

Structural studies of mitochondrial DNA polymerase γ

How mutations and small-molecule modulators affect
POL γ activity

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Cover illustration: “Scientific Progress Goes ‘Boink’ – in Mitochondria”
Artwork by Valle

The title of the cover serves as an homage to the brilliant work by Bill Watterson

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“You can't just turn on creativity like a faucet. You have to be in the
right mood.

What mood is that?

Last-minute panic.”

— Calvin and Hobbes (Bill Watterson)

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ABSTRACT

Mitochondria are essential eukaryotic organelles that generate most of the cell's adenosine triphosphate (ATP), the energy currency used to power cellular activities. Because mitochondria are descendants of once free-living bacteria that formed an endosymbiotic relationship with an archaeal host cell, mitochondria contain a small but well-preserved genome. Mitochondrial DNA (mtDNA) encodes 13 proteins that are crucial for ATP production, and proper maintenance of this genome is therefore essential for the cell. mtDNA replication is carried out by DNA polymerase γ (POL γ), a heterotrimeric complex composed of the catalytic subunit POL γ A (*POLG*) and the accessory dimer POL γ B (*POLG2*). Pathogenic *POLG* variants are among the most common causes of inherited mitochondrial disease, yet the underlying mechanisms remain poorly defined, and no effective therapies exist. This thesis integrates biochemical analysis, cryogenic electron microscopy (cryo-EM), cell assays, and mouse

models to expand the mechanistic understanding of POL γ function and dysfunction.

In **Paper I**, we identified small molecules that can restore polymerization activity in mutant POL γ complexes, both *in vitro* and in patient-derived fibroblasts. Our findings position these compounds as potential therapeutic candidates for *POLG*-related disease.

In **Paper II**, we generated and characterized mouse models to study common disease-causing *POLG* variants. *In vitro*, mouse Poly displays greater catalytic efficiency than the human enzyme, which results in milder phenotypes in mice. This observation is in part due to a more potent mouse accessory subunit, and our findings establish POL γ B as a critical determinant of phenotypic severity in *POLG* mouse models.

In **Paper III**, we combined cryo-EM and biochemical assays to elucidate how the small-molecule modulators identified in Paper I allosterically activate POL γ by stabilizing it in the polymerase state.

Collectively, these studies provide important mechanistic insight into POL γ function and dysfunction, establish characterized mouse models, and lay the foundation for developing targeted therapies to treat mitochondrial disorders caused by *POLG* mutations.

Keywords: POL γ , mitochondria, mtDNA, DNA replication

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SAMMANFATTNING PÅ SVENSKA

Mitokondrier är essentiella organeller i eukaryota celler och ansvarar för merparten av produktionen av cellens adenosintrifosfat (ATP), den energivaluta som driver alla cellens processer. Eftersom mitokondrier härstammar från en fritt levande bakterie som bildade en endosymbiotisk relation med en större värdcell, innehåller de också sitt eget DNA. Mitokondriellt DNA (mtDNA) kodar för 13 proteiner som är nödvändiga för mitokondriens funktion inom ATP-produktion, och korrekt underhåll av detta DNA är därför avgörande för cellens överlevnad. Replikationen av mtDNA utförs av DNA-polymeras γ ($POL\gamma$), ett komplex bestående av den katalytiska subenheten $POL\gamma A$ ($POLG$) och två subenheter av den accessoriska $POL\gamma B$ ($POLG2$). Patogena varianter i $POLG$ är bland de vanligaste orsakerna till ärftliga mitokondriella sjukdomar, men de bakomliggande mekanismerna är fortfarande inte fullt förstådda och effektiva behandlingar saknas. Denna avhandling kombinerar biokemiska experiment, kryoelektronmikroskopi (cryo-EM), cellbaserade analyser och musmodeller för att utöka förståelsen av $POL\gamma$:s funktion och dysfunktion.

I den första studien identifierade vi små molekyler som kan återställa polymerasaktiviteten hos mutanta $POL\gamma$ -komplex, både *in vitro* och i fibroblaster från patienter. Våra resultat visar att dessa molekyler är potentiella terapeutiska kandidater för $POLG$ -relaterade sjukdomar.

I den andra studien genererade och karakteriserade vi musmodeller för att studera vanliga sjukdomsorsakande $POLG$ -varianter. *In vitro* uppvisar musens Poly högre katalytisk effektivitet än det humana enzymet, vilket resulterar i mildare fenotyper hos möss. Denna skillnad beror delvis på en mer potent accessorisk subenhet, och våra resultat fastställer att $POL\gamma B$ är en avgörande faktor för den mildare sjukdomsbilden i $POLG$ -musmodeller.

I den tredje studien kombinerade vi cryo-EM och biokemiska analyser för att klargöra hur de små molekylerna vi identifierade i det första arbetet allosteriskt aktiverar POL γ genom att stabilisera enzymet i dess polymerasaktiva tillstånd.

Sammantaget ger dessa studier viktiga mekanistiska insikter i POL γ :s funktion och dysfunktion, visar hur musmodeller kan användas för att studera *POLG*-varianter, och lägger grunden för utvecklingen av terapier mot mitokondriella sjukdomar orsakade av *POLG*-mutationer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Small molecules restore mutant mitochondrial DNA polymerase activity.**

Valenzuela S, Zhu X, Macao B, Stamgren M, Geukens C, Charifson PS, Kern G, Hoberg E, Jenninger L, Gruszczyk AV, Lee S, Johansson KAS, Miralles Fusté J, Shi Y, Kerns SJ, Arabanian L, Martinez Botella G, Ekström S, Green J, Griffin AM, Pardo-Hernández C, Keating TA, Küppers-Munther B, Larsson NG, Phan C, Posse V, Jones JE, Xie X, Giroux S, Gustafsson CM, Falkenberg M.

Nature. 2025;642(8067):501-7.

II. **Modelling *POLG* mutations in mice unravels a critical role of POLyB in regulating phenotypic severity.**

Corrà S*, Zuppardo A*, Valenzuela S*, Jenninger L*, Cerutti R, Sillamaa S, Hoberg E, Johansson KAS, Rovsniik U, Volta S, Silva-Pinheiro P, Davis H, Trifunovic A, Minczuk M, Gustafsson CM, Suomalainen A, Zeviani M, Macao B, Zhu X, Falkenberg M, Viscomi C.

Nature Communications. 2025;16(1):4782.

III. **Structural basis for allosteric activation of human POLy via polymerase-state stabilization.**

Valenzuela S*, Hoberg E*, Sillamaa S, Stamgren M, Pardo-Hernández C, Jenninger L, Macao B, Zhu X, Săcultanu M, Miralles Fusté J, Keating TA, Giroux S, Gustafsson CM, Falkenberg M.

Manuscript.

*Contributed equally.

ADDITIONAL PUBLICATIONS

Publications not part of this thesis.

- I. **DNA polymerase gamma mutations that impair holoenzyme stability cause catalytic subunit depletion.**
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Nucleic Acids Research. 2021;49(9):5230-48.
- II. **Non-coding 7S RNA inhibits transcription via mitochondrial RNA polymerase dimerization.**
Zhu X, Xie X, Das H, Tan BG, Shi Y, Al-Behadili A, Peter B, Motori E, Valenzuela S, Posse V, Gustafsson CM, Hällberg BM, Falkenberg M.
Cell. 2022;185(13):2309-23.e24.
- III. **Pathological variants in TOP3A cause distinct disorders of mitochondrial and nuclear genome stability.**
Erdinc D, Rodríguez-Luis A, Fassad MR, Mackenzie S, Watson CM, Valenzuela S, Xie X, Menger KE, Sergeant K, Craig K, Hopton S, Falkous G, Genomics England Research Consortium, Poulton J, Garcia-Moreno H, Giunti P, de Moura Aschoff CA, Morales Saute JA, Kirby AJ, Toro C, Wolfe L, Novacic D, Greenbaum L, Eliyahu A, Barel O, Anikster Y, McFarland R, Gorman GS, Schaefer AM, Gustafsson CM, Taylor RW, Falkenberg M, Nicholls TJ.
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- IV. **The disease-causing mutation p.F907I reveals a novel pathogenic mechanism for POL γ -related diseases.**
Erdinc D, Macao B, Valenzuela S, Lesko N, Naess K, Peter B, Bruhn H, Wedell A, Wredenberg A, Falkenberg M.
Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2023;1869(7):166786.
- V. **Resin acids play key roles in shaping microbial communities during degradation of spruce bark.**
Sörensen Ristinmaa A, Tafur Rangel A, Idström A, Valenzuela S, Kerkhoven EJ, Pope PB, Hasani M, Larsbrink J.
Nature Communications. 2023;14(1):8171.
- VI. **Expanding the genetic and phenotypic spectrum of *POLRMT*-related mitochondrial disease.**
Fassad MR, Valenzuela S, Oláhová M, Collier JJ, Knowles CVY, Mavraki E, Elbracht M, Güzel N, Herberhold T, Kurth I, Maier A, Mattern L, Saunders C, McCullagh H, Őunap K, Wortmann SB, Reis A, Zhang L, Gustafsson CM, McFarland R, Taylor RW.
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ABBREVIATIONS

ADP	Adenosine diphosphate
AID	Accessory-interacting determinant (subdomain)
ATP	Adenosine triphosphate
bp	Base pair
Cryo-EM	Cryogenic electron microscopy
dAMP	Deoxyadenosine monophosphate
dATP	Deoxyadenosine triphosphate
D-loop	Displacement loop
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
Exo ⁻	Exonuclease-deficient POL γ
Exo site	Catalytic site for exonuclease activity
gp5	T7 DNA polymerase
HSP	Heavy-strand promoter
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IP	Intrinsic processivity (subdomain)

kDa	Kilodalton
LSP	Light-strand promoter
mtDNA	Mitochondrial DNA
mtSSB	Mitochondrial single-stranded DNA-binding protein
NCR	Non-coding region
nt	Nucleotides
OMM	Outer mitochondrial membrane
OriH/O _H	Origin of heavy-strand replication
OriL/O _L	Origin of light-strand replication
OXPHOS	Oxidative phosphorylation
MDDS	Mitochondrial DNA depletion syndrome
POL γ	Mitochondrial DNA polymerase γ
Pol site	Catalytic site for DNA polymerization
POLRMT	Mitochondrial RNA polymerase
rRNA	Ribosomal RNA
ssDNA	Single-stranded DNA
TLC	Thin-layer chromatography
tRNA	Transfer RNA

1 INTRODUCTION

Ever since Charles Darwin's publication of *On the Origin of Species* (1), the scientific study of life has been fundamentally shaped by the concept of evolution and the idea that all life on Earth shares a common origin, tracing back to a single-celled prokaryote ancestor (2). One of the most significant evolutionary milestones was the emergence of eukaryotic cells, which are the foundation of all multicellular organisms (3). While traditional evolutionary theory explains the diversity of life through the gradual accumulation of genetic changes over time, driven by natural selection, the creation of eukaryotic cells represents an exceptional case of evolution (4). Eukaryotic cells are far more complex than prokaryotes and are thought to have resulted from a rare evolutionary leap known as symbiogenesis, or the endosymbiotic theory (4-10). Rather than evolving solely through gradual mutations and selection, eukaryotic cells were formed by the fusion of two free-living prokaryotes, where an ancestral archaeal host cell engulfed an aerobic eubacterium (11, 12). This is known as the first major endosymbiotic event, and the eubacterium endosymbiont became the ancestor of modern mitochondria (13, 14). In a second endosymbiotic event, the eukaryotic ancestor of plants and algae engulfed a photosynthetic cyanobacterium, which was retained and evolved into modern chloroplasts (14).

