. *et al.* Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. *EMBO J.* **23**, 3735–3746 (2004).

24. Rehling, P. *et al.* Protein insertion into the mitochondrial inner membrane by a twin-pore translocase. *Science* **299**, 1747–1751 (2003).

25. Koehler, C. M. New developments in mitochondrial assembly. *Annu. Rev. Cell Dev. Biol.* **20**, 309–335 (2004).

26. Wiedemann, N. *et al.* Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* **424**, 565–571 (2003).

27. Kozjak, V. *et al.* An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. *J. Biol. Chem.* **278**, 48520–48523 (2003).

28. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T. & Pfanner, N. Importing mitochondrial proteins: machineries and mechanisms. *Cell* **138**, 628–644 (2009).

29. Schmidt, O., Pfanner, N. & Meisinger, C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat. Rev. Mol. Cell Biol.* **11**, 655–667 (2010).

30. Nolfi-Donegan, D., Braganza, A. & Shiva, S. Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. *Redox Biol.* **37**, 101674 (2020).

31. Lesnefsky, E. J. & Hoppel, C. L. Oxidative phosphorylation and aging. *Ageing Res. Rev.* **5**, 402–433 (2006).

32. Juan, C. A., Pérez De La Lastra, J. M., Plou, F. J. & Pérez-Lebeña, E. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. *Int. J. Mol. Sci.* **22**, 4642 (2021).

33. Kowaltowski, A. J., De Souza-Pinto, N. C., Castilho, R. F. & Vercesi, A. E. Mitochondria and reactive oxygen species. *Free Radic. Biol. Med.* **47**, 333–343 (2009).

34. Zhao, R., Jiang, S., Zhang, L. & Yu, Z. Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *Int. J. Mol. Med.* https://doi.org/10.3892/ijmm.2019.4188 (2019) doi:10.3892/ijmm.2019.4188.

35. Turrens, J. F. Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552**, 335–344 (2003).

36. Palma, F. R. *et al.* ROS production by mitochondria: function or dysfunction? *Oncogene* **43**, 295–303 (2024).

37. Averill-Bates, D. Reactive oxygen species and cell signaling. Review. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1871**, 119573 (2024).

38. Spinelli, J. B. & Haigis, M. C. The multifaceted contributions of mitochondria to cellular metabolism. *Nat. Cell Biol.* **20**, 745–754 (2018).

39. Rizzuto, R., De Stefani, D., Raffaello, A. & Mammucari, C. Mitochondria as sensors and regulators of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **13**, 566–578 (2012).

40. Giorgi, C., Marchi, S. & Pinton, P. The machineries, regulation and cellular functions of mitochondrial calcium. *Nat. Rev. Mol. Cell Biol.* **19**, 713–730 (2018).

41. Jouaville, L. S., Pinton, P., Bastianutto, C., Rutter, G. A. & Rizzuto, R. Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 13807–13812 (1999).

42. McCormack, J. G., Halestrap, A. P. & Denton, R. M. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol. Rev.* **70**, 391–425 (1990).

43. McCormack, J. G. & Denton, R. M. The effects of calcium ions and adenine nucleotides on the activity of pig heart 2-oxoglutarate dehydrogenase complex. *Biochem. J.* **180**, 533–544 (1979).

44. Mills, E. L., Kelly, B. & O'Neill, L. A. J. Mitochondria are the powerhouses of immunity. *Nat. Immunol.* **18**, 488–498 (2017).

45. Zhong, F., Liang, S. & Zhong, Z. Emerging Role of Mitochondrial DNA as a Major Driver of Inflammation and Disease Progression. *Trends Immunol.* **40**, 1120–1133 (2019).

46. West, A. P., Shadel, G. S. & Ghosh, S. Mitochondria in innate immune responses. *Nat. Rev. Immunol.* **11**, 389–402 (2011).

47. Nakahira, K. *et al.* Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat. Immunol.* **12**, 222–230 (2011).

48. West, A. P. *et al.* Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* **520**, 553–557 (2015).

49. Angajala, A. *et al.* Diverse Roles of Mitochondria in Immune Responses: Novel Insights Into Immuno-Metabolism. *Front. Immunol.* **9**, 1605 (2018).

50. Liu, Y. & Ma, Y. Finite-time non-fragile extended dissipative control for T-S fuzzy system via augmented Lyapunov-Krasovskii functional. *ISA Trans.* **117**, 1–15 (2021).

51. Sena, L. A. & Chandel, N. S. Physiological Roles of Mitochondrial Reactive Oxygen Species. *Mol. Cell* **48**, 158–167 (2012).

52. Bogenhagen, D. & Clayton, D. A. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. *J. Biol. Chem.* **249**, 7991–7995 (1974).

53. D'Erchia, A. M. *et al.* Tissue-specific mtDNA abundance from exome data and its correlation with mitochondrial transcription, mass and respiratory activity. *Mitochondrion* **20**, 13–21 (2015).

54. Satoh, M. Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. *Exp. Cell Res.* **196**, 137–140 (1991).

55. Payne, B. A. I. *et al.* Universal heteroplasmy of human mitochondrial DNA. *Hum. Mol. Genet.* **22**, 384–390 (2013).

