Mitochondrial DNA Mutagenesis in Metazoa: From Phenotypes to Rescue

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It is thus that, except for the rare cases of plastid inheritance, the inheritance of all known characters can be sufficiently accounted for by the presence of genes in the chromosome. In a word the cytoplasm may be ignored genetically. –Thomas H. Morgan (Am.Nat. 1926)

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ABBREVIATIONS

2D-AGE	Two-dimensional agarose gel electrophoresis
AHSCT	Allogenic hematopoietic stem cell transplantation
AMPK	AMP-dependent kinase
BAC	Bacterial artificial chromosome
B[a]P	Benzo[a]pyrene
BER	Base excision repair
BrdU	5-bromo-2'-deoxyuridine
CEETP	Carrier erythrocyte-entrapped deoxythymidine phosphorylase
CoRR	Colocalization for redox regulation
COX	Cytochrome c oxidase
CS	Citrate synthase
CytC	Cytochrome C
CSB1-3	Conserved sequence block 1-3
da	daughterless
Dah	Dahomey
DGUOK	Deoxyguanosine kinase
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
dsDNA	Double-stranded DNA
EM	Electron microscopy
EMS	Ethyl methanesulfonate
EMSA	Electrophoretic mobility shift assay
ENU	N-ethyl-N-nitrosourea
ETF	Electron-transferring-flavoprotein dehydrogenase
FACS	Fluorescence activated cell sorting
FAD	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FRDA	Friedreich's ataxia
FRT	Flippase recognition site
FSC	Follicle stem cell
GPA	Beta-guanidinopropionic acid
ISC	Intestinal stem cell
KO	Knockout
L/D	Light/Dark
LECA	Last eukaryotic common ancestor
MGME1	Mitochondrial genome maintenance exonuclease 1
MIM	Mitochondrial inner membrane
MMR	Mismatch repair
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger RNA
MRO	Mitochondrion-related organelle
mtDNA	Mitochondrial DNA
MTERF	Mitochondrial transcription termination factor
mtSSB	Mitochondrial single-stranded DNA binding protein
NAD^+	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NER	Nucleotide excision repair
NR	Nicotinamide riboside
NRTI	Nucleotide analog HIV reverse transcriptase inhibitor
NUMT	Nuclear copy of mitochondrial DNA
OXPHOS	Oxidative phosphorylation

PAH	Polycyclic aromatic hydrocarbon
PARP1	Poly [ADP-ribose] polymerase 1
pBS	pBlueScript II SK(+) phagemid vector
PGC1α	Peroxisome proliferator-activated receptor-γ1α
PPAR	Peroxisomal proliferator activator receptor
PQQ	Pyrroloquinoline quinone
ROS	Reactive oxygen species
RRF	Ragged-red fiber
rRNA	Ribosomal RNA
SDM	Strand-displacement model
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT1	Sirtuin 1
ssDNA	Single-stranded DNA
SYA	Sucrose/Yeast/Agar
TALEN	Transcription activator-like effector nuclease
TFAM	Mitochondrial transcription factor A
TK2	Thymidine kinase 2
TOR	Target of rapamycin
tRNA	Transfer RNA
UV	Ultraviolet

Amino Acid	Abbreviation	Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ZUSAMMENFASSUNG

Alle Metazoen besitzen Mitochondrien, subzelluläre Organellen α-proteobaketeriellen Ursprungs, welche eine Vielzahl zellulärer Prozesse wie zum Beispiel Zellatmung, Apoptose, Signalkaskaden und Fettsäureoxidation regulieren. Die meisten mitochondrialen Proteine sind zellkernkodiert und werden posttranslational importiert. Mitochondrien besitzen auch ein eigenes zirkuläres und doppelsträngiges Multikopie-Genom (mtDNA), welches für 13 essentielle Proteine der oxidativen Phosphorylierung (OXPHOS), sowie mitochondrienspezifische Transfer RNAs (tRNAs) und ribosomale RNAs (rRNAs) kodiert, welche zur Translation der OXPHOS-Proteine benötigt werden. Aufgrund dessen ist die Aufrechterhaltung und Regulation der mtDNA essentiell für die Funktion der Mitochondrien und damit auch der Lebensfähigkeit der Zelle.

Mitochondriale Dysfunktion kann in verschiedenen Organismen Alterungsprozesse beschleunigen und zur Entstehung von alterungsassoziierten Krankheiten beitragen. Eine Klasse dieser mitochondrialen Krankheiten wird durch zufällig auftretende Replikationsfehler der mitochondrialen DNA polymerase (POLγ) induziert. Mutationen in der mitochondrialen DNA korrelieren positiv mit steigendem Alter im Menschen und ein vielfach erhöhter mtDNA-Mutationslevel in Säugetieren, wie der Hausmaus, führt zur Entstehung eines einen Progerie-Phänotyps. Einige Aspekte der zugrundeliegenden molekularen Ursachen sind jedoch unerforscht, so ist zum Beispiel nicht bekannt ob somatische mtDNA Mutationen die Lebensdauer von kurzlebigen Organismen, wie der Fruchtfliege, direkt beeinflussen. Zur Beantwortung dieser Frage wurden im Rahmen dieser Doktorarbeit zunächst POLγ Varianten mit gesteigerter oder reduzierter Korrektur- bzw. Polymeraseaktivität *in vitro* charakterisiert. Um die Funktion dieser POLγ Varianten *in vivo* zu untersuchen wurden diese mittels gentechnischer Methoden in das Genom der Fruchtfliege integriert. Die transgenen Fruchtfliegen mit erhöhtem mtDNA Mutationslevel waren weder physiologisch noch hinsichtlich ihrer der Lebenserwartung negativ beeinflusst. Auch die Kreuzverpaarung mehrerer Fruchtfliegen-Filialgenerationen induzierte keinen vorzeitigen Alterungsphänotyp. Dies basiert auf der relativ langsamen Akkumulation von mtDNA Mutationen innerhalb des kurzen Lebenszyklus der Fruchtfliege. Diese Ergebnisse zeigen, dass mtDNA Mutationen wahrscheinlich nicht die Lebenserwartung von Wildtyp-Fruchtfliegenpopulationen beeinflussen.

Aufgrund der Multikopien Natur der mtDNA können Mutationen in einigen wenigen mtDNA Molekülen der Zelle präsent sein (Heteroplasmie) oder in allen (Homoplasmie), wobei der Heteroplasmiegrad als der prozentuale Anteil an mtDNA Molekülen mit Mutationen definiert ist. Es ist bekannt, dass mtDNA Mutationen hohe Heteroplasmiegrade erreichen müssen um mitochondriale Dysfunktion auszulösen und um phänotypisch in Erscheinung zu treten. Es stellt sich jedoch weiterhin die Frage, ob der relative Heteroplasmiegrad oder die absolute Anzahl von Wildtyp-mtDNA Kopien in der gesamten Zelle für die phänotypische Erscheinung von mitochondrialen Krankheiten verursacht.

Um diese Hypothese zu untersuchen wurde die sogenannte mtDNA-Mutator Maus, welche eine korrekturdefiziente POLγ Variante exprimiert und durch männliche Infertilität charakterisiert ist, mit einer Maus verpaart welche den mitochondrialen Transkriptionsfaktor A (TFAM) überexperimiert. Interessanterweise, konnte der Grad der mitochondrialen Dysfunktion und die Infertilität männlicher mtDNA-Mutator Mäuse, ausgelöst durch mtDNA Mutationen, partiell durch die Überexpression von TFAM reduziert werden. Diese Ergebnisse zeigen zum ersten Mal, dass die absolute Anzahl an Wildtyp-mtDNA Molekülen entscheidend für die phänotypische Ausprägung von mitochondrialen Krankheiten ist und ermöglicht die Entwicklung neuer Strategien zur Behandlung von mtDNA Mutationen-basierter Krankheiten.

ABSTRACT

All metazoans possess subcellular organelles of α-proteobacterial origin called mitochondria. These organelles participate in various cellular functions including cellular respiration, apoptosis, cell signaling and fatty acid oxidation. Although most mitochondrial proteins are encoded by the nuclear DNA and imported into mitochondria post-translationally, mitochondria still retain a small circular double-stranded multicopy genome (mtDNA) which encodes 13 essential proteins of the oxidative phosphorylation system (OXPHOS) in addition to the transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) needed to translate the mtDNA-encoded messenger RNAs (mRNAs) within mitochondria. Therefore, faithful maintenance of mtDNA is essential for mitochondrial function and organismal viability.

Mitochondrial dysfunction has been linked to ageing and ageing-associated diseases in various organisms. One group of dysfunctions is caused by mtDNA mutations, which are thought to originate from random replication errors of the mitochondrial DNA polymerase (POLγ). The amount of mtDNA mutations increases with age in humans and experimentally increasing mtDNA mutation load in mice results in a progeria phenotype. However, it is still unclear whether mtDNA mutations are limiting the lifespans of short-lived organisms such as fruit flies. To answer this question we first characterized POLγ variants with modified proofreading or polymerase activities *in vitro*. These variants were thereafter introduced into fruit flies using genetic engineering. Surprisingly, increasing mtDNA mutation load had no effect on fly physiology or ageing due to the slow accumulation of mtDNA mutations within the short lifespan of fruit flies and even across generations. These results suggest that mtDNA mutations are not limiting the lifespan of wild type fruit fly populations.