Today, there is a myriad of evidence supporting the endosymbiotic theory for the origin of the first eukaryotic cell (12-21), although the exact mechanism is still up for debate (11, 22). Nevertheless, the vast cellular complexity and metabolic diversity seen across eukaryotic life today stems from the symbiogenesis that formed chloroplasts and mitochondria (14).

1.1 THE MITOCHONDRIA

1.1.1 The origin of mitochondria

Following the primary endosymbiotic event, evidence indicates that the proto-mitochondrion was formed when an aerobic α -proteobacterium was engulfed and assimilated by an archaeal host cell, eventually transforming into a permanent organelle in eukaryotic cells (13, 18, 21). Although the reason for the original symbiotic relationship between the host cell and the eubacterium remains debated (11, 22), it is generally accepted that mitochondria offer a critical bioenergetic benefit to the cell by performing aerobic respiration (12, 23, 24). This function provided the early eukaryotic cell with a significant advantage in energy production, which in turn supported an expansion in genome size and cellular complexity (12). As a result, eukaryotes have been able to evolve novel gene families and protein folds, as well as longer proteins compared to prokaryotes (25-27). Mitochondria have effectively been a prerequisite for eukaryotic innovation and a key step toward the evolution of multicellular life (12), and all eukaryotes either have mitochondria or descend from ancestors that once possessed them (28, 29). In contrast, prokaryotes have remained relatively simple, likely due to bioenergetic limitations that constrain their ability to support larger genomes and more complex cellular machinery (12).

During the evolution of mitochondria, most of the ancestral bacterial genome became either redundant and was lost or was incorporated into the nuclear DNA of the host cell (13). Hence, the eukaryotic genome is chimeric, with both archaeal and bacterial ancestry (13, 30). Interestingly, the function of eukaryotic genes also shows a strong correlation with their evolutionary origins (16). Genes responsible for informational processes, such as nuclear DNA replication, transcription, and translation are most closely related to archaeal homologs, whereas operational genes that govern

cellular and energy metabolism, are more closely related to genes of eubacterial origin (16).

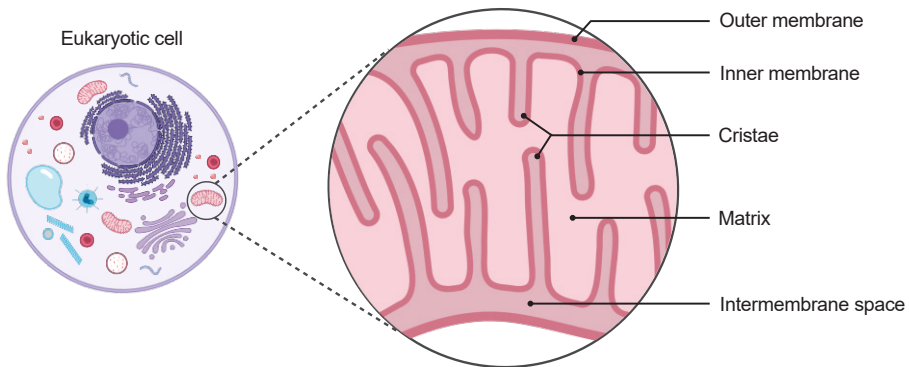


Figure 1. The characteristic double-membrane structure of mitochondria, showing the outer membrane, inner membrane with cristae, and the matrix. Created with BioRender.com.

1.1.2 Architecture of mitochondria

Mitochondria are characterized by a special double-membrane structure (Figure 1), composed of two phospholipid bilayers, which is essential for their role in cellular energy metabolism (31). The outer mitochondrial membrane (OMM) is relatively permeable and allows transport of ions, metabolites, and other small molecules, via channels created by porins, into the intermembrane space (IMS) (32, 33). In contrast, the inner mitochondrial membrane (IMM) is highly impermeable and folded into unique structures called cristae (24, 33). Since most mitochondrial proteins are nuclear-encoded (>1100 proteins) (34), these proteins must be actively transported across the membranes via dedicated translocases, mainly the TOM and TIM23 complexes (35). To ensure import, mitochondrial proteins are tagged with mitochondrial targeting sequences (MTSs). These sequences are often found in the N-terminal and typically cleaved upon import (31, 35). At the core of mitochondria is the matrix, which is where many of the mitochondrial functions take place (24, 35).

1.1.3 Mitochondrial functions

As a testament to their aerobic eubacterial origin, the main function of mitochondria is their role in energy production by generating adenosine triphosphate (ATP) through aerobic respiration via the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS) (24, 36). OXPHOS is the most efficient cellular pathway for ATP production, generating approximately 30 ATP per glucose molecule (37). This process relies on mitochondria's double-membrane structure, where the ETC complexes (I-IV) are embedded in the cristae structures in the IMM (24, 38). The ETC actively pumps protons from the mitochondrial matrix into the IMS, thus creating an electrochemical and pH gradient across the IMM. This proton motive force is what provides the energy required for ATP synthase to convert ADP and inorganic phosphate into ATP (36, 39).

The main mechanism for OXPHOS is the stepwise transfer of electrons in the ETC, which are obtained from the two electron donors NADH and FADH₂ (36). These are generated mainly through glycolysis and β -oxidation of fatty acids, either directly or indirectly via the formation of acetyl-CoA, which feeds the citric acid cycle to produce additional NADH and FADH₂ (24). In the ETC, Complex I and Complex II serve as the entry points for the electrons by oxidizing NADH or FADH₂, respectively (24). While Complex I actively pumps protons across the inner IMM, Complex II does not contribute to the proton gradient (24). Electrons from Complexes I and II are then transferred via ubiquinone (CoQ) to Complex III, which also translocates protons to the IMS (36). From there, electrons are passed to cytochrome c, a mobile electron carrier that shuttles them to Complex IV (36). Complex IV transfers the electrons to molecular oxygen, which acts as the final electron acceptor, and pumps additional protons into the IMM (36). The buildup of potential energy, caused by the proton gradient across the IMM, is harnessed by Complex V (ATP synthase) and

used to synthesize ATP from ADP and inorganic phosphate, as protons flow back into the mitochondrial matrix (Figure 2) (24, 36, 39).

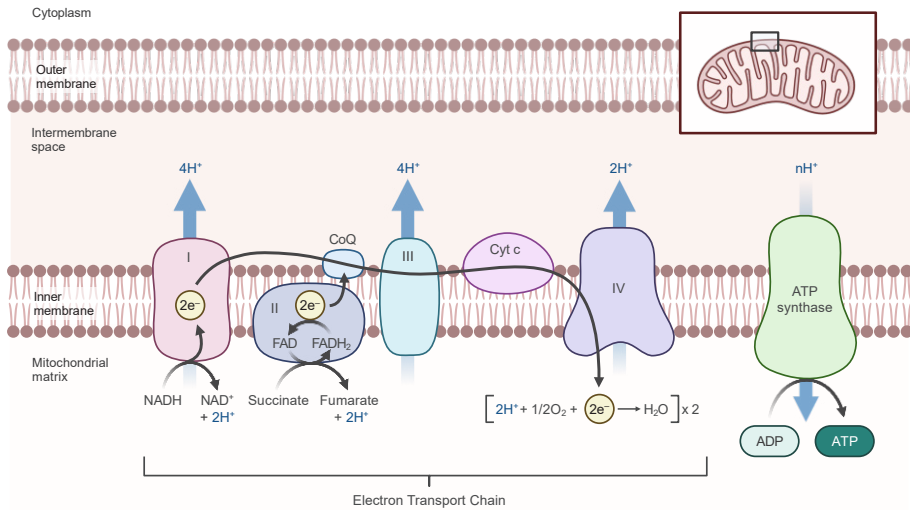


Figure 2. Schematic illustration of the OXPHOS system, showing the electron transport chain complexes (I–IV) and ATP synthase in the inner mitochondrial membrane. Created with BioRender.com.

In addition to their role in OXPHOS, mitochondria perform several other essential functions, most of which occur in the mitochondrial matrix. These include the citric acid cycle, β -oxidation of fatty acids, and amino acid metabolism (24). Mitochondria also act as central regulators of apoptosis, releasing pro-apoptotic factors such as cytochrome c, which initiate programmed cell death pathways (40). Furthermore, mitochondria contribute to the biosynthesis of key biomolecules, including steroids, heme, and iron-sulfur clusters (41–43). They also play a role in managing reactive oxygen species (ROS) (44). The mitochondrial matrix also contains mitochondrial DNA (mtDNA), and serves as the site for mtDNA replication, transcription, and translation, enabling the synthesis of essential mitochondrial proteins (45, 46).

1.2 MITOCHONDRIAL DNA

The mitochondrial genome is a small circular DNA molecule located within the mitochondrial matrix, and each mitochondrion contains multiple copies of this DNA (24, 47, 48). The number of mitochondria per cell varies depending on the energy demands of different cell types but ranges between 100-2000 in most cells (49). Kidney, heart, skeletal muscle, and nerve cells have high mitochondrial content to meet their energy requirements, while red blood cells completely lack mitochondria (49). As a result of the varying abundance of mitochondria, the copy number of mtDNA per cell also varies, typically ranging from 100 to 6,000 copies (50). Importantly, mtDNA is strictly maternally inherited (51), which is due to oocytes containing an exceptionally high number of mtDNA copies, exceeding 100,000 per cell (52), and the active degradation of paternal mitochondria and mtDNA following fertilization (53).

1.2.1 The mitochondrial genome

In humans, the mtDNA is only 16,569 bp long and encodes 37 genes, including 13 protein-coding genes, 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (rRNAs) (47). The compact nature of mtDNA is a consequence of the endosymbiotic evolution of mitochondria, where all other mitochondrial proteins have become nuclear encoded and must be transferred into the mitochondria (34). The 13 mitochondria-encoded proteins are all essential components of the OXPHOS system, encoding for seven Complex I subunits (*ND1-6*, *ND4L*), one Complex III subunit (*CYTB*), three Complex IV subunits (*COX1-3*), and two Complex V subunits (*ATP6*, *ATP8*) (47, 54). The reason why these genes must be expressed within mitochondria is not fully understood, but one plausible explanation is that these proteins are highly hydrophobic and cannot be imported from the cytosol (55). mtDNA has also retained the genes for the

tRNAs and the 12S and 16S rRNAs required for translation of mitochondrial mRNAs inside mitochondria (46, 47).