56. Marshall, A. S. & Jones, N. S. Discovering Cellular Mitochondrial Heteroplasmy Heterogeneity with Single Cell RNA and ATAC Sequencing. *Biology* **10**, 503 (2021).

57. Stewart, J. B. & Chinnery, P. F. Extreme heterogeneity of human mitochondrial DNA from organelles to populations. *Nat. Rev. Genet.* **22**, 106–118 (2021).

58. Stewart, J. B. & Chinnery, P. F. The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. *Nat. Rev. Genet.* **16**, 530–542 (2015).

59. Anderson, S. *et al.* Sequence and organization of the human mitochondrial genome. *Nature* **290**, 457–465 (1981).

60. Chomyn, A. *et al.* URF6, Last Unidentified Reading Frame of Human mtDNA, Codes for an NADH Dehydrogenase Subunit. *Science* **234**, 614–618 (1986).

61. Chomyn, A. *et al.* Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. *Nature* **314**, 592–597 (1985).

62. Taanman, J.-W. The mitochondrial genome: structure, transcription, translation and replication. *Biochim. Biophys. Acta BBA - Bioenerg.* **1410**, 103–123 (1999).

63. Corneo, G., Zardi, L. & Polli, E. Human mitochondrial DNA. *J. Mol. Biol.* **36**, 419–423 (1968).

64. Dong, D. W. *et al.* Association of G-quadruplex forming sequences with human mtDNA deletion breakpoints. *BMC Genomics* **15**, 677 (2014).

65. Oliveira, P. H., Lobato Da Silva, C. & Cabral, J. M. S. An Appraisal of Human Mitochondrial DNA Instability: New Insights into the Role of Non-Canonical DNA Structures and Sequence Motifs. *PLoS ONE* **8**, e59907 (2013).

66. Bharti, S. K. *et al.* DNA Sequences Proximal to Human Mitochondrial DNA Deletion Breakpoints Prevalent in Human Disease Form G-quadruplexes, a Class of DNA Structures Inefficiently Unwound by the Mitochondrial Replicative Twinkle Helicase. *J. Biol. Chem.* **289**, 29975–29993 (2014).

67. Doimo, M. *et al.* Enhanced mitochondrial G-quadruplex formation impedes replication fork progression leading to mtDNA loss in human cells. *Nucleic Acids Res.* **51**, 7392–7408 (2023).

68. Kukat, C. *et al.* Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA. *Proc. Natl. Acad. Sci.* **108**, 13534–13539 (2011).

69. Gilkerson, R. *et al.* The mitochondrial nucleoid: integrating mitochondrial DNA into cellular homeostasis. *Cold Spring Harb. Perspect. Biol.* **5**, a011080 (2013).

70. Bogenhagen, D. F., Rousseau, D. & Burke, S. The Layered Structure of Human Mitochondrial DNA Nucleoids. *J. Biol. Chem.* **283**, 3665–3675 (2008).

71. Kaufman, B. A. *et al.* The Mitochondrial Transcription Factor TFAM Coordinates the Assembly of Multiple DNA Molecules into Nucleoid-like Structures. *Mol. Biol. Cell* **18**, 3225–3236 (2007).

72. Farge, G. *et al.* In Vitro-Reconstituted Nucleoids Can Block Mitochondrial DNA Replication and Transcription. *Cell Rep.* **8**, 66–74 (2014).

73. Shutt, T. & Gray, M. Bacteriophage origins of mitochondrial replication and transcription proteins. *Trends Genet.* **22**, 90–95 (2006).

74. Robberson, D. L. & Davidson, N. Covalent coupling of ribonucleic acid to agarose. *Biochemistry* **11**, 533–537 (1972).

75. Brown, T. A., Cecconi, C., Tkachuk, A. N., Bustamante, C. & Clayton, D. A. Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. *Genes Dev.* **19**, 2466–2476 (2005).

76. Clayton, D. A. Replication of animal mitochondrial DNA. *Cell* **28**, 693–705 (1982).

77. Miralles Fusté, J. *et al.* In Vivo Occupancy of Mitochondrial Single-Stranded DNA Binding Protein Supports the Strand Displacement Mode of DNA Replication. *PLoS Genet.* **10**, e1004832 (2014).

78. Phillips, A. F. *et al.* Single-Molecule Analysis of mtDNA Replication Uncovers the Basis of the Common Deletion. *Mol. Cell* **65**, 527-538.e6 (2017).

79. Clayton, D. A. Replication and transcription of vertebrate mitochondrial DNA. *Annu. Rev. Cell Biol.* **7**, 453–478 (1991).

80. Falkenberg, M., Larsson, N.-G. & Gustafsson, C. M. Replication and Transcription of Human Mitochondrial DNA. *Annu. Rev. Biochem.* **93**, 47–77 (2024).

81. Fusté, J. M. *et al.* Mitochondrial RNA Polymerase Is Needed for Activation of the Origin of Light-Strand DNA Replication. *Mol. Cell* **37**, 67–78 (2010).