Due to the multicopy nature of mtDNA, mutations can be present in only some of the molecules (heteroplasmy) or all of the molecules (homoplasmy) in a cell or tissue. It has been reported that pathogenic mtDNA mutations only cause mitochondrial dysfunction if they are present above a certain threshold level that depend on the type of mutation and the energetic requirement of the affected tissue. The heteroplasmy level is defined as the percentage of all molecules carrying the mutation. However, it has been also argued that the heteroplasmy level is, to some extent, irrelevant and that it is the absolute number of WT mtDNA copies that determines disease penetrance. To test this theory we took advantage of the mtDNA mutator mouse model, which carries proofreading-deficient POLγ and presents with male sterility. Overexpressing mitochondrial transcription factor A (TFAM), a well-characterized regulator of mtDNA copy number, was sufficient to partially rescue mitochondrial dysfunction and to cure the male infertility of this mouse model without affecting the mtDNA mutation load. These results provide the first experimental evidence that the absolute number of WT mtDNA molecules determines disease penetrance and offers a strategy to rescue mitochondrial dysfunction originating from mtDNA mutations.

INTRODUCTION

1.1 MITOCHONDRIA: THE ORIGIN

The mitochondrion is a cellular organelle, which originates from the engulfment of an α-proteobacterium by an archaeon forming an endosymbiotic partnership. This integration was further deepened by eukaryogenesis whereby ~600 of the 3000 – 5000 genes of the original α-proteobacterium moved into the nucleus of the archaeon slowly converting the bacterium into a mitochondrion (Boussau et al., 2004). Most genes of the α-proteobacterium were therefore either lost or replaced by genes from other prokaryotes or eukaryotes. Indeed, the mitochondrial genome of the last eukaryotic common ancestor (LECA) was thought to encode only ~70 proteins. To some extent this substantial decrease in the number of genes encoded by the LECA is expected because some genes important for the free-living α-proteobacterium would not be needed anymore once it became a genetic endosymbiont. These dispensable genes would include among others genes needed to make the bacterial cell wall and genes involved in many metabolism pathways. Currently, mammalian mitochondria have been estimated to contain ~1200 proteins (Calvo et al., 2016) and only 20% of those are of α-proteobacterial origin (Gray, 2015). Interestingly several proteins required for replication and transcription of mtDNA are of bacteriophage origin (Oliveira et al., 2015), showing that metazoan mitochondria are composed of proteins of several origins.

In most metazoans mtDNA encodes only 13 proteins, which are core components of the OXPHOS, and 13 tRNAs and 2 rRNAs needed to translate the mRNAs encoding these proteins. It has been a long-standing question why mitochondria still retain their own genetic material. After all, translocating genes from mitochondria into nuclear DNA (nDNA) should have several advantages owing to recombination and sexual reproduction to avoid Muller's ratchet (irreversible accumulation of deleterious mutations) and faster fixing of beneficial mutations (Adams and Palmer, 2003). Integration of mtDNA into nDNA is unlikely the limiting step in this process as nuclear copies of mtDNA (NUMTs) are known to be abundant (Dayama et al., 2014, Rogers and Griffiths-Jones, 2012) and even cause diseases when disrupting nDNA encoded genes (Hazkani-Covo et al., 2010). Indeed, gene transfers between mtDNA and nDNA are unidirectional with a few possible exceptions (Szafranski, 2017, Pont-Kingdon et al., 1998). Three main hypotheses have been put forward to explain why mitochondria still retain some genetic material. According to the first theory, the 13 protein coding genes of mtDNA have not translocated into nDNA due to differences in the genetic code between nucleus and mitochondria. However, the fact that hundreds of genes of the α-proteobacterium have already translocated from mitochondria into the nucleus suggests this process can take place successfully. The second theory suggests that proteins still encoded by mtDNA are so hydrophobic that it would not be possible to import and sort them post-translationally from the cytosol into mitochondria if they were encoded by the nDNA, and that these proteins could even have toxic effects

if mislocalized to wrong subcellular compartments (Björkholm et al., 2015). The third hypothesis, also known as the colocalization for redox regulation (CoRR) hypothesis (Allen, 2015), suggests that the intramitochondrial control of the production of these 13 core components of the OXPHOS system allows individual mitochondria to dynamically optimize their function in response to changing conditions. It should be noted that these theories are not necessarily mutually exclusive. A recent analysis of more than 2000 mtDNA genomes suggested that hydrophobicity and the CoRR hypothesis have the highest explanatory power for current mtDNA gene compositions (Johnston and Williams, 2016).

1.2 MITOCHONDRIA: FUNCTION

One central question behind endosymbiosis remains still unanswered: *cui bono*? From the point-of-view of the α-proteobacterium endosymbiosis can be seen as an efficient way to outsource many of its functions to the host, but it is unclear how this arrangement benefits the host. Mitochondria are often referred as the powerhouses of the cell because they are known to produce most of cell's ATP. Indeed, it has been argued that owing to the compartmentalized mitochondrial energy production an eukaryotic nuclear gene controls almost 5000 times more energy flux in comparison with a prokaryotic gene (Lane and Martin, 2010). Additionally, a great part of this difference in the energetic flux between prokaryotes and eukaryotes is contributed to the presence and proximity of mtDNA to the OXPHOS system, which enables eukaryotes to rapidly react in response to changes in environment as suggested by the CoRR hypothesis (Allen, 2015, Lane and Martin, 2010). Therefore the endosymbiosis can be seen as highly beneficial and it seems that all eukaryotes have once had mitochondria although some carry only relict mitochondria; called mitochondrion-related organelles (MROs); and others have completely lost them (Makiuchi and Nozaki, 2014). Interestingly, eukaryotes that have lost mtDNA have also lost the ability to carry out OXPHOS (Adams and Palmer, 2003) further emphasizing the importance of mtDNA in controlling OXPHOS.

Some eukaryotes lack OXPHOS but nevertheless possess mitochondria suggesting that mitochondria have some additional functions besides OXPHOS. In fact, one of the most important processes taking place in mitochondria and MROs is the synthesis of iron-sulfur (Fe-S) clusters. Fe-S clusters have unique biochemical properties like the ability to accept and donate single electrons, perform oxidation/reduction (redox) reactions, function as redox sensors, catalyze ligation reactions and stabilize protein structures (Paul et al., 2017, Rouault, 2015). Studies have shown this cofactor to be essential for various cellular functions in- and outside of mitochondria. These processes include multiple metabolic processes, antiviral responses, genome maintenance, and protein translation (Braymer and Lill, 2017). Because Fe-S cluster synthesis takes place essentially in all eukaryotes, it is sometimes considered as the "minimal" function of mitochondria. Why does this biosynthesis pathway reside within mitochondria? It has been suggested that mitochondria functions as an iron storage

organelle. In support of this mitochondria are known to contain 20% of total cellular iron and most of the metabolically available iron (Paul et al., 2017). Moreover, through the control of Fe-S cluster synthesis mitochondria can also control cellular iron uptake and thereby control cellular iron levels (Lill et al., 2012). Therefore, mitochondria function as subcellular compartments to control iron storage and availability in the eukaryotic cell. Another reason for mitochondrial Fe-S cluster synthesis could be the lower oxygen concentration in mitochondria relative to the rest of the cell (Kurokawa et al., 2015). Indeed, Fe-S clusters can be assembled *in vitro* but only in in anaerobic conditions because oxygen oxidizes iron in Fe-S clusters resulting in disassembly of the cluster (Rouault, 2015). Thirdly, metabolically free iron can react with the reactive oxygen species (ROS) such as hydrogen peroxide and convert it into highly reactive hydroxyl radical (Halliwell and Gutteridge, 1992). Therefore, concentrating Fe-S cluster synthesis into mitochondria could limit cellular ROS damage to this organelle. In summary, it seems that mitochondria can sequester iron, especially metabolically active iron, for cofactor synthesis and also to minimize unwanted cellular damage.

Several mitochondrial metabolic pathways are linked, including Fe-S-cluster synthesis and fatty acid synthesis (Boniecki et al., 2017). Fatty acids are important constituents of membranes, precursors of hormones and a major energy source. One common function of mitochondria is fatty acid oxidation (FAO)/beta-oxidation which is a major energy source for some of the most energy demanding tissues such as heart, skeletal muscle and kidney (Houten et al., 2016). Additionally, liver uses FAO to produce ketones for brain in the absence of glucose. In any given tissue, the level of FAO dependency is not fixed but instead varies during development and in response to nutritional changes. In FAO flavin adenine dinucleotide (FAD) is reduced to FADH2, which can feed electrons to ubiquinone. In addition to FAO feeding electrons to OXPHOS, these two processes might be even more linked as several FAO enzymes are known to interact with OXPHOS complexes (Nsiah-Sefaa and McKenzie, 2016). The significance of these interactions is currently a heavily studied subject.