1.2.2 mtDNA organization

The two strands of mtDNA are commonly referred to as the heavy (H) and light (L) strands. This designation is derived from their differing nucleotide compositions, with the H-strand being more guanine-rich and thus heavier than the L-strand (47, 56). In mtDNA, the distribution of the 37 genes is asymmetric on the two strands (Figure 3), with the H-strand encoding 12 proteins, two rRNAs, and 14 tRNAs, while the L-strand encodes only one protein and eight tRNAs (47). Furthermore, most of the protein and rRNA genes in mtDNA are flanked by the tRNA sequences (47). Since mtDNA transcription produces polycistronic mRNA, excision of these tRNA sequences also serves as an essential processing step to release the individual mRNA transcripts (57). Afterwards, the mRNAs are polyadenylated, a modification which is required in some of the transcripts to create a stop codon necessary for correct translation (47, 57).

Most regulatory elements in mtDNA are clustered in a location termed the non-coding region (NCR) (Figure 3), including the promoters for each strand (H-strand promoter; HSP, and L-strand promoter; LSP), three conserved sequence blocks (CSBI–III), the origin of replication for the H-strand (OriH), and a termination-associated sequence (TAS) (58, 59). Recently, a second light-strand promoter (LSP2) has been identified in the NCR, although its operational significance remains unclear (60). The only functional element not found in the NCR is the origin of replication for the L-strand (OriL), which instead is located in the center of a tRNA gene-cluster (47). Transcription from the LSP is particularly important, as it also generates the RNA primer used for mtDNA replication (61, 62). This mechanism involves premature transcription termination caused by the

formation of an RNA G-quadruplex near the CSBII element (63). An RNA-DNA hybrid, known as the R-loop, is formed during these termination events and it is processed by RNase H1 into the active primer for replication (64-66). Another premature termination event occurs downstream of CSBI and produces a non-coding polyadenylated transcript

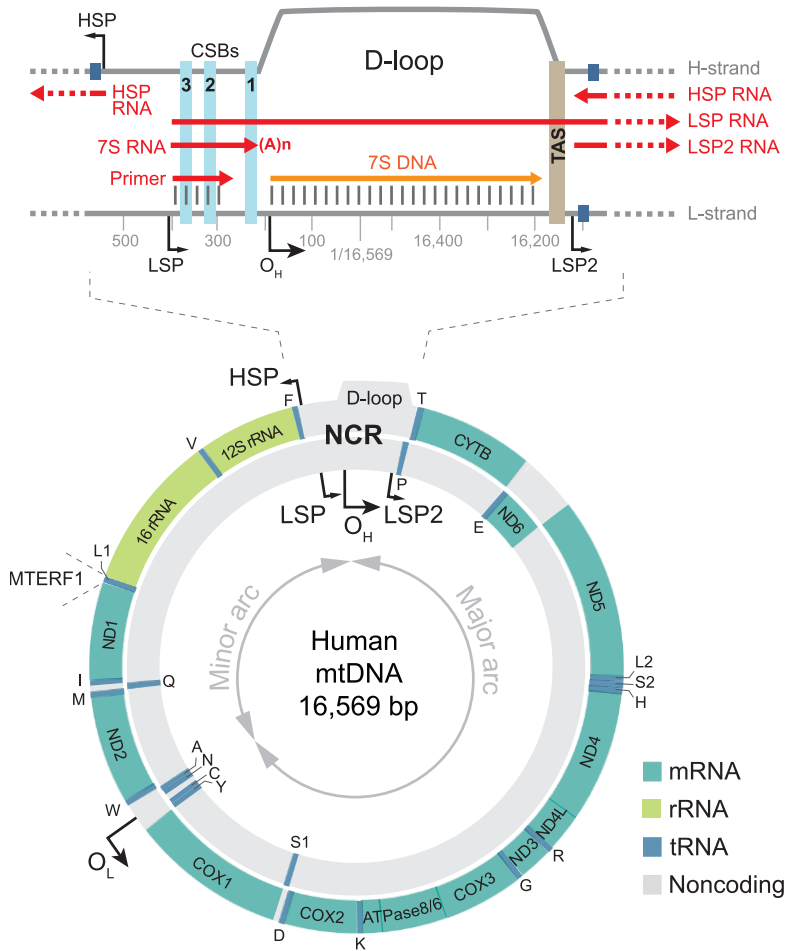


Figure 3. Schematic overview of the human mtDNA and a close-up of the non-coding region (NCR), showing promoters (LSP, LSP2, HSP), conserved sequence blocks (CSBs), and the D-loop region. Adapted from (45) with permission from Jennifer Uhler. Licensed under CC BY 4.0 (<http://creativecommons.org/licenses/by/4.0/>).

known as 7S RNA (45, 61). Interestingly, 7SRNA regulates transcription via a negative feedback loop, since it induces the RNA polymerase to dimerize, thus inactivating it (67). Premature termination also occurs during mtDNA replication, which results in a ~650 nt long DNA molecule called 7S DNA, which remains bound to the L-strand and creates a triple-stranded displacement loop (D-loop) (45). The function of the D-loop is poorly understood but is believed to play a role in regulating full-length mtDNA replication (45, 68).

1.3 MITOCHONDRIAL DNA REPLICATION

Accurate mtDNA maintenance is crucial for mitochondrial function, as mtDNA encodes key subunits of the respiratory chain complexes, and several replication factors are required to ensure correct mtDNA replication. These components, along with the widely accepted strand-displacement model of mtDNA replication, will be described in this section.

1.3.1 DNA polymerase γ

The mitochondrial DNA polymerase (POL γ) is the sole DNA polymerase responsible for replicating mtDNA in mammalian cells, synthesizing both strands of mtDNA (69). The enzyme consists of a heterotrimeric complex composed of a single ~140 kDa catalytic subunit (POL γ A) and two ~55 kDa accessory subunits (POL γ B) (45). POL γ is discussed in detail in Section 1.4.

1.3.2 TWINKLE helicase

TWINKLE is the primary replicative helicase in mammalian mitochondria and is functionally related to the T7 bacteriophage helicase-primase gp4 (70, 71). Despite this evolutionary relationship, TWINKLE has lost the primase activity found in gp4 (72), and primer synthesis is instead performed by POLRMT (61, 73, 74). The helicase operates in a 5' to 3' direction and needs

to hydrolyze ATP to unwind dsDNA (71, 75, 76). Structurally, TWINKLE is a ~70 kDa protein that assembles into hexameric rings, and can adopt either open or closed conformations, depending on ATP binding (77). While the hexamer is the catalytically active form, TWINKLE can also form heptamers (78, 79). Notably, many disease-related mutations have been shown to induce various oligomeric states, highlighting the importance of correct oligomerization for the function of TWINKLE (77).

1.3.3 Mitochondrial single-stranded DNA-binding protein

Unlike the other main factors in the mtDNA maintenance machinery, the mitochondrial single-stranded DNA-binding protein (mtSSB) is not structurally similar to its bacteriophage T7 counterpart, but instead homologous to the *Escherichia coli* SSB protein (80, 81). It functions as a tetramer, with each subunit approximately 16 kDa in size, and as the name suggests, mtSSB plays a role in binding single-stranded DNA (ssDNA) during mtDNA replication (82). mtSSB coats the displaced H-strand during mtDNA synthesis, thus preventing unspecific priming by POLRMT, formation of secondary structures, and reannealing with the template strand (83). This activity of mtSSB stimulates both the processivity of POL γ and the helicase activity of TWINKLE, ensuring efficient DNA synthesis (71, 76). Recently, disease-causing mutations in the *SSBPI* gene, which encodes mtSSB, have also been identified in patients with mitochondrial DNA deletions, further underlining the crucial role of mtSSB in maintaining mtDNA integrity (84, 85).

1.3.4 POLRMT

The human mitochondrial RNA polymerase (POLRMT) is a ~140 kDa protein structurally similar to the T7 bacteriophage RNA polymerase (RNAP), particularly in the C-terminal polymerase domain which harbors the catalytic core (45, 86). POLRMT is responsible for transcribing both the

H-strand and L-strand of mtDNA, initiating transcription at defined promoter regions called the H-strand promoter (HSP) or the L-strand promoter (LSP) (59). POLRMT requires two additional factors to initiate transcription at these promoters called mitochondrial transcription factors A and B2 (TFAM, TFB2M) (45).

POLRMT is also essential for mtDNA replication as it produces the primers used by POL γ to initiate replication at the two mtDNA origins of replication (45). At the origin of H-strand replication (OriH), LSP transcripts are used to prime replication (61), and depletion of POLRMT leads to reduction in 7S DNA and mtDNA levels (87). In contrast, there is no promoter sequence at the origin of L-strand replication (OriL), however, POLRMT can independently initiate transcription and produce short transcripts on ssDNA using ATP as the initiating nucleotide (74). As the ssDNA loop, in the OriL hairpin structure, consists of a Poly(dT) sequence it can therefore serve as a priming site for POLRMT (73, 88), thus enabling L-strand DNA synthesis. Recently, disease-causing mutations in POLRMT have been identified, leading to impaired mitochondrial transcription and secondary mtDNA depletion, further highlighting the essential role of POLRMT in maintaining mtDNA integrity (89, 90).

1.3.5 Other mtDNA maintenance factors

In addition to the core components of the mitochondrial replisome, several additional factors are required to ensure proper maintenance of mtDNA. One such factor is MGME1, a mitochondrial nuclease implicated in processing 7S DNA and removing displaced flap structures (91, 92), thereby facilitating successful ligation of the newly synthesized strand by DNA ligase III (93). RNaseH1 degrades RNA strands hybridized to DNA and plays an essential role in processing the R-loop into the primer used to initiate mtDNA replication at OriH (65). MTERF1, binds to a specific DNA

sequence located just downstream of the 16S rRNA gene (Figure 3), serving as a termination site for transcription initiated from LSP. It may also act as a replication fork pause site, possibly preventing collisions between transcription and replication machineries as they traverse the mitochondrial genome (94, 95). Mitochondrial topoisomerases such as TOP1MT and TOP3A are required to resolve DNA topological stress during replication, with TOP3A being specifically needed for decatenation of newly replicated daughter molecules at the termination of mtDNA replication (96, 97). Furthermore, TFAM, a transcription factor essential for mitochondrial transcription initiation, regulates both transcription and replication by promoting primer formation and by compacting mtDNA into mitochondrial nucleoids (98, 99).

1.3.6 Strand-displacement model

The mechanism of mtDNA replication is generally believed to follow the strand-displacement model (Figure 4), in which replication is initiated at the OriH, and DNA synthesis of the nascent H-strand is carried out by POL γ (45, 69, 100, 101). As POL γ has limited strand-displacement ability, the TWINKLE helicase is required to unwind duplex DNA ahead of the polymerase (71). The DNA synthesis then proceeds continuously, while the displaced parental H-strand is coated and stabilized by mtSSB (71, 83). When replication has advanced approximately two thirds of the genome (~11 kb), the OriL becomes single-stranded and folds into a conserved hairpin structure, composed of a GC-rich stem and a 12 nt loop containing a T-stretch (73, 88). This configuration prevents mtSSB binding and provides a recognition site for POLRMT, which synthesizes a short RNA primer of about 25 nt (73, 101). POL γ then replaces POLRMT at the primer terminus to initiate L-strand synthesis (73). Thus, in contrast to H-strand synthesis, which requires TWINKLE to unwind duplex DNA ahead of the fork, L-strand synthesis uses the already displaced parental H-strand

as a template and proceeds without TWINKLE. Replication of both strands continues until two complete daughter molecules are produced, notably without the formation of Okazaki fragments (45). The strand-displacement model is supported by a plethora of data in the form of *in vitro* reconstitution (73, 74), single-molecule analyses (102), mutagenesis studies in mice (88), and genome-wide mapping of mtSSB occupancy *in vivo* (83).