82. Yakubovskaya, E., Chen, Z., Carrodeguas, J. A., Kisker, C. & Bogenhagen, D. F. Functional Human Mitochondrial DNA Polymerase γ Forms a Heterotrimer. *J. Biol. Chem.* **281**, 374–382 (2006).

83. Gray, H. & Wong, T. W. Purification and identification of subunit structure of the human mitochondrial DNA polymerase. *J. Biol. Chem.* **267**, 5835–5841 (1992).

84. Carrodeguas, J. A., Theis, K., Bogenhagen, D. F. & Kisker, C. Crystal Structure and Deletion Analysis Show that the Accessory Subunit of Mammalian DNA Polymerase γ, PolγB, Functions as a Homodimer. *Mol. Cell* **7**, 43–54 (2001).

85. Longley, M. J., Nguyen, D., Kunkel, T. A. & Copeland, W. C. The Fidelity of Human DNA Polymerase γ with and without Exonucleolytic Proofreading and the p55 Accessory Subunit. *J. Biol. Chem.* **276**, 38555–38562 (2001).

86. Johnson, A. A. & Johnson, K. A. Exonuclease Proofreading by Human Mitochondrial DNA Polymerase. *J. Biol. Chem.* **276**, 38097–38107 (2001).

87. Park, J., Herrmann, G. K., Mitchell, P. G., Sherman, M. B. & Yin, Y. W. Polγ coordinates DNA synthesis and proofreading to ensure mitochondrial genome integrity. *Nat. Struct. Mol. Biol.* **30**, 812–823 (2023).

88. Lamichhane, R., Berezhna, S. Y., Gill, J. P., Van Der Schans, E. & Millar, D. P. Dynamics of Site Switching in DNA Polymerase. *J. Am. Chem. Soc.* **135**, 4735–4742 (2013).

89. Longley, M. J., Prasad, R., Srivastava, D. K., Wilson, S. H. & Copeland, W. C. 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.* **95**, 12244–12248 (1998).

90. Pinz, K. G. & Bogenhagen, D. F. Characterization of a Catalytically Slow AP Lyase Activity in DNA Polymerase γ and Other Family A DNA Polymerases. *J. Biol. Chem.* **275**, 12509–12514 (2000).

91. Lim, S. E., Longley, M. J. & Copeland, W. C. 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**, 38197–38203 (1999).

92. Lee, Y.-S. *et al.* Each Monomer of the Dimeric Accessory Protein for Human Mitochondrial DNA Polymerase Has a Distinct Role in Conferring Processivity. *J. Biol. Chem.* **285**, 1490–1499 (2010).

93. Spelbrink, J. N. *et al.* 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**, 223–231 (2001).

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

95. Korhonen, J. A., Gaspari, M. & Falkenberg, M. TWINKLE Has 5′ → 3′ DNA Helicase Activity and Is Specifically Stimulated by Mitochondrial Single-stranded DNA-binding Protein. *J. Biol. Chem.* **278**, 48627–48632 (2003).

96. Riccio, A. A. *et al.* Structural insight and characterization of human Twinkle helicase in mitochondrial disease. *Proc. Natl. Acad. Sci.* **119**, e2207459119 (2022).

97. Shutt, T. E. & Gray, M. W. Twinkle, the Mitochondrial Replicative DNA Helicase, Is Widespread in the Eukaryotic Radiation and May Also Be the Mitochondrial DNA Primase in Most Eukaryotes. *J. Mol. Evol.* **62**, 588–599 (2006).

98. Farge, G. *et al.* The N-terminal domain of TWINKLE contributes to single-stranded DNA binding and DNA helicase activities. *Nucleic Acids Res.* **36**, 393–403 (2007).

99. Milenkovic, D. *et al.* TWINKLE is an essential mitochondrial helicase required for synthesis of nascent D-loop strands and complete mtDNA replication. *Hum. Mol. Genet.* **22**, 1983–1993 (2013).

100. Suomalainen, A. *et al.* Autosomal dominant progressive external ophthalmoplegia with multiple deletions of mtDNA: Clinical, biochemical, and molecular genetic features of the 1Oq-linked disease. *Neurology* **48**, 1244–1253 (1997).

101. Curth, U. *et al.* Single-stranded-DNA-binding proteins from human mitochondria and *Escherichia coli* have analogous physicochemical properties. *Eur. J. Biochem.* **221**, 435–443 (1994).

102. Yang, C., Curth, U., Urbanke, C. & Kang, C. Crystal structure of human mitochondrial single-stranded DNA binding protein at 2.4 Å resolution. *Nat. Struct. Biol.* **4**, 153–157 (1997).

103. Jiang, M. *et al.* The mitochondrial single-stranded DNA binding protein is essential for initiation of mtDNA replication. *Sci. Adv.* **7**, eabf8631 (2021).

104. Chang, D. D. & Clayton, D. A. Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 351–355 (1985).

105. Wang, Y., Lyu, Y. L. & Wang, J. C. Dual localization of human DNA topoisomerase IIIalpha to mitochondria and nucleus. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12114–12119 (2002).

106. Nicholls, T. J. *et al.* Topoisomerase 3α Is Required for Decatenation and Segregation of Human mtDNA. *Mol. Cell* **69**, 9-23.e6 (2018).