Endotherms (warm-blooded animals) are able to maintain a constant body temperature. This heat originates from both uncontrolled exothermic reactions and regulated thermogenic reactions taking place in the body. The main form of regulated thermogenesis takes place within mitochondria of the brown adipose tissue in the form of proton leak across the mitochondrial inner membrane. Instead of using the proton gradient to produce ATP, proton leakage dissipates the membrane potential resulting in heat production. The main regulator of this process is uncoupling protein 1 (UCP1), which is a transporter of protons and fatty acid anions (Bertholet and Kirichok, 2017). UCP1 knockout mice do not have adaptive thermogenesis showing that UCP1 is essential in controlling body temperature (Golozoubova et al., 2001).

Another interesting function of eukaryotic mitochondria is the activation of intrinsic pathway of apoptosis. The apoptotic machinery is composed of several evolutionary conserved caspases, adaptor proteins and B-cell lymphoma 2 (Bcl-2) family of proteins. However, the actual steps and importance

of various players varies between organisms. For instance, in mammals the mitochondrial outer membrane permeabilization (MOMP) followed by the release of cytochrome C (CytC) is an essential requirement for the activation of caspases and apoptosis (Tait and Green, 2013). In contrast, studies done in fruit flies suggest that instead of mitochondria releasing apoptotic factors, such as CytC, fruit fly mitochondria participate in apoptosis by concentrating several regulators of apoptosis on the outer membrane (Clavier et al., 2016). Therefore mitochondria from flies to mammals play a different yet an important role in programmed cell death

In summary, mitochondria have multiple important functions that take place in many tissues (apoptosis, Fe-S cluster synthesis, OXPHOS, ROS signaling) and also tissue specific functions (thermogenesis, hormone synthesis). These functional differences are reflected at the mitochondrial proteome as only 57% of mitochondrial proteins are common between different tissues (Mootha et al., 2003). Similar tissue specific differences can be seen in the mitochondrial lipidome (Fleischer et al., 1967) which together contribute to tissue specific mitochondrial ultrastructures (Benard et al., 2006). Only the last of the mitochondrial macromolecules, mtDNA, does not vary, at least qualitatively, between various tissues.

1.3 MITOCHONDRIAL DNA

1.3.1 Structure of mtDNA

MtDNA was first visualized in chick embryos already in the 60's by electron microscopy (Nass and Nass, 1963), several years before the Lynn Marguli's (then Sagan) seminal paper on endosymbiont hypothesis (Sagan, 1967). With a few known exceptions (some classes of cnidarians), metazoan mtDNA is a circular double-stranded DNA generally between 15-20 kb of size (Boore, 1999). MtDNA is extremely gene dense with several genes overlapping and minimal intergenic regions. Extensive sequencing of metazoan mtDNAs has shown that the gene content, but not necessarily the gene order, is highly conserved. In mammals, the two mtDNA strands have been named heavy (H) and light (L) strands because they can be separated in $CsCl_2$ gradients due to differences in guanine content (Berk and Clayton, 1974). In fruit flies the corresponding heavy and light strands are called minor- and major coding strands, respectively (Garesse, 1988). In mammals, most genes are encoded by the heavy strand whereas in fruit flies the genes are more evenly distributed between the two strands (Fig. 1.1). In most metazoans, mtDNA encodes 11 mRNAs (two of which are polycistronic) translated to 13 proteins as well as two rRNAs and 22 tRNAs that are necessary for mitochondrial translation.

The highest variability in mtDNA is in the single large non-coding control region, which, in mammals, contains one of the replication origins (O_H) and both the heavy and light strand promoters for transcription (HSP, LSP) (Fig. 1.1 and 1.2). Despite the high variability among mammals the control region includes several conserved regions such as the conserved sequence blocks (CSB1-3) and

termination-associated sequence (TAS). In addition, mammalian mtDNA contains a third DNA strand in the control region, also known as the 7S DNA, creating a displacement loop (D-loop) structure extending from the O_H to the TAS (Gustafsson et al., 2016)(Fig. 1.2). The percentage of mtDNA molecules containing annealed 7S DNA varies from 8% to 55% between cell types (Brown et al., 1978) and the precise function of the 7S DNA is not known as most 7S DNA molecules do not engage in replication but are constantly synthesized and turned over (Bogenhagen and Clayton, 1978, Berk and Clayton, 1974). The 7S DNA may have some regulatory function to control mtDNA replication (Nicholls and Minczuk, 2014).

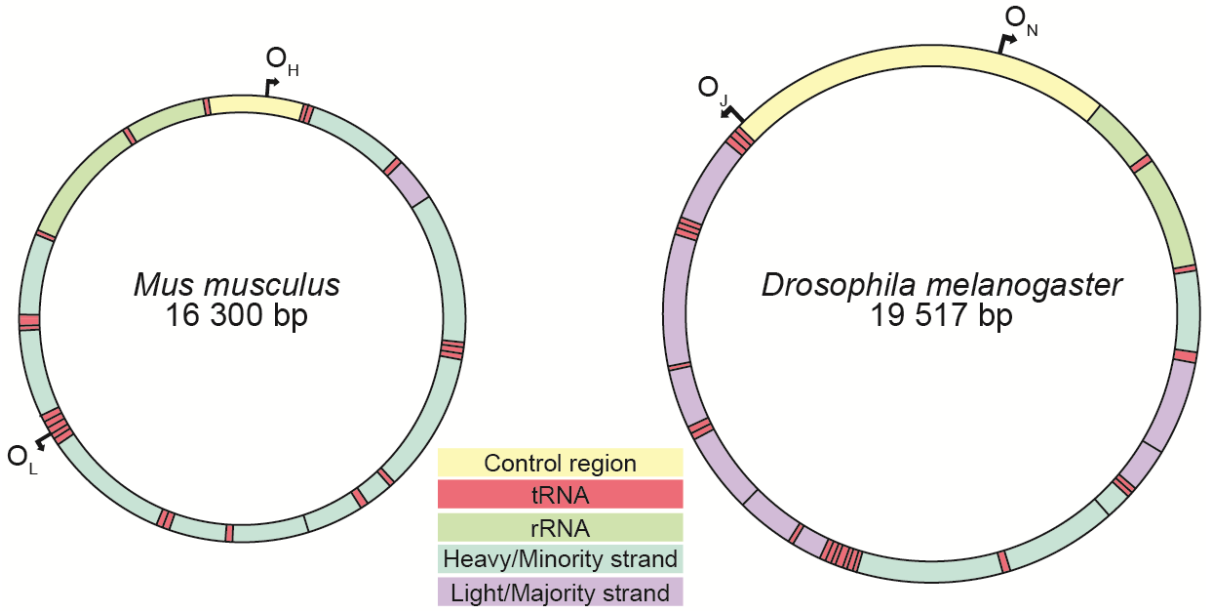


Figure 1.1: Schematic views of the mtDNAs of *Mus musculus* and *Drosophila melanogaster*.
The heavy (O_H) and light (O_L) strand origins of replication are depicted in the mouse mtDNA. In fruit flies the corresponding replication origins are designated the minority (O_N) and majority (O_J) strand origins of replication. Note that in mouse mtDNA the rRNA genes are the last ones to be replicated whereas in fruit flies they are the first ones. In mice the O_H is located in the control region and O_L is located within a tRNA cluster. In contrast, in fruit flies both replication origins have been mapped to the control region.

We have a poor understanding of the structure and function of fruit fly control region owing largely to the high adenine and thymine content of the fly mtDNA (82%), particularly in the control region (90-95%), which is also known as the A+T-rich region (Garesse and Kaguni, 2005, Lewis et al., 1995). The fruit fly control regions vary in size between 1 kb to 4.8 kb depending on *Drosophila* subspecies in question and there is even variability within any given species (Salminen et al., 2017) some of which is caused by the intracellular microbe Wolbachia (Chen et al., 2012). Neither the mammalian control region elements nor the 7S DNA are conserved in flies (Tsujino et al., 2002, Goddard and Wolstenholme, 1980, Goddard and Wolstenholme, 1978, Rubenstein et al., 1977) which instead have type I and type II repeat elements in addition to long poly-dT stretches that are thought to control mtDNA transcription and replication (Fig. 1.2). Support for poly-dT stretches as regulators of mtDNA replication and transcription comes from studies done in nucleus where homopolymeric stretches are known to be important for the transcriptional control of many nuclear genes and origins of replication in the vicinity of these structures are more likely to be used in DNA replication (Segal and Widom,

2009). In addition, in mammalian mitochondria poly-dT stretches are known to stimulate the primer synthesis by mitochondrial RNA polymerase (POLRMT) (Fusté et al., 2014), which in mammals is known to prime mtDNA replication (Gustafsson et al., 2016). Furthermore, in fruit flies, free 5'-ends of mtDNA have been mapped to these poly-dT stretches suggesting a function as replication origins (Saito et al., 2005). Although there are substantial differences between the control region structures of mammalian and fruit fly mtDNA, the proteins responsible for the replication of mtDNA are conserved from flies to humans (Oliveira et al., 2015).