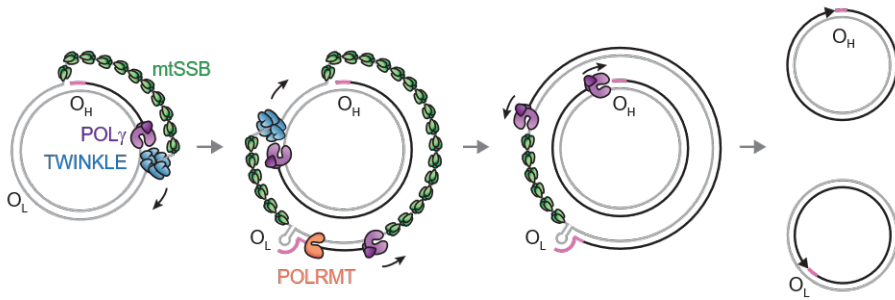


Figure 4. Schematic representation of the strand-displacement model of mtDNA replication, showing leading-strand synthesis initiated at OriH (O_H), followed by delayed initiation at OriL (O_L). Adapted from (103) with permission from Jennifer Ubler. Licensed under CC BY-NC-ND 4.0 (<https://creativecommons.org/licenses/by-nc-nd/4.0/>).

1.4 FUNCTION AND STRUCTURE OF POL γ

As the sole DNA polymerase responsible for replication of mtDNA, POL γ is essential for maintaining the mitochondrial genome (45, 69). POL γ forms a heterotrimeric complex (Figure 5), consisting of one catalytic subunit (POL γ A, encoded by *POLG*) and a homodimer of accessory subunits (POL γ B, encoded by *POLG2*) (45, 104). The catalytic subunit performs the polymerase and exonuclease activities required for DNA synthesis and proofreading, while the accessory subunits enhance DNA binding affinity and replication rate (104, 105). Complete loss of either POL γ A or POL γ B results in embryonic lethality in mice (106, 107), highlighting their essential

roles in early development and the critical requirement of mitochondrial genome maintenance.

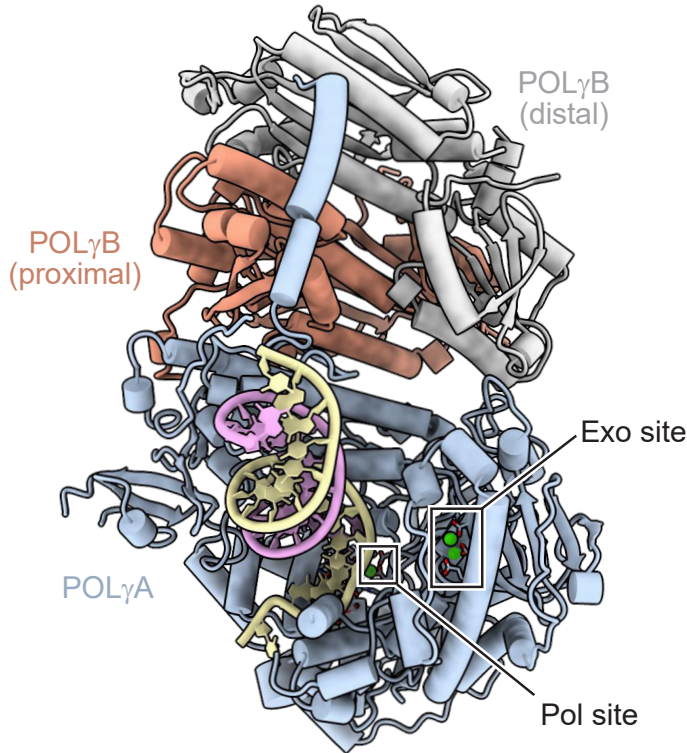


Figure 5. Overview of POL γ with DNA bound to the catalytic Pol site (PDB: 9SA0, Paper III). The proximal and distal POL γ B subunits are shown interacting with the POL γ A-DNA complex.

1.4.1 POL γ A

The catalytic subunit, POL γ A, belongs to the family A DNA polymerases, and shares ancestry with bacterial DNA polymerase I (Pol I), as well as bacteriophage polymerases such as the T7 DNA polymerase (also known as gene 5 protein, gp5) (108, 109). Although the sequences of these polymerases have diverged during evolution, they have retained the

canonical “right-hand” architecture characteristic of family A polymerases (Figure 6), composed of palm, fingers, and thumb subdomains that coordinate DNA synthesis and nucleotide selection (110, 111). They also contain an exonuclease domain that enables proofreading of misincorporated nucleotides (109). Consequently, POL γ A, Pol I, and gp5 each possess both the 5′–3′ polymerase and 3′–5′ exonuclease proofreading activities, which together ensure high-fidelity DNA synthesis (109, 112).

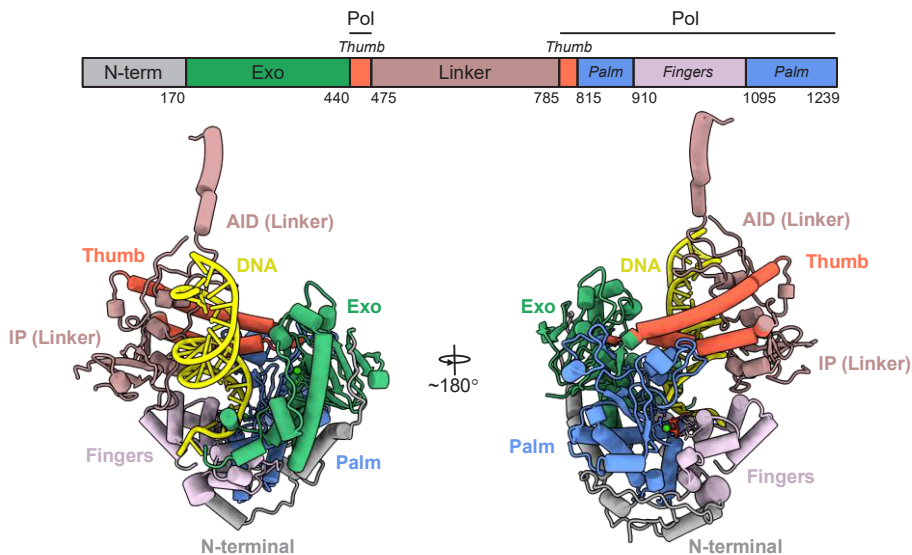


Figure 6. Schematic representation and structure of the different domains in the catalytic subunit POL γ A, showing the N-terminal, exonuclease (Exo), linker, and polymerase (Pol) domains bound to DNA (PDB: 9SA0, Paper III).

A key distinction between POL γ A and other family A polymerases is the presence of a unique linker region of approximately 400 residues, which contains two specialized subdomains; the intrinsic processivity (IP) subdomain and the accessory-interacting determinant (AID) subdomain (Figure 6) (113). Both contribute to stabilizing POL γ A’s interaction with DNA, but the AID subdomain’s function is dependent on binding to the accessory subunit, POL γ B (113). In the absence of POL γ B, the AID

subdomain remains flexible and becomes a target for degradation by the mitochondrial protease LONP1 (114). Furthermore, POL γ A shares strong structural similarity with gp5, and both rely on accessory factors to enhance processivity (POL γ B and thioredoxin, respectively). However, gp5 lacks the IP subdomain and contains a thioredoxin-binding loop in place of the AID subdomain (113).

1.4.2 POL γ B

POL γ B forms a stable homodimer that binds to the catalytic subunit (Figure 5), to create the fully functional enzyme complex (113, 115, 116). POL γ B plays a crucial stabilizing role, enhancing DNA binding affinity, processivity, and overall replication efficiency (105, 113). Each POL γ B monomer contributes differently to this function, where the subunit closest to POL γ A (proximal subunit) stabilizes interactions with the DNA, while the other (distal subunit) mainly interacts with POL γ A during elongation to stimulate the polymerization rate (117). POL γ B is also capable of binding dsDNA independently of POL γ A, and this activity has been shown to be important for replisome function (118, 119). The mechanism and function of POL γ B dsDNA binding is not fully understood, however, POL γ B can form a trimer of dimers when interacting with dsDNA, which targets forked DNA structures resembling the D-loop (120). Structurally, POL γ B resembles class IIa aminoacyl-tRNA synthetases, and is thus not homologous to thioredoxin, which acts as a processivity factor for gp5 in T7 DNA replication (115, 121). Interestingly, not all eukaryotes possess the accessory subunit in dimeric form. For example, *Saccharomyces cerevisiae* lacks POL γ B entirely (122), while *Drosophila melanogaster* has only a monomeric form of the subunit (123).

1.4.3 Polymerization activity

To synthesize DNA, POL γ functions through the coordinated action of its three conserved polymerase subdomains (palm, fingers, and thumb) (111, 113). The palm subdomain houses the catalytic core, containing conserved acidic residues that coordinate two divalent magnesium ions (Mg^{2+}) to catalyze phosphodiester bond formation in the catalytic polymerase (Pol) site (111, 124). The fingers subdomain is responsible for nucleotide selection and positioning and undergoes a conformational “closing” movement upon correct base pairing, aligning the incoming dNTP with the template strand near the primer terminus (125, 126). This mechanism enhances substrate specificity and minimizes misincorporation (126). The thumb subdomain stabilizes binding of the DNA duplex during the replication process (113).

The two-metal-ion mechanism is a conserved catalytic strategy used by DNA and RNA polymerases, including family A polymerases (Figure 7) (127-130). In this configuration, the active site contains two divalent metal ions, typically magnesium ions coordinated by conserved acidic residues (D890 and D1135 in POL γ A) located within the palm domain (124, 127). The two metal ions have different functions, where metal ion A has the primary catalytic role by being positioned near the 3'-hydroxyl group of the primer terminus (127). It lowers the pK_a of this hydroxyl group, enabling it to perform a nucleophilic attack on the α -phosphate of the incoming dNTP (127). Metal ion B has a stabilizing role by interacting with the β - and γ -phosphates of the incoming dNTP, stabilizing the transition state and the leaving pyrophosphate group (127, 131).