107. Erdinc, D. *et al.* Pathological variants in TOP3A cause distinct disorders of mitochondrial and nuclear genome stability. *EMBO Mol. Med.* **15**, e16775 (2023).

108. Zhang, H. *et al.* Human mitochondrial topoisomerase I. *Proc. Natl. Acad. Sci.* **98**, 10608–10613 (2001).

109. Douarre, C. *et al.* Mitochondrial Topoisomerase I is Critical for Mitochondrial Integrity and Cellular Energy Metabolism. *PLoS ONE* **7**, e41094 (2012).

110. Menger, K. E. *et al.* Two type I topoisomerases maintain DNA topology in human mitochondria. *Nucleic Acids Res.* **50**, 11154–11174 (2022).

111. Lima, W. F. *et al.* Human RNase H1 Discriminates between Subtle Variations in the Structure of the Heteroduplex Substrate. *Mol. Pharmacol.* **71**, 83–91 (2007).

112. Karlowicz, A. *et al. In vitro* reconstitution reveals a key role of human mitochondrial EXOG in RNA primer processing. *Nucleic Acids Res.* **50**, 7991–8007 (2022).

113. Kazak, L. *et al.* A Cryptic Targeting Signal Creates a Mitochondrial FEN1 Isoform with Tailed R-Loop Binding Properties. *PLoS ONE* **8**, e62340 (2013).

114. Uhler, J. P. & Falkenberg, M. Primer removal during mammalian mitochondrial DNA replication. *DNA Repair* **34**, 28–38 (2015).

115. Nicholls, T. J. *et al.* Linear mtDNA fragments and unusual mtDNA rearrangements associated with pathological deficiency of MGME1 exonuclease. *Hum. Mol. Genet.* **23**, 6147–6162 (2014).

116. Kornblum, C. *et al.* Loss-of-function mutations in MGME1 impair mtDNA replication and cause multisystemic mitochondrial disease. *Nat. Genet.* **45**, 214–219 (2013).

117. Uhler, J. P. *et al.* MGME1 processes flaps into ligatable nicks in concert with DNA polymerase γ during mtDNA replication. *Nucleic Acids Res.* **44**, 5861–5871 (2016).

118. Lakshmipathy, U. & Campbell, C. The Human DNA Ligase III Gene Encodes Nuclear and Mitochondrial Proteins. *Mol. Cell. Biol.* **19**, 3869–3876 (1999).

119. Lakshmipathy, U. Antisense-mediated decrease in DNA ligase III expression results in reduced mitochondrial DNA integrity. *Nucleic Acids Res.* **29**, 668–676 (2001).

120. Khrapko, K. *et al.* Mitochondrial mutational spectra in human cells and tissues. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13798–13803 (1997).

121. Alexeyev, M., Shokolenko, I., Wilson, G. & LeDoux, S. The maintenance of mitochondrial DNA integrity--critical analysis and update. *Cold Spring Harb. Perspect. Biol.* **5**, a012641 (2013).

122. Gorman, G. S. *et al.* Mitochondrial diseases. *Nat. Rev. Dis. Primer* **2**, 16080 (2016).

123. Clayton, D. A., Doda, J. N. & Friedberg, E. C. The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **71**, 2777–2781 (1974).

124. Miyaki, M., Yatagai, K. & Ono, T. Strand breaks of mammalian mitochondrial DNA induced by carcinogens. *Chem. Biol. Interact.* **17**, 321–329 (1977).

125. Svilar, D., Goellner, E. M., Almeida, K. H. & Sobol, R. W. Base excision repair and lesion-dependent subpathways for repair of oxidative DNA damage. *Antioxid. Redox Signal.* **14**, 2491–2507 (2011).

126. Chattopadhyay, R. *et al.* Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells. *Nucleic Acids Res.* **34**, 2067–2076 (2006).

127. Sia, E. A. *et al.* Analysis of microsatellite mutations in the mitochondrial DNA of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **97**, 250–255 (2000).

128. Chi, N. W. & Kolodner, R. D. Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. *J. Biol. Chem.* **269**, 29984–29992 (1994).

129. De Souza-Pinto, N. C. *et al.* Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. *DNA Repair* **8**, 704–719 (2009).

130. Bacman, S. R., Williams, S. L. & Moraes, C. T. Intra- and inter-molecular recombination of mitochondrial DNA after in vivo induction of multiple double-strand breaks. *Nucleic Acids Res.* **37**, 4218–4226 (2009).

131. Zhou, J., Liu, L. & Chen, J. Mitochondrial DNA heteroplasmy in Candida glabrata after mitochondrial transformation. *Eukaryot. Cell* **9**, 806–814 (2010).

132. Davila, J. I. *et al.* Double-strand break repair processes drive evolution of the mitochondrial genome in Arabidopsis. *BMC Biol.* **9**, 64 (2011).

133. Manchekar, M. *et al.* DNA recombination activity in soybean mitochondria. *J. Mol. Biol.* **356**, 288–299 (2006).

134. Furda, A. M., Marrangoni, A. M., Lokshin, A. & Van Houten, B. Oxidants and not alkylating agents induce rapid mtDNA loss and mitochondrial dysfunction. *DNA Repair* **11**, 684–692 (2012).