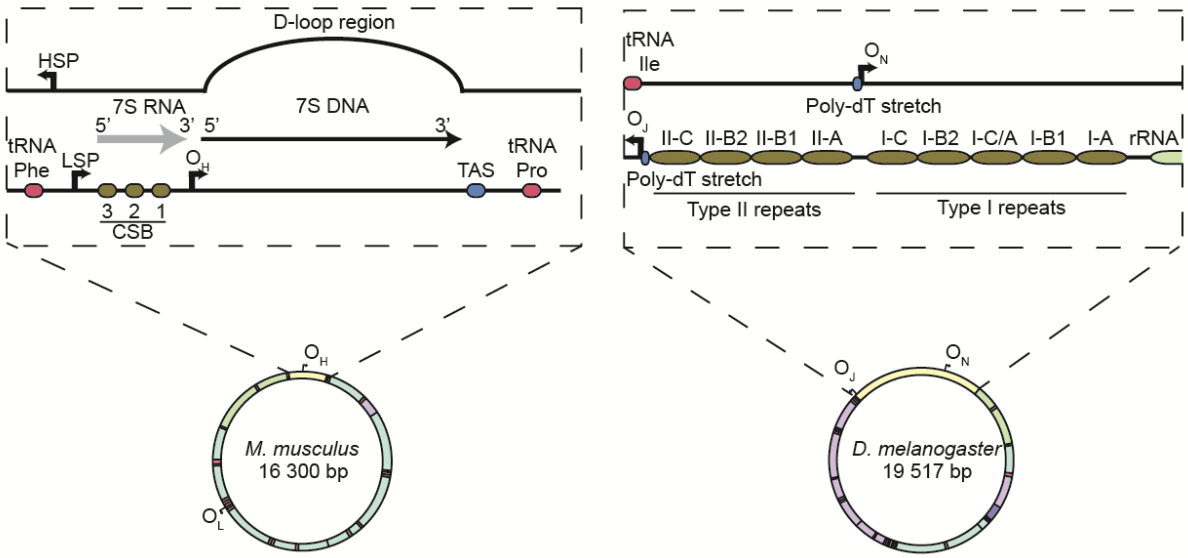


Figure 1.2: Schematic view of the mtDNA control regions (A+T-rich region) of *Mus musculus* and *Drosophila melanogaster*.
The replication of the heavy strand of mouse mtDNA begins by creating a RNA primer by POLRMT using the light strand promoter (LSP). This RNA primer is used by POLγA to produce the 7S DNA, which in most cases stops at the TAS region and is degraded. Although the control mechanism is yet unclear, sometimes replication extends pass the TAS region and engages in productive replication. In flies, the A+T-rich region does not contain any triple-stranded DNA structures and both replication origins have been mapped to the control region. The poly-dT stretches in the fly control region are believed to be used to initiate the minor (O_N) and major (O_J) strand replications.

MtDNA is not composed only of deoxyribonucleotides (dNTPs) because mtDNA extracted from HeLa or mouse L cells is sensitive to alkaline conditions, suggesting that mtDNA contains ribonucleotides (Margolin et al., 1981, Wong-Staal et al., 1973). A more recent thorough analysis of ribonucleotides in mtDNA suggested that they are evenly distributed between both strands and occur randomly throughout the mtDNA (Berglund et al., 2017). These ribonucleotides incorporated into mtDNA could have severe consequences due to the inherent instability of ribonucleotides, and may be especially relevant in diseases with changes in mitochondrial nucleotide pools (Berglund et al., 2017). Although a completely unexplored area, it will be interesting to see in the future how the tissue- and cell type specific variations in mitochondrial nucleotide pools (Gandhi and Samuels, 2011b, Bradshaw and Samuels, 2005) affect ribonucleotide incorporation and thereby maintenance and expression of mtDNA.

1.3.2 Mitochondrial nucleoids

Nuclear DNA is packed into regularly spaced nucleosomes. In contrast, DNA-protein crosslinking studies have suggested that mtDNA from flies to humans is packed more randomly into dense

nucleoprotein particles also known as nucleoids (Pardue et al., 1984, Potter et al., 1980, DeFrancesco and Attardi, 1981, Albring et al., 1977). Recent advances in high-resolution microscopy have provided us with insights to the size, shape and distribution of these particles (Kukat et al., 2015, Kukat et al., 2011, Brown et al., 2011). The size of nucleoids is somewhat elongated with the mean size of ~100 nm and most nucleoids have been estimated to contain only a single mtDNA molecule (Kukat et al., 2011). This one mtDNA per nucleoid state seems to be regulated as experimentally increasing mtDNA copy number increases the number of nucleoids but not the number of mtDNA molecules per nucleoid (Kukat et al., 2015).

If mtDNA is not wrapped by histones, what then are the constituents of mitochondrial nucleoids? A plethora of putative nucleoid proteins have been shown to colocalize with nucleoids (Rajala et al., 2015, Bogenhagen et al., 2008, Wang and Bogenhagen, 2006), although most of these interactions are likely transient or experimental artifacts caused by strong overexpression of labeling proteins (Han et al., 2017). Both *in vitro* and *in vivo* studies have suggested that a single protein, mitochondrial transcription factor A (TFAM), is sufficient to pack mtDNA (Kukat et al., 2015, Farge et al., 2014, Kaufman et al., 2007). TFAM has two high mobility group (HMG)-box domains that bind DNA with some specificity towards mitochondrial transcription promoters but also without sequence specificity inducing occasionally sharp U-turns (Ngo et al., 2014, Ngo et al., 2011, Rubio-Cosials et al., 2011). TFAM is highly abundant in cells (1000:1 TFAM to mtDNA ratio) and covers the whole mtDNA evenly (Fusté et al., 2014, Wang et al., 2013, Takamatsu et al., 2002). Studies in model organisms have shown it to be an essential protein involved both in mtDNA transcription and mtDNA maintenance (Ekstrand et al., 2004, Larsson et al., 1998).

1.3.3 Mitochondrial DNA polymerase (POLγ)

The minimal mitochondrial replisome (Korhonen et al., 2004) is formed by three proteins: mitochondrial DNA polymerase (POLγ), mitochondrial single-stranded DNA binding protein (mtSSB) and mitochondrial DNA helicase (TWINKLE)(Fig. 1.3). POLγ is the main DNA-dependent DNA polymerase in mitochondria and is absolutely necessary for mtDNA replication from yeast to mice (Bratic et al., 2015, Bratic et al., 2009, Hance et al., 2005, Iyengar et al., 1999, Genga et al., 1986). Recently, a new dual-localized (mitochondria and nucleus) primase polymerase (PRIMPOL) was described in mammals (Bailey and Doherty, 2017). The mouse PRIMPOL knockout is viable without any obvious phenotype suggesting this polymerase is not needed for mtDNA replication. Several species, including yeast and fruit flies, have even lost this protein. Therefore POLγ is thought to be the only polymerase responsible for replication and repair of mtDNA. The structure of the POLγ holoenzyme varies from the monomeric yeast (MIP1) and nematode (polg-1) polymerases to the heterodimeric DmPOLγA/DmPOLγB and heterotrimeric POLγA/POLγB_2 polymerases of fruit flies and vertebrates, respectively (Oliveira et al., 2015). The catalytic subunit of POLγ (POLγA) belongs to the family A DNA polymerases, which also includes the DNA polymerase I of *E. coli* and

bacteriophage T7 DNA polymerase. POLγA possesses a DNA-dependent DNA polymerase activity for DNA synthesis, a 3'-5' exonuclease activity for proofreading and a 5'-deoxyribose phosphate lyase activity, which might participate in base excision repair (BER) (Kaguni, 2004). POLγA can also use short stretches of RNA as a template for DNA synthesis (Kaguni, 2004) suggesting that ribonucleotides in mtDNA would not stall the polymerase.