In addition to the catalytic aspartates, several other residues contribute to the organization of the polymerase active site, including E895, R943, K947,

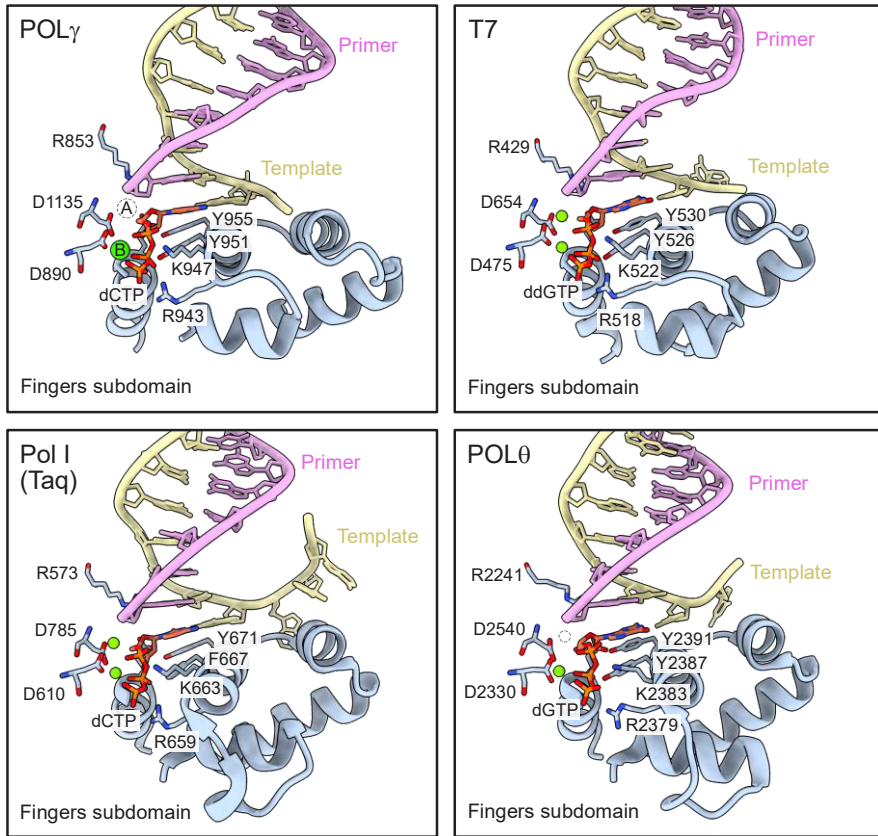


Figure 7. Comparison of the catalytic Pol site in Family A DNA polymerases, highlighting the well-conserved architecture of the two-metal-ion mechanism. The A-metal is missing in the POL γ and POL θ structures as Ca²⁺ ions or ddATP was used, respectively, which prevents metal-ion coordination and stalls the polymerase. POL γ (PDB: 9SA0) (Paper III), T7 DNA polymerase (PDB: 1T7P) (132), *Thermus aquaticus* (Taq) Pol I (PDB: 3RTV) (133), and human POL θ (PDB: 9AU5) (134).

Y951, and Y955, all of which participate in coordinating the incoming dNTP (124, 135). Among these, the Y951 residue has been implicated as a steric gate, which plays a key role in ribonucleotide discrimination by blocking the ribose 2'-hydroxy group (135, 136). The Y951 residue also contributes to relaxed discrimination against dideoxynucleotides (ddNTPs), while replacing Y951 with phenylalanine restores effective

exclusion of ddNTPs without affecting the enzyme activity (137). Similarly, Pol I strongly exclude ddNTPs due to the presence of phenylalanine at the corresponding position, whereas substitution with tyrosine abolishes this selectivity (138).

1.4.4 Exonuclease activity

POL γ possesses an intrinsic 3'–5' exonuclease activity to maintain high-fidelity mtDNA replication by excising misincorporated nucleotides (45). However, the exonuclease (Exo) site is located approximately 32 Å away from the Pol site, requiring the primer terminus to be repositioned once a mismatch is detected (139). During this proofreading process, the three terminal nucleotides of the primer strand are unwound and repositioned into the Exo site, where the incorrect base is removed (139). The active site contains conserved acidic residues (D198, E200, D274, and D399) which coordinate two divalent metal ions, typically Mg²⁺, and function through a two-metal-ion catalytic mechanism similar to the one employed in the Pol site (127, 129-131, 139). In the Exo site, metal A activates a water molecule, generating a hydroxide ion that attacks the phosphodiester bond of the terminal nucleotide, while metal B stabilizes the negatively charged transition state and promotes release of the excised product (130, 131). The mechanism and coordination of metal ions by catalytic residues are conserved in many family A polymerases, including the gp5 and the Klenow fragment of Pol I (Figure 8) (109, 127, 129-131).

The exonuclease activity of POL γ can be decreased or completely inactivated by substituting one or several of the catalytic residues to alanines, as this affects the coordination of the metal ions (139-142). The importance of POL γ 's exonuclease activity has been highlighted in a study where exonuclease-deficient (Exo⁻) POL γ (D257A, corresponding to D274A in human) resulted in a drastic increase in point mutations and caused

premature aging phenotypes in mice (143). The exonuclease function is also essential for the formation of ligatable ends during mtDNA replication, as Exo⁻ POL γ (D274A) exhibits enhanced strand-displacement (144). This activity produces unligatable 5' termini, which results in the formation of linear mtDNA fragments in POL γ Exo⁻ mice (143, 144).

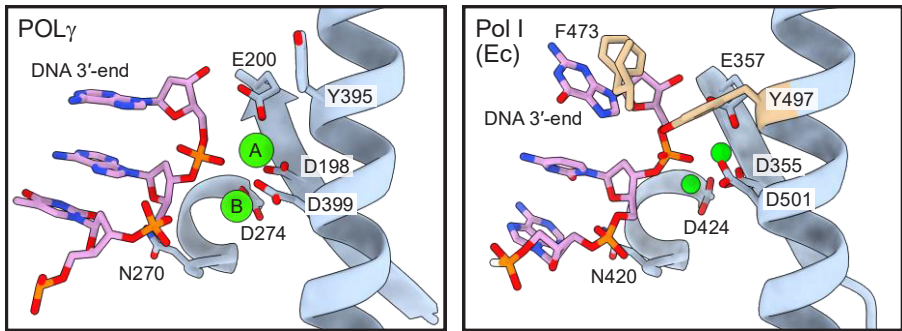


Figure 8. Comparison of the catalytic Exo site in POL γ (PDB: 9SB8) (Paper III) and *Escherichia coli* (Ec) Pol I (PDB: 1KFS) (145). The catalytic residues are well-conserved, however, in Pol I additional residues (F473 and Y497) contribute to the coordination of the DNA.

1.4.5 The balance between Pol and Exo activities

Effective replication of mtDNA by POL γ relies on the coordinated action of its polymerase and exonuclease activities to maintain both efficiency and fidelity (112). In single-nucleotide incorporation experiments, the misincorporation rate of POL γ has been estimated to be about 1 in 440,000 incorporated nucleotides, whereas the exonuclease activity increases the fidelity to 1 in $1.8\text{--}37 \times 10^6$ incorporations (112, 140, 146). However, these experiments may overestimate the *in vivo* fidelity as they do not catch the behavior of POL γ when replicating longer stretches of DNA. In another study where primed M13mp2 ssDNA was used as a template, the mutation frequency has been estimated to be 1 in $1 \times 10^5\text{--}10^6$, indicating that the fidelity of POL γ is lower on longer DNA stretches (147).

The balance between Pol and Exo activities in polymerases is not only dependent on detection of mismatches, but also on dNTP concentration (148). High dNTP levels favor the polymerization reaction as substrate will be readily available. However, low dNTP levels or complete absence of dNTPs will lead to an increase in exonuclease activity, even without of a mismatch at the 3' terminus (148). The Exo site cannot distinguish between correctly or incorrectly paired nucleotides and will excise either if positioned in the active site (146, 149). Thus, the decision to initiate excision is determined at the Pol site, where low dNTP levels increase the likelihood that the polymerase shifts the primer terminus to the Exo site, as less substrate is available (148, 149). The leading model to explain how polymerases selectively remove incorrect nucleotides, while preserving correctly paired ones, is the kinetic partitioning model (149, 150). According to this model, a mismatched primer terminus is more likely to be redirected to the Exo site due to stalling in the Pol site caused by the unfavorable geometry of the mismatch (149, 150). This increases the time window for the polymerase to shuttle the primer terminus to the Exo site. Correctly paired nucleotides do not cause such stalling and are therefore more rarely excised (149, 150).

Based on the kinetic partition model it is possible to calculate the “cost of proofreading”, which is a measure of the energetic expense associated with exonuclease activity. This cost reflects how often a polymerase excises a correctly incorporated nucleotide, thereby wasting energy (151). In single-nucleotide incorporation experiments, the cost of proofreading has been estimated to be about 0.08% for gp5 and 0.14% for POL γ (146, 149). However, several studies have demonstrated that proofreading polymerases remove correctly incorporated nucleotides far more frequently than these estimates suggest (151-153). For gp5, excision of nucleotides has been shown to be between 3–20% of the total incorporation events (152, 153).

Similar behavior has been observed for *Escherichia coli* Pol III and *Saccharomyces cerevisiae* Mip1 (mitochondrial DNA polymerase in yeast) (151, 153). This shows that polymerases transfer the DNA to the Exo site more often than required, since these excision events occur several orders of magnitude more frequently than the actual misincorporation rate of these polymerases (154-156). The reason for this excessive removal of nucleotides is not fully understood, however, in the absence of the T7 single-stranded DNA binding protein (gp2.5), gp5 experience an increase in excision rate (153). This observation is likely due to the formation of secondary structures in the DNA, which in turn causes stalling events and promotes shuttling from Pol to Exo site (149, 153).

1.5 MITOCHONDRIAL DISORDERS

Mitochondrial disorders comprise a diverse group of diseases that typically develop as a result from defects in mitochondrial ATP production (157). These disorders can result either from pathogenic variants in nuclear genes encoding mtDNA replication and maintenance factors, or from mutations directly affecting mtDNA itself (157-159). A common underlying cause is mitochondrial DNA depletion syndrome (MDDS), which encompasses a group of inherited disorders characterized by a severe reduction in mtDNA copy number, leading to impaired oxidative phosphorylation and energy deficiency in high-demand tissues such as liver, muscle, and brain (157, 159-161). MDDS is caused by defective mtDNA maintenance, involving abnormalities in the replication machinery, dNTP metabolism, or related mitochondrial processes (157, 159, 162). Consequently, pathogenic variants in genes encoding the core mtDNA replication factors, including POL γ , TWINKLE, and mtSSB, have been directly linked to human disease (84, 158, 163). These defects disrupt replication fidelity and genome stability, leading to mtDNA depletion, multiple deletions, or accumulation of point

mutations, which in turn compromise respiratory chain and ATP synthase functions (158, 162). Clinically, such replication-related disorders encompass a wide spectrum of phenotypes ranging from progressive external ophthalmoplegia (PEO) and Alpers-Huttenlocher syndrome to IOSCA (infantile-onset spinocerebellar ataxia) and MNGIE (mitochondrial neurogastrointestinal encephalomyopathy) (157). Other conditions include MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes), SANDO (sensory ataxic neuropathy, dysarthria, and ophthalmoparesis), MIRAS (mitochondrial recessive ataxia syndrome), and MEMSA (myoclonic epilepsy myopathy sensory ataxia) (157).