135. Shokolenko, I., Venediktova, N., Bochkareva, A., Wilson, G. L. & Alexeyev, M. F. Oxidative stress induces degradation of mitochondrial DNA. *Nucleic Acids Res.* **37**, 2539–2548 (2009).

136. Shokolenko, I. N., Wilson, G. L. & Alexeyev, M. F. Persistent damage induces mitochondrial DNA degradation. *DNA Repair* **12**, 488–499 (2013).

137. Moretton, A. *et al.* Selective mitochondrial DNA degradation following double-strand breaks. *PLOS ONE* **12**, e0176795 (2017).

138. Peeva, V. *et al.* Linear mitochondrial DNA is rapidly degraded by components of the replication machinery. *Nat. Commun.* **9**, 1727 (2018).

139. Nissanka, N., Bacman, S. R., Plastini, M. J. & Moraes, C. T. The mitochondrial DNA polymerase gamma degrades linear DNA fragments precluding the formation of deletions. *Nat. Commun.* **9**, 2491 (2018).

140. Longley, M. J. *et al.* Mutant POLG2 disrupts DNA polymerase gamma subunits and causes progressive external ophthalmoplegia. *Am. J. Hum. Genet.* **78**, 1026–1034 (2006).

141. Van Goethem, G., Dermaut, B., Löfgren, A., Martin, J. J. & Van Broeckhoven, C. Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nat. Genet.* **28**, 211–212 (2001).

142. Carrozzo, R. *et al.* Multiple mtDNA deletions features in autosomal dominant and recessive diseases suggest distinct pathogeneses. *Neurology* **50**, 99–106 (1998).

143. Horvath, R. *et al.* Phenotypic spectrum associated with mutations of the mitochondrial polymerase gamma gene. *Brain J. Neurol.* **129**, 1674–1684 (2006).

144. Hudson, G. *et al.* Mutation of OPA1 causes dominant optic atrophy with external ophthalmoplegia, ataxia, deafness and multiple mitochondrial DNA deletions: a novel disorder of mtDNA maintenance. *Brain J. Neurol.* **131**, 329–337 (2008).

145. Nissanka, N. & Moraes, C. T. Mitochondrial DNA heteroplasmy in disease and targeted nuclease-based therapeutic approaches. *EMBO Rep.* **21**, e49612 (2020).

146. Mariotti, C. *et al.* Defective respiratory capacity and mitochondrial protein synthesis in transformant cybrids harboring the tRNA(Leu(UUR)) mutation associated with maternally inherited myopathy and cardiomyopathy. *J. Clin. Invest.* **93**, 1102–1107 (1994).

147. Wallace, D. C. *et al.* Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science* **242**, 1427–1430 (1988).

148. Nishioka, T., Soemantri, A. & Ishida, T. mtDNA/nDNA ratio in 14484 LHON mitochondrial mutation carriers. *J. Hum. Genet.* **49**, 701–705 (2004).

149. Moraes, C. T. *et al.* Atypical clinical presentations associated with the MELAS mutation at position 3243 of human mitochondrial DNA. *Neuromuscul. Disord. NMD* **3**, 43–50 (1993).

150. Goto, Y. *et al.* Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutation. *Neurology* **42**, 545–550 (1992).

151. Yu-Wai-Man, P. *et al.* Somatic mitochondrial DNA deletions accumulate to high levels in aging human extraocular muscles. *Invest. Ophthalmol. Vis. Sci.* **51**, 3347–3353 (2010).

152. Chinnery, P. F. *et al.* Risk of developing a mitochondrial DNA deletion disorder. *The Lancet* **364**, 592–596 (2004).

153. Bua, E. *et al.* Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. *Am. J. Hum. Genet.* **79**, 469–480 (2006).

154. Trifunovic, A. Mitochondrial DNA and ageing. *Biochim. Biophys. Acta BBA - Bioenerg.* **1757**, 611–617 (2006).

155. Chen, T., He, J., Huang, Y. & Zhao, W. The generation of mitochondrial DNA large-scale deletions in human cells. *J. Hum. Genet.* **56**, 689–694 (2011).

156. Schon, E. A. *et al.* A direct repeat is a hotspot for large-scale deletion of human mitochondrial DNA. *Science* **244**, 346–349 (1989).

157. Mancuso, M. *et al.* Redefining phenotypes associated with mitochondrial DNA single deletion. *J. Neurol.* **262**, 1301–1309 (2015).

158. Ross, F. A., MacKintosh, C. & Hardie, D. G. AMP-activated protein kinase: a cellular energy sensor that comes in 12 flavours. *FEBS J.* **283**, 2987–3001 (2016).

159. Herzig, S. & Shaw, R. J. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nat. Rev. Mol. Cell Biol.* **19**, 121–135 (2018).

160. Scarpulla, R. C. Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1813**, 1269–1278 (2011).

161. Reznick, R. M. & Shulman, G. I. The role of AMP-activated protein kinase in mitochondrial biogenesis. *J. Physiol.* **574**, 33–39 (2006).