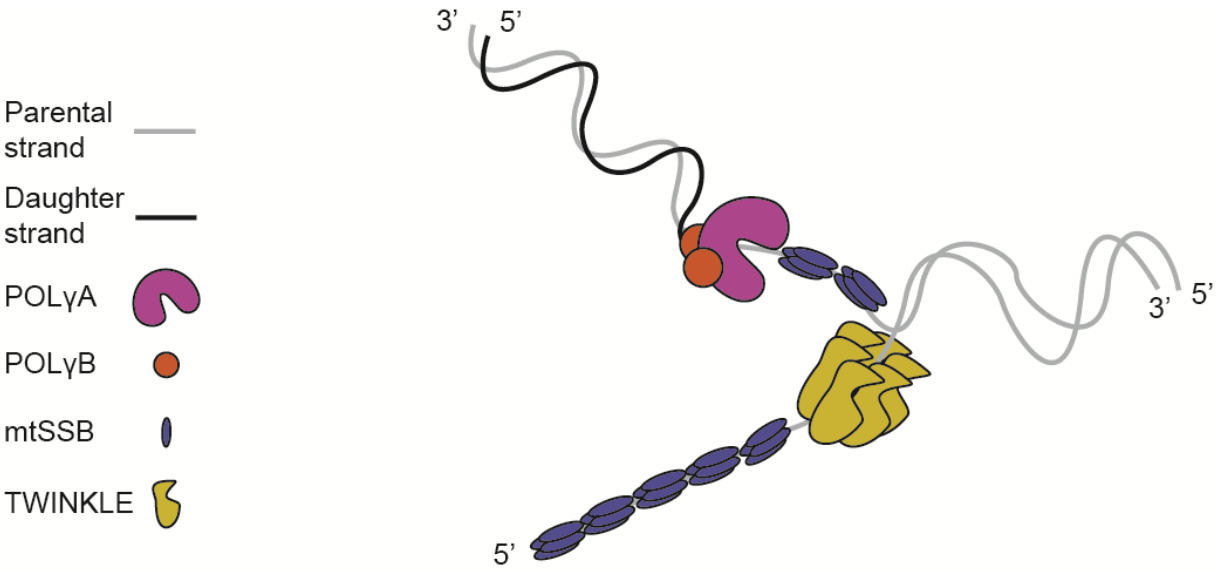


Figure 1.3: Schematic view of the minimal mtDNA replisome
The catalytic (POLγA) and accessory (POLγB) subunits of POLγ, mtSSB and TWINKLE constitute the minimum number of proteins needed to produce full length replication products *in vitro*. TWINKLE unwinds the dsDNA into 5' to 3' direction, whereas heterodimeric POLγAB (fly) or heterotrimeric POLγAB_2 (vertebrates) uses the other strand as a template for DNA synthesis. MtSSB increases the activity of both POLγ and TWINKLE and protects the single-stranded portions of DNA. TWINKLE and POLγ have been also shown to stimulate each other.

The bacteriophage T7 DNA polymerase contains an accessory subunit, thioredoxin (Lee and Richardson, 2011). The thioredoxin does not directly interact with DNA but instead reorganizes to flexible regions of the T7 DNA polymerase to increase protein-DNA interaction surface and thus functions as a processivity factor. In a similar manner the accessory subunit of POLγ (POLγB) has been shown to function as a processivity factor in mtDNA replication (Fig. 1.3) and is essential for mtDNA maintenance in all species studied (Euro et al., 2017, Humble et al., 2013, Iyengar et al., 2002). Structurally POLγB resembles class IIa aminoacyl-tRNA synthetases but it is not able to bind ATP or recognize anticodons (Carrodeguas et al., 2001). POLγB is interesting from the evolutionary point-of-view as it is not orthologous to the two-subunit tRNA synthetases present in α-proteobacterium. Instead POLγB was likely gained through horizontal gene transfer from another group of bacteria by a later eukaryotic lineage explaining why it is not present in yeast, nematodes and fungi (Wolf and Koonin, 2001). The ability to form homodimers seems to also be unique to vertebrate POLγB owing to the acquisition of a new domain (Oliveira et al., 2015). This is in line with the results showing that only POLγB homodimer, but not the fruit fly monomer, has the ability to bind double stranded DNA (dsDNA) though the biological relevance of this, if any, is unknown (Lim et al., 1999).

1.3.4 Mitochondrial DNA helicase (TWINKLE)

POLγ has no strand-displacement activity and cannot therefore use dsDNA as a substrate for replication (Farge et al., 2007, Korhonen et al., 2004, Wernette and Kaguni, 1986). Instead, mtDNA has to be unwound first for mtDNA replication to proceed. This is performed by the hexameric mtDNA helicase (TWINKLE) which unwinds DNA in the 5' to 3' direction in a NTP-dependent manner (Fig. 1.3) and is essential for mtDNA maintenance (Milenkovic et al., 2013). TWINKLE has evolved from bifunctional primase-helicases found in T-odd bacteriophages such as the gp4 helicase of T7 bacteriophage (Shutt and Gray, 2006). In most metazoans, residues needed for the primase activity are not conserved and therefore TWINKLE retains only the helicase activity (Kaguni and Oliveira, 2016). In T7 bacteriophage, the primase-helicase gp4 is known to interact with the T7 DNA polymerase increasing the processivity of both enzymes (Lee and Richardson, 2011). Similarly, TWINKLE alone is a slow DNA helicase but its activity can be stimulated by both POLγ and mtSSB (Korhonen et al., 2004) probably through a specific interactions (Euro et al., 2017). Of note, the insect TWINKLE is an interesting anomaly among the metazoan mitochondrial DNA helicases in that it coordinates an Fe-S cluster not found in other metazoans (Stiban et al., 2014). Further studies are needed to understand whether this Fe-S cluster has a regulatory function or whether it is only a passive structural element.

Other DNA helicases in addition to TWINKLE have been reported to localize in mitochondria. One of them is the dual-localized PIF1 helicase (Bannwarth et al., 2016). The mouse PIF1 knockout is viable without any changes in mtDNA copy number (Bannwarth et al., 2016) suggesting it is not essential to mtDNA maintenance. DNA2 is yet another dual-localized helicase/nuclease which has been suggested to participate in mtDNA replication and repair (Zheng et al., 2008). In support of its function in mitochondria, mutations in DNA2 have been shown to lead to mtDNA instability (Ronchi et al., 2013). Importantly, neither of these helicases can compensate for the absence of TWINKLE emphasizing that TWINKLE is the main replicative mitochondrial DNA helicase (Milenkovic et al., 2013).

1.3.5 Mitochondrial single-stranded DNA binding protein (mtSSB)

Unwinding of dsDNA to its single-stranded constituents by dedicated DNA helicases makes DNA vulnerable to chemical and nucleolytic attacks that can lead to DNA mutations and increase DNA instability. Single-stranded DNA (ssDNA) binding proteins (SSBs) are important for the replication, recombination and repair of DNA because they can prevent DNA damage by covering any ssDNA regions (Shereda et al., 2008). They have a passive function in protecting DNA but they can also interact with multitude of proteins to recruit them to DNA. Mitochondria have their own SSB (mtSSB) (Fig. 1.3) and similar to POLγ and TWINKLE it is also essential for mtDNA maintenance (Maier et al., 2001). Interestingly, while POLγ and TWINKLE resemble bacteriophage enzymes, mtSSB shares structural and biochemical similarities with the SSB of *E. coli* (Qian and Johnson, 2017, Morin et al.,

2017). MtSSB binds mtDNA as a homotetramer and it is a highly abundant protein (mtSSB:mtDNA ratio is 3000:1) to the extent that in can coat any ssDNA regions during mtDNA replication (Fusté et al., 2014, Takamatsu et al., 2002). It has been also observed that mtSSB is enriched in the D-loop region (Fusté et al., 2014) where it might participate in regulating the 7S DNA (Ruhanen et al., 2010, Takamatsu et al., 2002) and it is excluded from a secondary DNA structure in the O_L probably to allow POLRMT to prime mtDNA replication (Fusté et al., 2014). As elucidated before, although mtSSB has no intrinsic enzymatic activity, it has an important function in stimulating both POLγ and TWINKLE (Ciesielski et al., 2015, Korhonen et al., 2003). The interactions between these proteins are specific as distinct mutations on the surface of mtSSB can impair its ability to stimulate POLγ without affecting the unwinding activity of TWINKLE and vice versa (Oliveira and Kaguni, 2011).

1.3.6 MtDNA replication

MtDNA has to be replicated in mitotic tissues to enable sufficient mtDNA transmission for both progeny cells in cytokinesis but in addition mtDNA is replicated in post-mitotic tissues due to constant mtDNA turnover. The estimations for mtDNA turnover rates vary widely depending on the method of choice. For instance, mtDNA half-life in rat heart was estimated to be 7 days using tritium labeled dT (Gross et al., 1969) and 350 days using heavy water labeling (Collins et al., 2003). Electron microscopy (EM) studies have suggested that in flies 1-3% of all mtDNA molecules are engaged in replication at any given moment (Goddard and Wolstenholme, 1980). Original pulse-chase labeling studies suggested that the complete replication time of mtDNA in mouse cells is ~120 min (270 bp/min), which is close to the observed *in vitro* rates of 180 bp/min (Korhonen et al., 2003, Berk and Clayton, 1974). Significantly faster replication times (<15 min) have been reported in fruit flies (Rubenstein et al., 1977).