While nuclear gene defects cause secondary mtDNA abnormalities, primary mtDNA mutations represent a distinct group of disorders directly affecting the mitochondrial genome itself. These mutations are subject to heteroplasmy, meaning that mutated and wild-type genomes may coexist within the same cell (164). The proportion of mutant mtDNA must surpass a critical threshold to clinically manifest, and the threshold varies depending on the nature of the mutation as well as the energetic requirements of the affected tissue (164). The threshold effect is not fixed and can differ among individuals carrying the same mutation but also between different tissues within a single patient, thus contributing to the variable onset, progression, and severity of mitochondrial disorders with rarely any genotype-phenotype correlations (157, 164). Because of their wide variability in clinical presentation, mitochondrial disorders are challenging to diagnose. Nevertheless, the prevalence of adult mitochondrial disease is estimated to be approximately 1 in 4,300 individuals (165).

1.5.1 POLG-related disorders

Mutations in *POLG*, the gene encoding the catalytic subunit of POL γ , are among the most common causes of inherited mitochondrial disease, with more than 300 pathogenic variants described to date (162). These mutations compromise POL γ function, resulting in mtDNA depletion and/or multiple deletions in affected individuals (162, 166). Most pathogenic variants are recessive and rare, however, three amino acid substitutions (A467T, W748S, and G848S) account collectively for around 70% of reported cases (167, 168). These variants are thought to be founder mutations of ancient European origin, passed down through descendants of the original carriers and resulting in a current carrier frequency of up to 1%

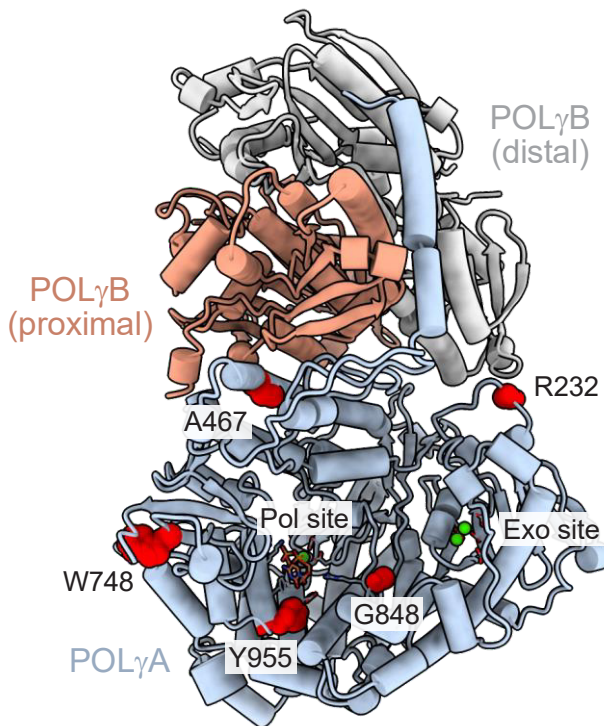


Figure 9. The common disease-related mutations in POL γ are dispersed in different regions and domains of the holoenzyme (PDB: 9SA0) (Paper III).

in certain populations (169). The A467T, W748S, and G848S variants all have impaired polymerase activities, thus resulting in defective mtDNA replication (170-172). Another relatively common recessive mutation, the R232H variant, resides in the exonuclease domain of POL γ , exhibiting both reduced polymerase and increased exonuclease activity (173). The most common dominant *POLG* mutation is the Y955C variant, which is found in the catalytic Pol site where it disrupts nucleotide binding and thus impairs DNA synthesis (Figure 9) (174, 175).

Although the genotype-phenotype relationships are difficult to identify in *POLG*-related disorders (162), there is a correlation between the age of onset and the clinical presentation, where early-onset disease (0–12 years) is the most severe form and characterized by severe mtDNA depletion, leading to rapid neurodegeneration, organ failure and short life expectancy (176). Juvenile to adult-onset forms (12–40 years) results in mtDNA depletion or deletions, leading to progressive neurological dysfunction (176). Late-onset presentations (after 40 years) show milder phenotypes associated primarily with accumulated mtDNA deletions and gradual neuromuscular decline (176). In all forms, patients with epilepsy or compound heterozygous *POLG* variants tend to have a significantly poorer prognosis (176-178).

1.5.2 Nucleotide metabolism disorders

While mutations in *POLG* are the most common genetic cause of MDDS, other genes, such as those involved in mitochondrial nucleotide synthesis, are also susceptible to pathogenic mutations that impair mtDNA maintenance (162, 179). This is because POL γ function is directly dependent on a sufficient and balanced supply of dNTPs within mitochondria (179). Maintaining these nucleotide pools is crucial because POL γ -catalyzed DNA synthesis follows Michaelis-Menten kinetics, meaning that dNTP concentrations directly influence the polymerization

rate and the equilibrium between polymerase and exonuclease activities (105, 149, 180). Mutations in genes involved in mitochondrial nucleotide metabolism, such as *DGUOK* (deoxyguanosine kinase, DGUOK) and *TK2* (thymidine kinase 2, TK2), disrupt the mitochondrial salvage pathways that regenerate dNTPs from deoxynucleosides (181-186). Thus, impaired DGUOK or TK2 functions lead to an imbalance or depletion of the mitochondrial dNTP pools, which in turn compromises DNA synthesis by POL γ and ultimately results in mtDNA depletion (183, 186). These metabolic defects illustrate that MDDS can arise not only from direct impairment of POL γ itself but also from perturbations in the substrate levels required for its activity, tightly linking nucleotide homeostasis to the maintenance of the mitochondrial genome (187). It is worth noting that nucleotides levels naturally vary in different cell types, with actively dividing cells having higher dNTP levels than postmitotic cells (188-190). Mutations in *POLG*, *DGUOK*, or *TK2* are therefore likely to have a more severe effect on postmitotic tissues, and this may explain why mutations causing severe MDDS can be tolerated during embryogenesis and fetal development but cause severe phenotypes after birth (162, 191). Experimental studies have also shown that deoxynucleoside supplementation can restore mitochondrial dNTP balance, enhance mtDNA replication, and partially rescue mtDNA depletion in cells and animal models harboring *POLG*, *DGUOK* or *TK2* mutations (187, 192-196). Furthermore, clinical trials of deoxynucleoside therapy in patients with TK2 deficiency have shown promising results, including improved muscle function and slowed disease progression (195, 197, 198). This therapeutic strategy highlights how maintaining adequate mitochondrial nucleotide pools is essential for POL γ function and overall mitochondrial genome stability (187).

2 AIMS

The overarching aim of this thesis is to elucidate the molecular mechanisms underlying POL γ function and dysfunction. Mutations in *POLG* are among the most common causes of inherited mitochondrial disease, leading to severe defects in energy metabolism for which no effective therapies currently exist. By integrating biochemical, structural, and *in vivo* approaches, this work seeks to:

Paper I: Identify and characterize small molecules capable of restoring or enhancing the enzymatic activity of mutant POL γ , thus offering potential therapeutic strategies for *POLG*-related mitochondrial disease.

Paper II: Characterize key human and mouse *POLG* variants and expand the available mouse models to study *POLG*-related mitochondrial dysfunction.

Paper III: Elucidate the molecular mechanisms underlying allosteric activation of POL γ .

3 METHODS

This chapter presents a general overview of the key experimental methods used. Detailed descriptions of specific procedures are provided in the corresponding papers.

3.1 BIOCHEMICAL ASSAYS

3.1.1 Single-strand synthesis assay

The single-strand synthesis assay is a type of primer extension assay used to study DNA synthesis (Figure 10). A radiolabeled primer is annealed to a ssDNA template, such as M13mp18 ssDNA, and mixed with POL γ and mtSSB, allowing POL γ extend the primer into full-length dsDNA (Figure 10) (71). The resulting DNA products are separated and visualized by agarose gel electrophoresis, enabling assessment of polymerase activity and replication efficiency (71). This method is widely used to investigate human POL γ function and to compare the enzymatic performance of wild-type and disease-associated mutant variants (71, 199, 200).

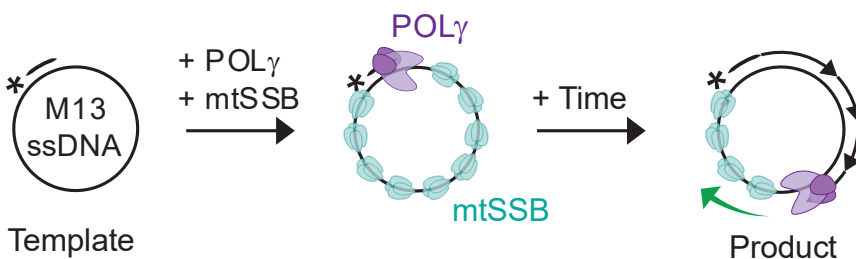


Figure 10. Schematic illustration of the single-strand synthesis assay used to assess POL γ activity. Adapted with permission from Jennifer Uhler and Springer Nature (201). Licensed under CC BY 4.0 (<http://creativecommons.org/licenses/by/4.0/>).

3.1.2 Rolling circle replication assay

The rolling circle assay is an *in vitro* system used to reconstitute and study the mitochondrial replisome and its components (71). In this assay, a circular double-stranded DNA template containing a preformed replication fork serves as the substrate (71). Unlike in the single-strand synthesis assay, POL γ cannot replicate the dsDNA template without the TWINKLE helicase, which unwinds the duplex DNA from the 5' tail (71). POL γ and TWINKLE are stimulated by mtSSB, which coats the displaced strand, and together these proteins assemble into a functional mitochondrial replisome capable of synthesizing DNA fragments exceeding the length of the mitochondrial genome (Figure 11) (71, 76). Moreover, when the DNA template contains the OriL sequence and both POLRMT and ribonucleotides are included, DNA synthesis can also be initiated in the opposite direction, consistent with the strand-displacement model of mtDNA replication (73). By using radiolabeled dNTPs, the DNA synthesis can be studied and visualized via agarose gel electrophoresis (71).

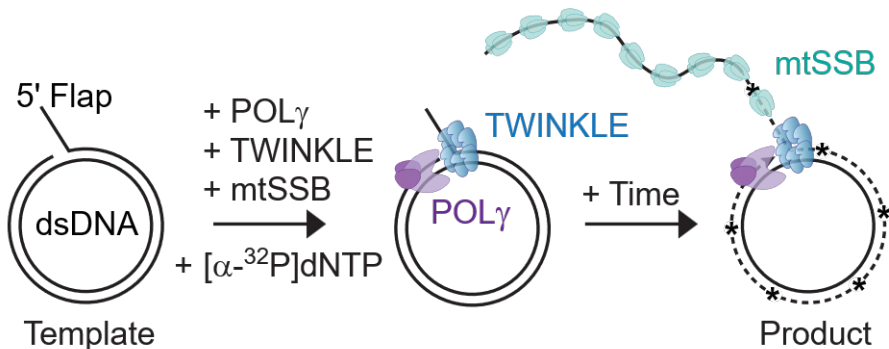


Figure 11. Schematic illustration of the double-stranded (rolling circle) synthesis assay used to analyze the coordinated activity of the mitochondrial replisome. Adapted with permission from Jennifer Ubler and Springer Nature (201). Licensed under CC BY 4.0 (<http://creativecommons.org/licenses/by/4.0/>).