162. Ducommun, S. *et al.* Motif affinity and mass spectrometry proteomic approach for the discovery of cellular AMPK targets: Identification of mitochondrial fission factor as a new AMPK substrate. *Cell. Signal.* **27**, 978–988 (2015).

163. Toyama, E. Q. *et al.* AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. *Science* **351**, 275–281 (2016).

164. Sinha, R. A. *et al.* Thyroid hormone induction of mitochondrial activity is coupled to mitophagy via ROS-AMPK-ULK1 signaling. *Autophagy* **11**, 1341–1357 (2015).

165. Egan, D. F. *et al.* Phosphorylation of ULK1 (hATG1) by AMP-Activated Protein Kinase Connects Energy Sensing to Mitophagy. *Science* **331**, 456–461 (2011).

166. Narendra, D. P. *et al.* PINK1 Is Selectively Stabilized on Impaired Mitochondria to Activate Parkin. *PLoS Biol.* **8**, e1000298 (2010).

167. Wang, B. *et al.* AMPKα2 Protects Against the Development of Heart Failure by Enhancing Mitophagy via PINK1 Phosphorylation. *Circ. Res.* **122**, 712–729 (2018).

168. Lazarou, M. *et al.* The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* **524**, 309–314 (2015).

169. García-Gómez, S. *et al.* PrimPol, an archaic primase/polymerase operating in human cells. *Mol. Cell* **52**, 541–553 (2013).

170. Torregrosa-Muñumer, R. *et al.* PrimPol is required for replication reinitiation after mtDNA damage. *Proc. Natl. Acad. Sci.* **114**, 11398–11403 (2017).

171. Guilliam, T. A., Bailey, L. J., Brissett, N. C. & Doherty, A. J. PolDIP2 interacts with human PrimPol and enhances its DNA polymerase activities. *Nucleic Acids Res.* **44**, 3317–3329 (2016).

172. Liu, L., Rodriguez-Belmonte, E. M., Mazloum, N., Xie, B. & Lee, M. Y. W. T. Identification of a Novel Protein, PDIP38, That Interacts with the p50 Subunit of DNA Polymerase δ and Proliferating Cell Nuclear Antigen*. *J. Biol. Chem.* **278**, 10041–10047 (2003).

173. Tissier, A. *et al.* Crosstalk between replicative and translesional DNA polymerases: PDIP38 interacts directly with Poleta. *DNA Repair* **9**, 922–928 (2010).

174. Maga, G. *et al.* DNA polymerase δ-interacting protein 2 is a processivity factor for DNA polymerase λ during 8-oxo-7,8-dihydroguanine bypass. *Proc. Natl. Acad. Sci.* **110**, 18850–18855 (2013).

175. Brown, D. I. *et al.* Poldip2 knockout results in perinatal lethality, reduced cellular growth and increased autophagy of mouse embryonic fibroblasts. *PloS One* **9**, e96657 (2014).

176. Lyle, A. N. *et al.* Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. *Circ. Res.* **105**, 249–259 (2009).

177. Datla, S. R. *et al.* Poldip2 controls vascular smooth muscle cell migration by regulating focal adhesion turnover and force polarization. *Am. J. Physiol. Heart Circ. Physiol.* **307**, H945-957 (2014).

178. Cheng, X. *et al.* PDIP38 associates with proteins constituting the mitochondrial DNA nucleoid. *J. Biochem. (Tokyo)* **138**, 673–678 (2005).

179. Kulik, A. A. *et al.* Crystal structure and molecular dynamics of human POLDIP2, a multifaceted adaptor protein in metabolism and genome stability. *Protein Sci. Publ. Protein Soc.* **30**, 1196–1209 (2021).

180. Hernandes, M. S., Lassègue, B. & Griendling, K. K. Polymerase δ-interacting protein 2: a multifunctional protein. *J. Cardiovasc. Pharmacol.* **69**, 335–342 (2017).

181. Strack, P. R. *et al.* Polymerase delta-interacting protein 38 (PDIP38) modulates the stability and activity of the mitochondrial AAA+ protease CLPXP. *Commun. Biol.* **3**, 1–12 (2020).

182. Shimuta, T. *et al.* Novel heat shock protein HspQ stimulates the degradation of mutant DnaA protein in *Escherichia coli*. *Genes Cells* **9**, 1151–1166 (2004).

183. Puri, N. & Karzai, A. W. HspQ Functions as a Unique Specificity-Enhancing Factor for the AAA+ Lon Protease. *Mol. Cell* **66**, 672-683.e4 (2017).

184. Nishimura, K. *et al.* Discovery of a Unique Clp Component, ClpF, in Chloroplasts: A Proposed Binary ClpF-ClpS1 Adaptor Complex Functions in Substrate Recognition and Delivery. *Plant Cell* tpc.15.00574 (2015) doi:10.1105/tpc.15.00574.

185. d'Alençon, E. *et al.* Isolation of a new hemimethylated DNA binding protein which regulates dnaA gene expression. *J. Bacteriol.* **185**, 2967–2971 (2003).