Even among mammals, where we have the best understanding of mtDNA replication, the actual mode of mtDNA replication has been debated for years (Ciesielski et al., 2016). Original studies suggested that replication starts sequentially from two origins of replication (Berk and Clayton, 1974, Robberson and Clayton, 1972)(Fig. 1.4). First, replication of the heavy strand is initiated from the O_H taking advantage of the RNA primer synthesized upstream starting from LSP by POLRMT. The replication proceeds unidirectionally with continuous displacement of the parental heavy strand and synthesis of the daughter strand, hence the name strand-displacement model (SDM) (Fig. 1.4). While single-stranded, the displaced heavy strand is coated by mtSSB likely protecting it from chemical or nucleolytic damage (Fusté et al., 2014). Once the replication reaches two thirds of the molecule the O_L in the middle of a tRNA cluster becomes single-stranded and is able to form a stem-loop structure (Fig. 1.4). This stem-loop structure is used by POLRMT to synthesize a short primer, which is further used to replicate the light strand. Most evidence to date suggests that SDM is the main replication mode in mammalian tissues: 1) There is a well-known mutation skew at the fourfold redundant sites of mtDNA which correlate with the duration some segments have to remain single-stranded during

replication (Faith and Pollock, 2003). 2) In support of the previous mutation skew, some fish that have inverted control region relative to other vertebrates show also inverted mutation skewness (Fonseca et al., 2014). 3) A recent study quantifying mtSSB binding to mtDNA showed a gradient along the displaced paternal heavy strand, another result fitting the SDM (Fusté et al., 2014). 4) A recent single molecule analysis of mtDNA replication using DNA combing also supported the SDM (Phillips et al., 2017). 5) Experimental studies using the mtDNA mutator mouse have shown how the O_L is essential for mtDNA maintenance as mutations in this element are under strong negative selection *in vivo* (Wanrooij et al., 2012) and 6) single large deletions of mtDNA always retain both O_H and O_L in human patients.

Two alternative replication models are based on studies done using two-dimensional agarose gel electrophoresis (2D-AGE). In the first alternative model known as the ribonucleotide incorporation throughout the lagging strand (RITOLS), the paternal heavy strand is coated by RNA instead of mtSSB (Yasukawa et al., 2006). However, it was recently shown that if separately purified mtDNA and mitochondrial transcripts are mixed in a test tube, similar RITOLS replication intermediates can be observed in 2D-AGE questioning the validity of this approach and therefore the RITOLS model (Fusté et al., 2014). The second alternative model, strand-coupled model (SCM), suggests that the replication of heavy and light strand is coupled (Holt et al., 2000). As listed above, however, most results using various methods are in support of the SDM.

Results regarding the mode on replication in fruit flies have also provided contradictive results. The first EM studies suggested that fruit flies have a minor strand origin of replication located in the middle of the control region (Goddard and Wolstenholme, 1980, Goddard and Wolstenholme, 1978)(Fig. 1.1, 1.2 and 1.4) and that the replication would use the similar SDM as observed in mammals. In fruit flies, however, replication of the minor strand seems to proceed up to 97% before the initiation of major strand replication, suggesting that in fruit flies both replication origins are located in the control region (Fig. 1.1, 1.2 and 1.4). This replication model is also supported by the findings that strand- and position specific nucleotide biases exist across the whole mtDNA coding region of several insects (Stewart and Beckenbach, 2006) and mapping of free 5'-ends to two conserved poly-dT stretches in the control regions likely representing the replication origins for the minor and major strands (Saito et al., 2005, Tsujino et al., 2002). In contrast to most EM studies and mapping of the free 5'-end, a recent 2D-AGE based study suggested that the fruit fly mtDNA would replicate using the SCM (Joers and Jacobs, 2013). Similar experiments to those done in mammalian systems (single-molecule analysis, mtSSB occupancy, *in vivo* mutagenesis) would clearly increase our understanding of the mtDNA replication in fruit flies if applied to this model organism.

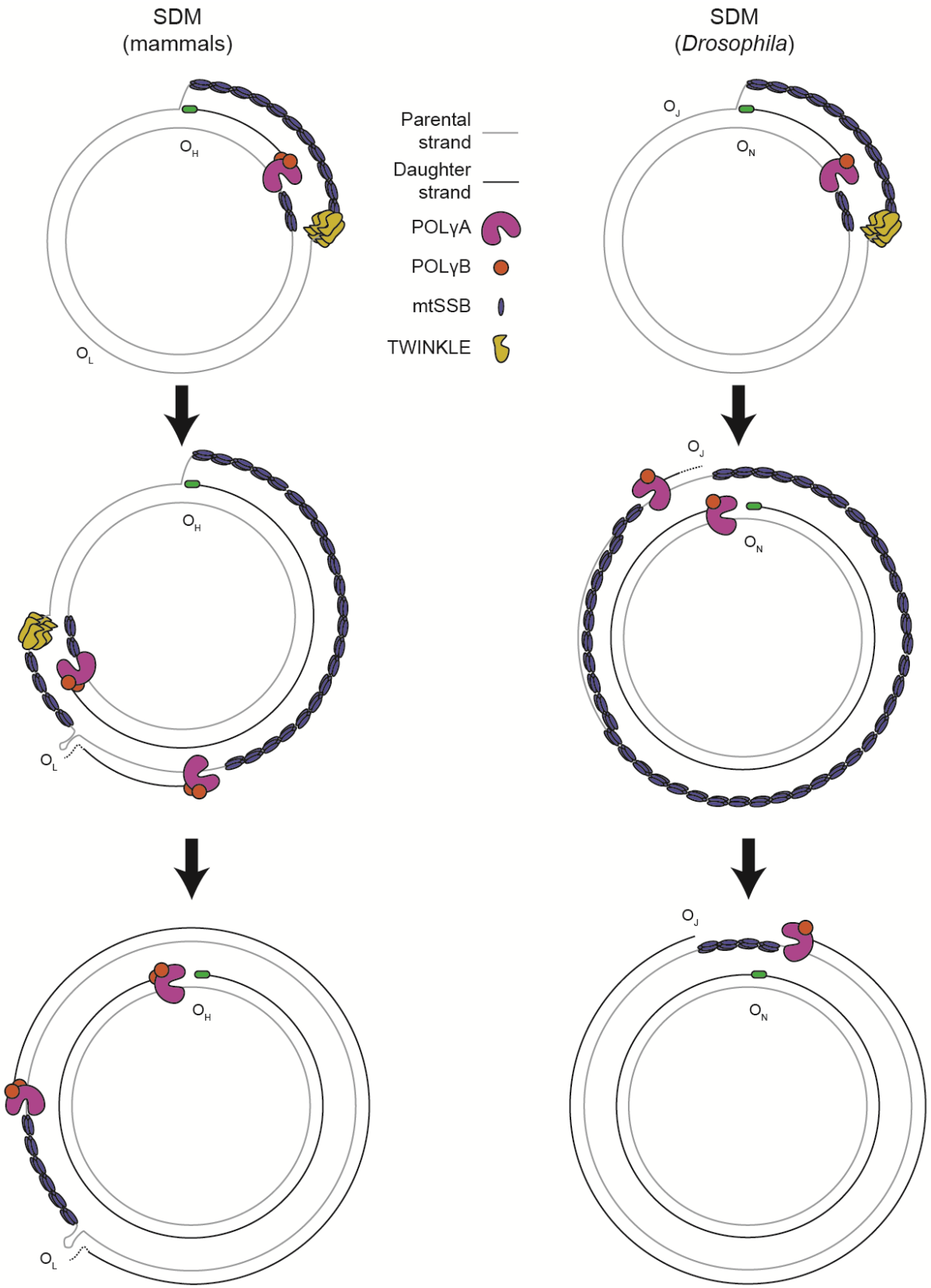


Figure 1.4: Schematic view of the strand-displacement model (SDM) of replication in vertebrates and fruit flies
In vertebrates, mtDNA replication begins from the O_H and continues in a strand-asynchronous manner towards O_L. The parental heavy strand displaced by TWINKLE is covered by mtSSB. After reaching O_L the parental heavy strand forms a hairpin structure which POLRMT uses to synthesize a short RNA primer. POLγ uses this primer to synthesize the light strand. In flies, mtDNA replication is similar with the difference that both replication origins are located in the control region. Therefore the heavy strand replication if nearly finished before the light strand replication engages.

It is poorly understood whether collisions between replication and transcription machineries take place in mitochondria, and if so, whether they are controlled. In other systems, these collisions are known to cause abortive DNA synthesis and mutagenesis (Lang et al., 2017) hence some mechanisms to control these collisions are likely to exist also in mitochondria. Mitochondria contain a family of mitochondrial transcription termination factor (MTERF) proteins that are participating in regulation of mitochondrial transcription, ribosome assembly and translation (Wredenberg et al., 2013, Terzioglu et al., 2013, Joers et al., 2013, Bruni et al., 2012, Cámara et al., 2011, Roberti et al., 2009, Park et al., 2007, Roberti et al., 2006, Roberti et al., 2005, Roberti et al., 2003). Interestingly, both in mammals and fruit flies, some of these proteins have been suggested to control replication-transcription collisions. For instance, MTERF2 and MTERF3 can bind mtDNA unspecifically and overexpression of either protein increases the amount of replication intermediates (Hyvärinen et al., 2011). A more interesting protein is MTERF1 which normally binds downstream of the ribosomal transcription unit and stops antisense transcription over the rRNA genes (Terzioglu et al., 2013) but in addition it can arrest mtDNA replication at the same site by inhibiting TWINKLE (Shi et al., 2016). The fruit fly ortholog of MTERF1, DmTTF, has two binding sites in fruit fly mtDNA where it can block mitochondrial transcription bidirectionally (Roberti et al., 2006, Roberti et al., 2005, Roberti et al., 2003). Fruit flies possess also an insect specific DmTTF paralog, MTERF5, which interacts with DmTTF binding sites and has the opposite effect on transcription in comparison with DmTTF (Bruni et al., 2012). It was recently shown that DmTTF and MTERF5 can affect mtDNA replication as DmTTF knockdown decreased whereas MTERF5 knockdown increased site-specific pausing (Joers et al., 2013). These results support the idea that mitochondria possess mechanism to avoid collisions between replication and transcription machineries. How common are these collisions, how they are resolved and what are their consequences is not yet known.