3.1.3 Thermal shift assay

A thermal shift assay (also known as a differential scanning fluorimetry assay) is a biophysical technique used to measure the thermal stability of proteins and to assess how factors such as mutations, cofactors, or ligands affect protein stability (202). In this method, a fluorescent dye, typically SYPRO Orange, is added to a reaction buffer containing the protein of interest. The dye fluoresces upon binding to hydrophobic regions that become exposed as the protein unfolds with increasing temperature. As the sample is gradually heated, the fluorescence signal increases until the protein is fully denatured, generating a characteristic sigmoidal curve (Figure 12) (202). The melting temperature (T_m) is defined as the midpoint of this transition, representing the temperature at which half of the protein population is unfolded. A higher T_m indicates greater thermal stability (202). When comparing protein variants or conditions, the change in melting temperature (ΔT_m) quantifies the stabilizing or destabilizing effect, where

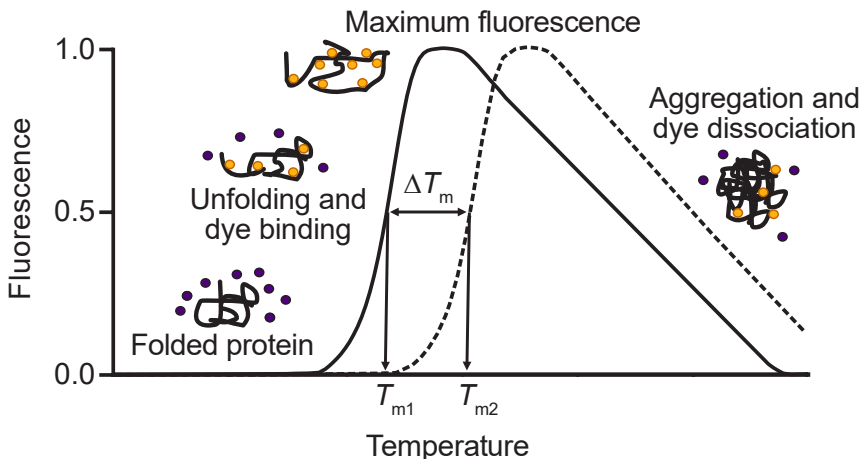


Figure 12. Representative schematic of a thermal shift assay with the same protein in two different conditions.

positive ΔT_m values indicate increased stability, while negative values suggest reduced stability (202, 203).

3.1.4 Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) is a biochemical technique used to study protein-nucleic acid interactions (204). A labeled DNA or RNA template is incubated with a purified protein or protein complex under controlled binding conditions (205). When the protein binds to the nucleic acid, it forms a larger complex that migrates more slowly through a non-denaturing polyacrylamide or agarose gel during electrophoresis compared to the free nucleic acid template (205). The resulting mobility shift is visualized, typically using radioactive or fluorescent detection to indicate binding. By varying the protein concentration, the dissociation constant (K_d) can be determined as a measure of binding affinity (205).

3.1.5 Thin-layer chromatography

Thin-layer chromatography (TLC) is an analytical technique used to separate and quantify small molecules, such as nucleotides, based on their differing affinities for a stationary phase and a mobile solvent (206). In a typical setup to separate nucleotides and DNA, samples are spotted onto a cellulose sheet treated with polyethylenimine (PEI) (the stationary phase) and developed in a solvent system containing LiCl (the mobile phase) (151, 206). As the solvent moves up the plate by capillary action, nucleotides migrate at different rates depending on their polarity and chemical interactions with the stationary phase (206). After development, the separated components can be visualized by UV light or autoradiography (if radiolabeled nucleotides are used) (206).

To study DNA polymerases, TLC can be applied to simultaneously monitor nucleotide incorporation and proofreading activities (151, 207). In

this approach, a single-strand DNA synthesis or rolling circle assay is performed using radiolabeled dNTPs, and the reaction products are subsequently separated by TLC (151). This allows quantification of both nucleotide incorporation and excision events, making it possible to study the balance between polymerization and proofreading (151).

3.2 CELL ASSAYS

3.2.1 mtDNA depletion and recovery in cells

mtDNA depletion and recovery experiments using ethidium bromide (EtBr) are a classical method for studying mtDNA replication dynamics in cells (208). EtBr is a DNA-intercalating agent that preferentially inhibits mtDNA replication over nuclear DNA synthesis. When cells are cultured in low concentrations of EtBr (typically 25–100 ng/mL) over several days, mtDNA is progressively depleted (209). To assess mtDNA recovery, EtBr is removed from the culture medium, and cells are maintained under standard conditions, allowing mitochondria to resume DNA replication (208). The rate of mtDNA repopulation can then be quantified by qPCR or Southern blotting, which reflects the efficiency of the mtDNA replication machinery (208-210).

3.2.2 Mitochondrial stress test (Seahorse XF)

The mitochondrial stress test (Seahorse XF) is a widely used analytical assay designed to evaluate mitochondrial energy production in living cells (211). It measures the oxygen consumption rate (OCR) as an indicator of mitochondrial respiration (212). The assay involves the sequential addition of specific inhibitors and uncouplers of the mitochondrial electron transport chain (211). Oligomycin, an ATP synthase inhibitor, is first added to determine the portion of oxygen consumption coupled to ATP synthesis (211). Subsequently, a chemical uncoupler (FCCP) is added, which

collapses the mitochondrial membrane potential, driving the electron transport chain to its maximal capacity and enabling the calculation of maximal respiration and spare respiratory capacity (211). Finally, rotenone and antimycin A, inhibitors of Complex I and Complex III respectively, completely block mitochondrial respiration, revealing non-mitochondrial oxygen consumption (Figure 13) (211). Collectively, these measurements provide a comprehensive assessment of mitochondrial performance and bioenergetic efficiency. The mitochondrial stress test is particularly valuable for investigating mitochondrial dysfunction, including those arising from *POLG* mutations as these will affect OXPHOS activity.

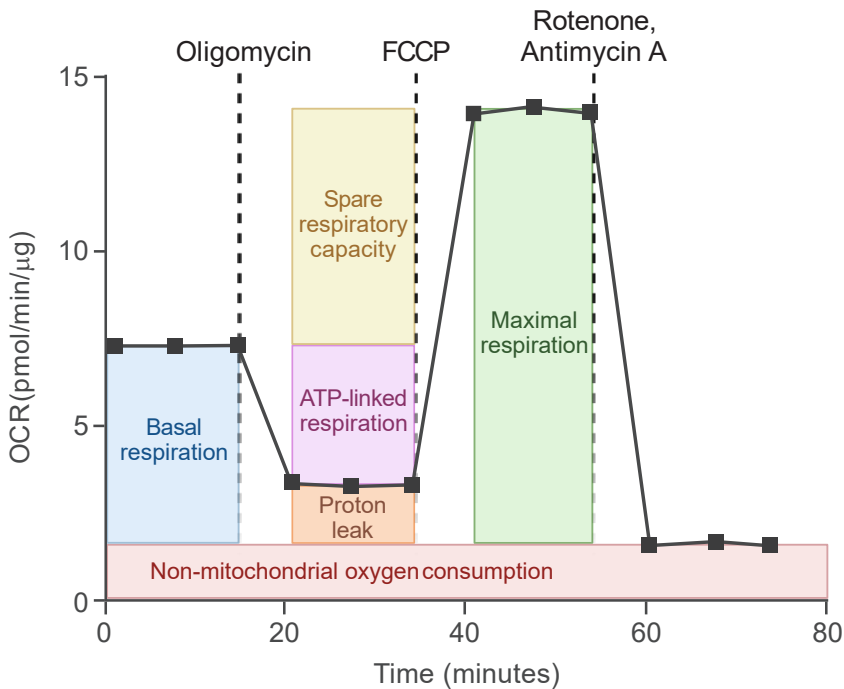


Figure 13. Typical profile of the mitochondrial stress test used to measure oxygen consumption rate (OCR) and assess mitochondrial respiratory function. Created with BioRender.com.

3.3 CRYOGENIC ELECTRON MICROSCOPY

Cryo-electron microscopy (cryo-EM) is a powerful structural biology technique used to visualize macromolecules in their near-native state at high resolution (213). In this method, purified biological samples such as proteins, nucleic acids, or larger complexes are rapidly frozen in a thin layer of vitreous ice, preserving their natural conformation without the need for chemical fixation or crystallization (214). The vitrified samples are then imaged using a transmission electron microscope operating at cryogenic temperatures, where an electron beam passes through the specimen to produce two-dimensional projection images (214). These images are recorded as stacks of movie frames, which contain the particles of interest in random orientations (214). By using sophisticated image-processing algorithms and schemes (single particle analysis), the particles can be

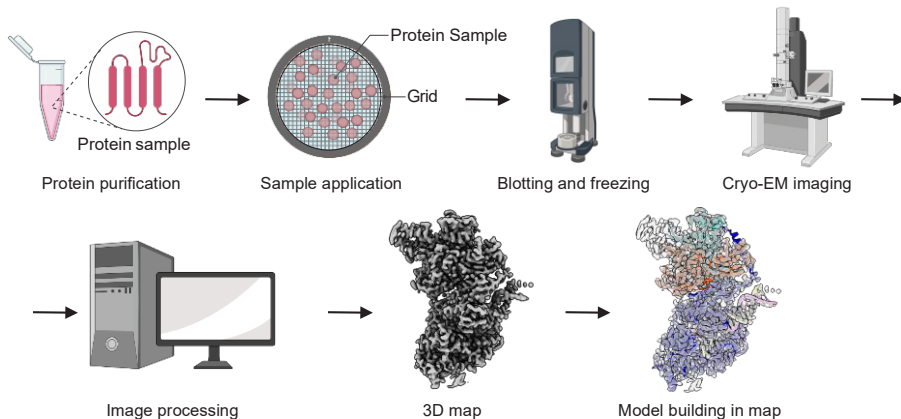


Figure 14. Cryo-EM overview. Samples are purified and applied to a grid, followed by blotting to remove excess liquid, and then immediately plunge-frozen. This process embeds the proteins in a thin layer of vitreous ice, preserving them in a near-native state while allowing the electron beam to pass through the sample. In contrast, crystalline ice can damage the specimen and will obstruct the electron beam. After imaging, the images are processed in multiple steps to generate a 3D map. This map is then used as a guide during model building of the protein. Created with BioRender.com.

computationally aligned and used to reconstruct a three-dimensional (3D) density map (215). This density map makes it possible to build accurate molecular models of the imaged macromolecule (Figure 14) (214).

4 RESULTS & DISCUSSION

4.1 PAPER I

Small molecules restore mutant mitochondrial DNA polymerase activity.

In this paper, we performed a high-throughput screening campaign to identify small molecules capable of stimulating DNA replication in POL γ . The initial screening was carried out against wild-type POL γ , resulting in a single hit with modest effect. This compound underwent repeated medicinal chemistry optimization cycles to develop PZL-A, a compound which showed improved ability to stimulate POL γ . We then picked four common disease-associated *POLG* mutations (R232H, A467T, W748S, and G848S), which all exhibit severely reduced polymerase activity, and investigated if PZL-A could restore their function.