186. Kasho, K. *et al.* A unique arginine cluster in PolDIP2 enhances nucleotide binding and DNA synthesis by PrimPol. *Nucleic Acids Res.* **49**, 2179–2191 (2021).

187. Ho, M. S., Tsai, P.-I. & Chien, C.-T. F-box proteins: the key to protein degradation. *J. Biomed. Sci.* **13**, 181–191 (2006).

188. Gibson, M. M., Bagga, D. A., Miller, C. G. & Maguire, M. E. Magnesium transport in Salmonella typhimurium: the influence of new mutations conferring Co2+ resistance on the CorA Mg2+ transport system. *Mol. Microbiol.* **5**, 2753–2762 (1991).

189. Bottoms, C. A., Smith, P. E. & Tanner, J. J. A structurally conserved water molecule in Rossmann dinucleotide-binding domains. *Protein Sci. Publ. Protein Soc.* **11**, 2125–2137 (2002).

190. Cicero, D. O. *et al.* Solution structure of ApaG from Xanthomonas axonopodis pv. citri reveals a fibronectin-3 fold. *Proteins* **67**, 490–500 (2007).

191. Krzysiak, T. C., Chen, B. B., Lear, T., Mallampalli, R. K. & Gronenborn, A. M. Crystal structure and interaction studies of the human FBxo3 ApaG domain. *FEBS J.* **283**, 2091–2101 (2016).

192. Sutliff, R. L. *et al.* Polymerase Delta Interacting Protein 2 Sustains Vascular Structure and Function. *Arterioscler. Thromb. Vasc. Biol.* **33**, 2154–2161 (2013).

193. Chian, C.-F. *et al.* Panels of tumor-derived RNA markers in peripheral blood of patients with non-small cell lung cancer: their dependence on age, gender and clinical stages. *Oncotarget* **7**, 50582–50595 (2016).

194. Chen, Y.-C. *et al.* Knockdown of POLDIP2 suppresses tumor growth and invasion capacity and is linked to unfavorable transformation ability and metastatic feature in non-small cell lung cancer. *Exp. Cell Res.* **368**, 42–49 (2018).

195. Xie, B. *et al.* Further characterization of human DNA polymerase delta interacting protein 38. *J. Biol. Chem.* **280**, 22375–22384 (2005).

196. Klaile, E., Kukalev, A., Obrink, B. & Müller, M. M. PDIP38 is a novel mitotic spindle-associated protein that affects spindle organization and chromosome segregation. *Cell Cycle Georget. Tex* **7**, 3180–3186 (2008).

197. Wong, A. *et al.* PDIP38 is translocated to the spliceosomes/nuclear speckles in response to UV-induced DNA damage and is required for UV-induced alternative splicing of MDM2. *Cell Cycle Georget. Tex* **12**, 3184–3193 (2013).

198. Paredes, F. *et al.* Poldip2 is an oxygen-sensitive protein that controls PDH and αKGDH lipoylation and activation to support metabolic adaptation in hypoxia and cancer. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 1789–1794 (2018).

199. Klaile, E. *et al.* The cell adhesion receptor carcinoembryonic antigen-related cell adhesion molecule 1 regulates nucleocytoplasmic trafficking of DNA polymerase delta-interacting protein 38. *J. Biol. Chem.* **282**, 26629–26640 (2007).

200. Arakaki, N. *et al.* Regulation of mitochondrial morphology and cell survival by Mitogenin I and mitochondrial single-stranded DNA binding protein. *Biochim. Biophys. Acta* **1760**, 1364–1372 (2006).

201. Iyer, L. M. Origin and evolution of the archaeo-eukaryotic primase superfamily and related palm-domain proteins: structural insights and new members. *Nucleic Acids Res.* **33**, 3875–3896 (2005).

202. Kobayashi, K. *et al.* Repriming by PrimPol is critical for DNA replication restart downstream of lesions and chain-terminating nucleosides. *Cell Cycle* **15**, 1997–2008 (2016).

203. Mourón, S. *et al.* Repriming of DNA synthesis at stalled replication forks by human PrimPol. *Nat. Struct. Mol. Biol.* **20**, 1383–1389 (2013).

204. Schiavone, D. *et al.* PrimPol Is Required for Replicative Tolerance of G Quadruplexes in Vertebrate Cells. *Mol. Cell* **61**, 161–169 (2016).

205. Guilliam, T. A. *et al.* Human PrimPol is a highly error-prone polymerase regulated by single-stranded DNA binding proteins. *Nucleic Acids Res.* **43**, 1056–1068 (2015).

206. Corydon, T. J. *et al.* A human homologue of Escherichia coli ClpP caseinolytic protease: recombinant expression, intracellular processing and subcellular localization. *Biochem. J.* **331 (Pt 1)**, 309–316 (1998).

207. de Sagarra, M. R. *et al.* Mitochondrial localization and oligomeric structure of HClpP, the human homologue of E. coli ClpP. *J. Mol. Biol.* **292**, 819–825 (1999).

208. Kang, S. G. *et al.* Functional proteolytic complexes of the human mitochondrial ATP-dependent protease, hClpXP. *J. Biol. Chem.* **277**, 21095–21102 (2002).