1.3.7 Regulation of mtDNA copy number

MtDNA is a multicopy genome within a eukaryotic cell and depending on the cell and tissue type its numbers can vary between 200 and 100 000 per nucleus (Wolff et al., 2013, Masuyama et al., 2005, Shmookler Reis and Goldstein, 1983, Bogenhagen and Clayton, 1974). It is still poorly understood what are the molecular factors controlling mtDNA copy number. On the one hand a certain amount of mtDNA is required to provide templates for mitochondrial gene expression and to ensure that each daughter cell receives sufficient amount of mtDNA during cytokinesis. On the other hand maintaining surplus of mtDNA could be considered energetically wasteful due to the high requirement of deoxynucleotides needed for mtDNA replication (Gandhi and Samuels, 2011a, Bradshaw and Samuels, 2005). Nevertheless, there are several examples in different species where changes in mtDNA integrity are known to lead to a compensatory upregulation of the total mtDNA copy number (Jiang et al., 2017a, Tsang and Lemire, 2002, Beziat et al., 1997, Bogenhagen and Clayton, 1974) pointing towards the existence of specific regulatory mechanisms. Because all proteins required to maintain mtDNA are encoded by the nDNA, the control of mtDNA copy number is bound to be under

nuclear control and we know already a great deal about the factors controlling the expression of these genes in multiple systems (Fernandez-Moreno et al., 2013, Scarpulla, 2008, Lefai et al., 2000b). Perhaps surprisingly, studies performed using the hemizygous *PolγA* mice suggest that at least in mice the control of *PolγA* expression is not under tight control as the expression from the remaining allele is not upregulated (Hance et al., 2005). POLγA is also constitutively expressed in the absence of mtDNA (Davis et al., 1996) and its overexpression has no effect or mtDNA copy number *in vitro* (Schultz et al., 1998) or can even lead to mtDNA depletion *in vivo* (Lefai et al., 2000a). Of course, the presence of nuclear control does not exclude the possibility of post-translational control mechanisms within mitochondria that could be used to fine tune mtDNA copy number in response to changes in cellular environment. It also noteworthy that mtDNA copy number does not necessarily correlate with mitochondrial transcript levels as 50% decrease in mtDNA copy number does not affect RNA steady state levels in mice or flies (Sanchez-Martinez et al., 2012, Larsson et al., 1998). This would suggest that mitochondria have yet uncharacterized mechanisms to either increase mtDNA transcription or decrease transcript degradation in response to changing mtDNA copy number.

Currently, there are only two proteins verified to regulate mtDNA copy number: TFAM and TWINKLE. As described above, mtDNA replication and transcription are connected because the transcription machinery is needed to synthesize the RNA primers used by the replication machinery. TFAM levels are known to correlate well with the mtDNA copy number with one known exception (Kuhl et al., 2016) but it has been difficult to separate whether this regulation is taking place through its effects on mitochondrial transcription or packaging of mtDNA. An interesting insight was provided by a study where human TFAM was expressed in mice. Human TFAM cannot interact properly with the mouse transcription machinery but was nevertheless able to increase mtDNA copy number suggesting that TFAM has a direct role in regulating mtDNA copy number independently of its function in mtDNA transcription (Ekstrand et al., 2004). Therefore, there has been a considerable interest to better understand how TFAM levels are controlled. Several studies have shown that TFAM levels seem to be regulated by a mitochondrial matrix protease Lon and two studies have described how unbound "free" TFAM would be targeted for degradation through phosphorylation. (Zhang et al., 2015, Lu et al., 2013, Matsushima et al., 2010). Interestingly, too high TFAM levels can have detrimental effects as high TFAM/mtDNA ratio can block mtDNA replication and transcription (Khan et al., 2016, Farge et al., 2014) and lead to developmental lethality in flies (Cagin et al., 2015) showing that the TFAM levels need to be controlled to ensure organelle function. It will be of high interest in the future to better understand what are the signaling pathways controlling TFAM phosphorylation, degradation and thereby mtDNA levels.

TWINKLE remains the only protein that, in addition to TFAM, can increase mtDNA copy number when overexpressed in mice (Ylikallio et al., 2010b, Tyynismaa et al., 2004). Because TWINKLE is part of the mitochondrial replisome (Fig. 1.3), these results would suggest TWINKLE is a limiting

factor in mtDNA replication. Interestingly, the effects of TWINKLE and TFAM overexpression on mtDNA copy number are additive resulting in ~5 fold increase in mtDNA levels (Ylikallio et al., 2010b). This double overexpression mouse presented with mtDNA deletions, decreased mitochondrial transcription and respiration showing that inducing high mtDNA copy number by overexpressing mtDNA maintenance proteins can have detrimental effects. One possibility for the observed mtDNA maintenance defects could stem from inadequate dNTP pools to support high mtDNA copy number (Gandhi and Samuels, 2011a, Bradshaw and Samuels, 2005), leading to replication stalling and mutagenesis. Not all studies support the view that TWINKLE is a limiting factor in mtDNA replication. For instance, overexpressing DmTWINKLE in fruit flies has no effect on mtDNA copy number (Sanchez-Martinez et al., 2012) suggesting organism or dosage specific differences.

In addition to the replisome proteins, mtDNA replication requires also dNTPs as building blocks and indeed nucleotide pool imbalances are known to cause numerous cellular problems (Pai and Kearsey, 2017, El-Hattab et al., 2017a, Da-Re et al., 2014, Bourdon et al., 2007). It has therefore been suggested that the size of nucleotide pools determines mtDNA copy number in any given cell type (Tang et al., 2000). This theory has gained recent support from transgenic models where overexpression of some of the enzymes involved in nucleotide pool maintenance led to increased mtDNA copy number (Tufi et al., 2014, Hosseini et al., 2007) but it should be noted that overexpression of other related enzymes has no effect (Krishnan et al., 2013) or can even lead to mtDNA depletion (Ylikallio et al., 2010a). Therefore, dNTPs as regulators of mtDNA copy number remains a controversial subject.

1.4 MITOCHONDRIA IN DISEASE AND AGEING

1.4.1 MtDNA point mutations

As described above, mitochondria have maintained only the minimal number of genes to produce some of the essential core components of the OXPHOS. Studies done almost 40 years ago counterintuitively showed that despite the importance of these genes, the mtDNA of primates evolves 10 times faster than the nuclear DNA (Brown et al., 1979). It was recognized already then that this rapid evolution could be caused by two, not mutually exclusive, processes; high mutation rate or high fixation rate of mutations. Although we have increased our understanding by leaps and bounds regarding the relative contributions of these mechanisms, even today both of these mechanisms remain highly studied subjects. The multicopy nature of mtDNA makes it a challenging target to study because mutations homoplasmic or heteroplasmic. Recent advances in next-generation sequencing (Stewart and Chinnery, 2015) have increased interest in this area of research as it has become apparent that all humans carry some level of heteroplasmic mtDNA mutations, many of which are pathogenic (Ye et al., 2014, Payne et al., 2013).

To better understand the mechanism affecting the formation of mtDNA mutations it is helpful to categorize the type of changes to extrinsic sources, such as environmental toxins, and intrinsic sources, such as DNA replication mistakes. Additionally, the contribution of both sources to mtDNA mutagenesis is further modulated by the presence, or in the case of mitochondria, absence of DNA repair pathways (Kazak et al., 2012). Extrinsic factors potentially leading to mtDNA mutations include ultraviolet (UV) radiation, environmental toxins and nucleotide analogs. However, *in vitro* studies have shown that POLγ rarely bypasses various types of DNA damage and in the rare cases POLγ bypasses adducts, it inserts the correct base (Table 1.1). Some data suggest that stalling of POLγ is not mutagenic. For instance, cigarette smoke and UV-light, both highly mutagenic in nDNA, do not increase mtDNA mutation load (Ju et al., 2014). Similarly, it was recently shown that although one type of PAH, benzo[a]pyrene (B[a]P) and an alkylating agent N-ethyl-N-nitrosourea (ENU), can both create DNA adducts both in nDNA and mtDNA, these adducts are converted into mutations only in nDNA (Valente et al., 2016). Although less studied, it has been also suggested that TWINKLE might have difficulties in bypassing bases with oxidative damage (Khan et al., 2016), which is expected to have similar consequences as the stalling of POLγ. Because mtDNA is a multicopy genome, stalling of the replisome likely results in degradation of the damaged DNA without further consequences. Although stalling of the mitochondrial replisome is not mutagenic *per se*, it might increase the clonal expansion of already existing mtDNA mutations as has been observed in HIV patients.