Stimulation could be clearly demonstrated in biochemical assays, where PZL-A enhanced DNA synthesis in all mutant POL γ variants, both over time and across a range of dNTP concentrations. Based on these assays, kinetic analyses demonstrated that PZL-A increases the k_{cat} four- to tenfold. The compound also promoted DNA synthesis in the fully reconstituted mitochondrial replisome (rolling circle assay) and increased polymerase processivity. In addition, PZL-A improved the stability of mutant POL γ complexes bound to DNA, particularly under idling conditions when nucleotide incorporation was limited. Moreover, the compound enhanced the thermal stability of POL γ A in complex with POL γ B, as reflected by higher melting temperatures (T_m), indicating a more stable holoenzyme assembly. Structural studies by cryo-EM revealed that PZL-A binds to an allosteric site between POL γ A and the proximal POL γ B subunit, which

explains the compound's effect on POL γ A melting temperature. Mutation of residues involved in compound binding also eliminated the stabilizing effect, confirming the site of binding.

In cell-based assays, using mtDNA depleted patient-derived fibroblasts and iPSC-derived neural stem cells carrying *POLG* mutations, treatment with PZL-A rescued mtDNA levels and improved respiratory chain complex activity. Stimulation was also observed in quiescent cells, which have lower dNTP levels, indicating that the compound is active in both dividing and postmitotic cells. Importantly, PZL-A did not affect the viability of the cells, indicating that the compound has no toxic effect on the cells.

Collectively, these results establish proof of concept that small-molecule allosteric activation can restore mtDNA replication both *in vitro* and in cells with mutant POL γ . Importantly, PZL-A can stimulate several distinct POL γ mutants, despite each mutation being located in different domains of the protein. These findings provide a novel pharmacological avenue, which offers a potential therapeutic approach for a broad spectrum of *POLG* mutations.

4.2 PAPER II

Modelling *POLG* mutations in mice unravels a critical role of POL γ B in regulating phenotypic severity.

In this study, we characterized the most common recessive and dominant *POLG* mutations (A467T, W748S, G848S, and Y955C) and compared the human variants to the corresponding mouse variants (A449T, W726S, G826S, and Y933C). The DNA replication activities of the mutants were evaluated in both single-strand synthesis and rolling-circle replication assays. Mixed recessive variants were also tested to simulate heterozygous

conditions. All variants, including mixing experiments, displayed reduced polymerase activity, but the effect was milder in the mouse enzymes. In human, the dominant Y955C mutant exhibited the most severe effect, followed by the recessive A467T, G848S, and W748S. The pattern was similar in mouse, except that A449T was slightly better tolerated than G826S.

Structural analysis by cryo-EM of mouse Pol γ revealed overall conservation of the two structures, especially in the replication conformation. However, in the proofreading conformation, there was a noticeable shift in the accessory subunit POL γ B. While the catalytic core and DNA positioning was identical, POL γ B and the accessory-interacting determinant (AID) subdomain of POL γ A was rotated about 10° compared to the human version. In mouse Pol γ , the POL γ B dimer forms a more rigid and stable interface with POL γ A, whereas the human complex exhibits greater conformational flexibility during the shift from replication to proofreading. This rigidity likely stabilizes the polymerase complex, thereby providing tolerance to destabilizing mutations, explaining the milder enzymatic defects in mice. Kinetic studies also confirmed that mouse Pol γ B is a more potent accessory subunit and was able to enhance nucleotide incorporation rates even when paired with the human catalytic subunit. Swapping experiments demonstrated that the accessory subunit, at least partially, accounts for the superior activity of the mouse enzyme.

To further investigate the pathogenesis of *POLG*-disorders, nine *Polg* mutant mouse models were generated, corresponding to the most common pathogenic *POLG* variants observed in humans. Among the homozygous mutants, G826S and Y933C were embryonically lethal, whereas A449T and W726S mice were viable but exhibited only mild, tissue-specific reductions in mtDNA content. Analysis of compound heterozygous genotypes revealed a gradient of severity, where A449T/W726S mice were viable and

showed no evident clinical or molecular abnormalities, A449T/G826S animals were also viable but displayed partial mtDNA depletion across multiple tissues and reduced OXPHOS activity, while W726S/G826S mutants were embryonically lethal. These phenotypes align closely with the corresponding human mutations, where compound heterozygous combinations such as A467T/G848S or W748S/G848S cause severe, early-onset mitochondrial disease. Interestingly, although *in vitro* assays indicated that A449T/G826S had a more profound effect on replication than W726S/G826S, as the latter proved to be embryonic lethal *in vivo*. This difference indicates that other factors play important roles in the observed phenotypes. These factors could be POL γ A stability, expression, or cellular compensatory responses, which may affect disease severity in ways not solely explained by enzymatic activity. Furthermore, heterozygous WT/Y933C mice, modeling the human dominant Y955C mutation, exhibited signs of late-onset neurodegeneration and ultrastructural abnormalities of mitochondria from skeletal muscle. However, while mtDNA deletions are a common observation in Y955C patients, deletions could not be observed in the Y933C mouse model, likely reflecting the shorter murine lifespan and limited time for deletion accumulation.

Overall, the study demonstrates that mouse Poly has better tolerance to the most common recessive mutations, and POL γ B is a critical determinant of this mutation tolerance and phenotypic outcome. The more robust POL γ A-POL γ B interaction in mice enhances enzyme stability, mitigating the effects of pathogenic mutations. These findings establish POL γ B as a key modulator of mitochondrial disease severity and highlight the value and limitations of murine models for understanding *POLG*-related disorders.

4.3 PAPER III

Structural basis for allosteric activation of human POL γ via polymerase-state stabilization.

In this paper, we used cryo-EM and biochemical analyses to investigate how the small molecule PZL-A affects the activity of wild-type POL γ . Like in the mutant structures, our data showed that PZL-A binds at the interface between the POL γ A and the proximal POL γ B accessory subunit. Binding was also observed in both polymerase and exonuclease states. PZL-A binding displaces water molecules from the pocket and creates bridging interactions between the two subunits. It promotes specific hydrogen-bonds between POL γ B and POL γ A, which stabilize the subunit interface during transition from replication to exonuclease mode. In the exonuclease state, PZL-A induces a distinct rotation of POL γ B toward a mouse-like conformation, restricting subunit movement and likely reduces idle transitions between polymerase and exonuclease modes.

Biochemical characterization showed that PZL-A does not affect POL γ 's ability to detect and remove misincorporated nucleotides. However, PZL-A strongly reduces the excessive exonuclease activity on correctly paired nucleotides which occur in the absence of substrate (dNTPs). This indicates that the compound stimulates DNA polymerization by preventing unnecessary transitions into the exonuclease mode. The effect was also observed at low substrate levels, where PZL-A induces POL γ to favor DNA synthesis over exonuclease activity. Nevertheless, PZL-A still allows proofreading and does not make wild-type POL γ behave as the Exo $^-$ variant (D274A), which experience severe stalling when encountering a mismatch due to its lack of proofreading capability.

With TLC experiments we were able to simultaneously monitor POL γ 's Pol and Exo activities by quantifying dATP, DNA, and excised dAMP levels during active DNA synthesis. This allowed us to calculate the ratio of Exo/Pol activities, which revealed that PZL-A shifts the equilibrium between Pol and Exo activities toward productive DNA synthesis, resulting in markedly fewer excessive excision events. In these experiments, mouse Poly also exhibited higher replication efficiency compared to the human enzyme, which is in line with our findings in Paper II. Furthermore, the Exo/Pol ratio was twofold lower than in human, suggesting that mouse Poly's superior replication activity stems from a shift in the balance of Pol and Exo activities. Interestingly, PZL-A did not stimulate the DNA synthesis rate of mouse Poly, with only a modest decreasing effect on the Exo/Pol ratio. Since we in Paper II showed that POL γ B is a more potent accessory factor, we also performed mixing experiments of human and mouse POL γ A/POL γ B subunits. Both combinations showed Exo/Pol ratios between those observed in the pure systems, with PZL-A reducing the number of excision events. Moreover, mouse PolyB could stimulate the replication rate of human POL γ A to levels similar to those observed with PZL-A. By combining these findings with our structural data, it is clear that a mouse-like positioning of the POL γ B subunits in the exonuclease state (either by mouse PolyB or PZL-A) correlates with an increased replication rate and decreased Exo/Pol balance. As such, PZL-A (and mouse PolyB) appears to raise the energetic barrier between the polymerase and exonuclease states, thus favoring replication.

To test if PZL-A also stabilized the polymerase state in *POLG* mutants, we performed the TLC experiments with the R232H, A467T, W748S, and G848S variants. In all four variants, the Exo/Pol ratio was distinctly shifted in favor of polymerization, explaining why their activity can be rescued despite being located in different regions of POL γ .

Together, these findings demonstrate that PZL-A acts as an allosteric stabilizer of POL γ , which strengthens subunit interactions and promotes efficient replication by preventing excessive switching to the exonuclease mode. The compound effectively allows human POL γ to mimic the more rigid and catalytically favorable conformation observed in mouse POL γ , as PZL-A compensates for the lower intrinsic stability of the human enzyme. These findings provide a mechanistic basis for compound modulation of POL γ and underscore the potential use of small-molecule activators such as PZL-A to restore mtDNA replication in disorders arising from impaired *POLG* function. Because PZL-A enhances POL γ 's ability to function under conditions of limited nucleotide availability, PZL-A and related compounds also become promising therapeutic candidates for mitochondrial disorders that impair nucleotide metabolism, including those caused by *DGUOK* or *TK2* mutations.

5 CONCLUDING REMARKS

Mutations in POL γ are among the most common causes of inherited mitochondrial disease, leading to progressive defects in energy metabolism that often affect the nervous system, muscles, and other high-demand tissues. Despite their clinical prevalence, the molecular mechanisms by which *POLG* mutations impair mtDNA replication have remained poorly understood, and no effective therapies currently exist. Understanding how these mutations alter enzyme structure, stability, and activity is therefore essential for uncovering the pathogenic basis of mitochondrial disorders and for guiding the development of targeted treatments.

This thesis provides a comprehensive molecular framework for understanding the function of POL γ in health and disease. The key finding is the discovery of PZL-A, a first-in-class activator of POL γ -associated DNA synthesis. This compound was shown to restore polymerase activity in multiple disease-associated *POLG* variants, both *in vitro* and in patient-derived cells, providing proof of concept that pharmacological activation of POL γ can counteract pathogenic mutations. Structural and biochemical analyses revealed that PZL-A binds at the interface between the catalytic and accessory subunits, which stabilizes the enzyme in its polymerase state and reduces excessive exonuclease activity. The data are consistent with the performance of the homologous mouse Pol γ , which is inherently more efficient than its human counterpart, and suggest that PZL-A induces human POL γ to mimic the behavior of the mouse enzyme. Together, these findings establish an allosteric mechanism by which small molecules can enhance the efficiency of mtDNA replication. This work lays the foundation for rational design of targeted therapies for *POLG*-associated mitochondrial disorders and related defects in nucleotide metabolism.

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