209. Ripstein, Z. A., Vahidi, S., Houry, W. A., Rubinstein, J. L. & Kay, L. E. A processive rotary mechanism couples substrate unfolding and proteolysis in the ClpXP degradation machinery. *eLife* **9**, e52158 (2020).

210. Dunn, C. D., Tamura, Y., Sesaki, H. & Jensen, R. E. Mgr3p and Mgr1p are adaptors for the mitochondrial i-AAA protease complex. *Mol. Biol. Cell* **19**, 5387–5397 (2008).

211. Micevski, D. *et al.* Insight into the RssB-Mediated Recognition and Delivery of σs to the AAA+ Protease, ClpXP. *Biomolecules* **10**, 615 (2020).

212. Kirstein, J., Molière, N., Dougan, D. A. & Turgay, K. Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nat. Rev. Microbiol.* **7**, 589–599 (2009).

213. Kuhlmann, N. J. & Chien, P. Selective adaptor dependent protein degradation in bacteria. *Curr. Opin. Microbiol.* **36**, 118–127 (2017).

214. Lowth, B. R. *et al.* Substrate recognition and processing by a Walker B mutant of the human mitochondrial AAA+ protein CLPX. *J. Struct. Biol.* **179**, 193–201 (2012).

215. Cottle, T., Joh, L., Posner, C., DeCosta, A. & Kardon, J. R. An adaptor for feedback regulation of heme biosynthesis by the mitochondrial protease CLPXP. Preprint at https://doi.org/10.1101/2024.07.05.602318 (2024).

216. Rahman, S. & Copeland, W. C. POLG-related disorders and their neurological manifestations. *Nat. Rev. Neurol.* **15**, 40–52 (2019).

217. Chan, S. S. L. & Copeland, W. C. DNA polymerase gamma and mitochondrial disease: understanding the consequence of POLG mutations. *Biochim. Biophys. Acta* **1787**, 312–319 (2009).

218. Bekheirnia, M. R. *et al.* POLG mutation in a patient with cataracts, early-onset distal muscle weakness and atrophy, ovarian dysgenesis and 3-methylglutaconic aciduria. *Gene* **499**, 209–212 (2012).

219. Steinberg, G. R. & Carling, D. AMP-activated protein kinase: the current landscape for drug development. *Nat. Rev. Drug Discov.* **18**, 527–551 (2019).

220. Jazayeri, M. *et al.* Inducible expression of a dominant negative DNA polymerase-gamma depletes mitochondrial DNA and produces a rho0 phenotype. *J. Biol. Chem.* **278**, 9823–9830 (2003).

221. Wanrooij, S., Goffart, S., Pohjoismäki, J. L. O., Yasukawa, T. & Spelbrink, J. N. Expression of catalytic mutants of the mtDNA helicase Twinkle and polymerase POLG causes distinct replication stalling phenotypes. *Nucleic Acids Res.* **35**, 3238–3251 (2007).

222. Sullivan, E. D., Longley, M. J. & Copeland, W. C. Polymerase γ efficiently replicates through many natural template barriers but stalls at the HSP1 quadruplex. *J. Biol. Chem.* **295**, 17802–17815 (2020).

223. Biffi, G., Tannahill, D., McCafferty, J. & Balasubramanian, S. Quantitative visualization of DNA G-quadruplex structures in human cells. *Nat. Chem.* **5**, 182–186 (2013).

224. Hänsel-Hertsch, R., Spiegel, J., Marsico, G., Tannahill, D. & Balasubramanian, S. Genome-wide mapping of endogenous G-quadruplex DNA structures by chromatin immunoprecipitation and high-throughput sequencing. *Nat. Protoc.* **13**, 551–564 (2018).

225. Lyu, J., Shao, R., Kwong Yung, P. Y. & Elsässer, S. J. Genome-wide mapping of G-quadruplex structures with CUT&Tag. *Nucleic Acids Res.* **50**, e13 (2022).

226. Wang, Z. *et al.* Poldip2 promotes mtDNA elimination during Drosophila spermatogenesis to ensure maternal inheritance. *EMBO J.* **44**, 1724–1748 (2025).

227. Huang, X. *et al.* CHCHD2 accumulates in distressed mitochondria and facilitates oligomerization of CHCHD10. *Hum. Mol. Genet.* https://doi.org/10.1093/hmg/ddy270 (2018) doi:10.1093/hmg/ddy270.

228. Funayama, M. *et al.* CHCHD2 mutations in autosomal dominant late-onset Parkinson's disease: a genome-wide linkage and sequencing study. *Lancet Neurol.* **14**, 274–282 (2015).

229. Gerlach, J. *et al.* The CHCHD2-CHCHD10 protein complex is modulated by mitochondrial dysfunction and alters lipid homeostasis in the mouse brain. *Cell Death Dis.* **16**, 693 (2025).

230. Meng, H. *et al.* Loss of Parkinson's disease-associated protein CHCHD2 affects mitochondrial crista structure and destabilizes cytochrome c. *Nat. Commun.* **8**, 15500 (2017).