Table 1.1: Publications reporting POLγ stalling at damaged sites

Damage	Effect	Publication
UV-damage	Stall/Bypass	(Kasiviswanathan et al., 2012)
8-oxoG	Stall/Bypass	(Stojkovic et al., 2016, Garcia-Gomez et al., 2013, Graziewicz et al., 2007, Pinz et al., 1995)
Abasic site	Stall	(Kozhukhar et al., 2016, Garcia-Gomez et al., 2013, Pinz et al., 1995)
Polycyclic aromatic hydrocarbons (PAH)	Stall	(Graziewicz et al., 2004)
Acrolein	Stall	(Kasiviswanathan et al., 2013)
Platinum-DNA-adduct	Stall	(Vaisman et al., 1999)

Some pharmacological compounds are known to inhibit POLγ such as nucleotide analog HIV reverse transcriptase inhibitors (NRTIs) leading to mitochondrial toxicity (Szymanski et al., 2015, Lewis and Dalakas, 1995, McKenzie et al., 1995). It was later observed that HIV patients treated with NRTIs show premature ageing phenotypes and that this might be caused by mtDNA mutations (Payne et al., 2011). Interestingly, this increase in mtDNA mutation load was not caused by increased mutagenesis but increased mtDNA turnover which in turns accelerates the random drift of already existing mtDNA mutations leading to focal mitochondrial dysfunctions. It will be interesting to see in the future whether other compounds inhibiting POLγ or mtDNA damage could also result in increased mtDNA mutation loads by affecting mtDNA turnover. This clonal expansion of low level mtDNA mutations

could be also relevant in the case of therapeutic manipulations of mtDNA as removing mtDNA molecules carrying a pathogenic mutation (Pereira and Moraes, 2017) could result in the expansion of these mutations.

One of the intrinsic causes for mtDNA mutations is imbalance in mitochondrial nucleotide pools. It is known that imbalances in nucleotide pools can decrease POLγ replication fidelity *in vitro*, cause mtDNA deletions in HeLa cells and mtDNA depletion in mice (Dalla Rosa et al., 2016, Ylikallio et al., 2010a, Song et al., 2005, Song et al., 2003, Kunkel and Soni, 1988). Nucleotide pools are different in various tissues and change depending on the cell cycle (Ferraro et al., 2005). In humans, imbalances in cellular nucleotide pools are known to cause several mitochondrial diseases (El-Hattab et al., 2017a, Da-Re et al., 2014). One of these diseases is mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). This disease is caused by mutations in thymidine phosphorylase (TP) which degrades thymidine. Decreased or abolished of TP activity leads to elevated dTTP levels relative to other nucleotides and in patients this nucleotide pool imbalance leads to mtDNA point mutations, deletions and depletion (González-Vioque et al., 2011, Nishigaki et al., 2003). It is still unclear how these dNTP pool differences lead to mtDNA instability and why certain fluctuations lead to mtDNA depletion whereas others cause mtDNA point mutations and deletions. It is also possible that the intrinsic dNTP pool asymmetries observed in different tissues might contribute to tissue specific mtDNA mutagenesis patterns and frequencies (Wheeler and Mathews, 2011, Ferraro et al., 2006, Song et al., 2005).

Nucleotide pool imbalances likely cause mutations through their effect on POLγ. In fact, increasing amount of evidence suggests that most mtDNA mutations originate from replication errors. Most of these studies are based of interpreting mtDNA mutation patterns as oxidative damage creates usually G>T transversion mutations whereas most polymerase errors are transition mutations (Zheng et al., 2006, Lindahl, 1993). A recent sequencing study of mtDNA from young and old individuals showed a clear increase in mtDNA mutation load with age but the mutation pattern observed was inconsistent with oxidative damage (Kennedy et al., 2013). Similar absence of hallmarks of oxidative damage has also been observed in tumors and ageing mice (Ju et al., 2014, Pereira et al., 2012, Ameur et al., 2011). In addition, a recent study quantified mtDNA mutations in a fly model with reduced SOD2 activity and OGG1-deficiency, which led to increased levels of ROS and decreased removal of oxidized guanosine, respectively (Itsara et al., 2014). There was no increase in transversion mutations suggesting that ROS is not a major player in mtDNA mutagenesis. In summary, most studies suggest that mtDNA mutations originate from replication errors (Szczepanowska and Trifunovic, 2017, Kauppila et al., 2017).

1.4.2 MtDNA deletions

The first mtDNA deletions were characterized already in 1988 in patients with mitochondrial myopathy (Holt et al., 1988) and it has been intensively researched ever since whether these deletions

are associated with decreased mitochondrial function in ageing tissues and what are the mechanisms leading to the formation of mtDNA deletions. In mitochondrial disease patients these deletions are often caused by mutations in nuclear genes necessary for mtDNA maintenance or nucleotide synthesis and transport (Ahmed et al., 2015). However, in many cases there is no clear nuclear trait to be identified. Mapping of the breakpoints of circular mtDNA deletions has provided us with hints on how these molecules might originate. Interestingly, the ends of these deletions are not randomly distributed along the mtDNA but instead show rather specific distributions (Dong et al., 2014, Samuels et al., 2004). Most of the deletions (~90%) are flanked by homologous or near-homologous repeats suggesting the involvement of replication slippage in the formation of these deletions. Additionally, the majority of the detected deletions are missing a part of the major arc of mtDNA probably for two reasons. First, the major arc encompasses most of the short homologous regions with the potential to cause a replication slippage (Krishnan et al., 2008). Secondly, for a deleted molecule to propagate it would have to include both origins of replications (O_H and O_L). Therefore a deletion lacking one or both origins of replication would never be able to accumulate to a significant level.

What is leading to replication slippage during mtDNA replication? There are two prerequisites for replication slippage to take place, namely the presence of a single-stranded template and pausing of the replication machinery. The presence of a single-stranded template allows the repeat regions to mispair. This mispairing can create various DNA secondary structures such as hairpins, cruciforms and G-quadruplex structures (Dong et al., 2014, Damas et al., 2012). In the strand-displacement model of replication, large portions of the mtDNA can be single-stranded enabling the formation of secondary structures than can induce stalling of the replisome (Bharti et al., 2014) although the formation of these structures should be prevented by mtSSB (Fusté et al., 2014). Stalling of the replisome would allow the end of the newly synthesized strand to reanneal with downstream repeats. It is largely an unexplored area, what factors cause the replisome to pause. One factor could be the formation of secondary structures in the template DNA. Another possibility would be stalling of the replisome due to various types of DNA damage although to date efforts to detect mtDNA deletions in the presence of for example increased oxidative stress have provided mixed results (Wanagat et al., 2015, Zhang et al., 2002). However, there seems to be a link between catecholamine metabolism and mtDNA deletions both in neurons and adrenals (Neuhaus et al., 2017, Neuhaus et al., 2014). As mentioned above, not all deletion breakpoints are localized with flanked repeats. Several other mechanisms can also contribute to the formation of these deletions such as replication products from partial mtDNA duplications and repair of double-strand breaks (Fukui and Moraes, 2009).

Studies reporting the presence of circular mtDNA molecules with deletions vary greatly in their deletion abundance estimations raising the question whether the deletions have any biochemical impact (Brierley et al., 1998). As is the case with mtDNA point mutations, deletions have to also reach a certain threshold level (~60%), often lower than that of point mutations (~80%), before impairing

mitochondrial function. Some of the variation between different studies can be at least partially explained by the different approaches used to quantify deletion levels as has been previously shown in humans (Brierley et al., 1998) and more recently in a mouse model with a mosaic mitochondrial deficiency (Baris et al., 2015). For instance, long-range PCR reactions using tissue homogenates often show that less than 1% of the mtDNA molecules carry deletions. More sensitive real-time PCR methods of the same tissue homogenate produce estimates in the 10-30% range. Furthermore, when deletions are quantified specifically from cytochrome c oxidase (COX) deficient muscle fibers their levels reach or even surpass the common threshold levels needed to cause a biochemical defect, underlining the importance of using single cell analysis as opposed to the use of tissue homogenates (Zambelli et al., 2017). These COX deficient regions of muscle fibers with high deletion levels are also accompanied by muscle atrophy (Wanagat et al., 2001, Cao et al., 2001). These results strongly suggest that the mtDNA deletions are the driving force behind these OXPHOS dysfunctions.

Two relevant mouse models have been engineered to study the biological significance of mtDNA deletions. The first model, also known as the mtDNA deletor mouse, expresses TWINKLE with dominant mutation leading to the accumulation of low level but large-scale mtDNA deletions and progressive OXPHOS deficiency (Tyynismaa et al., 2005). The second mouse model expresses mitochondrial targeted restriction enzyme (Fukui and Moraes, 2009). These mice accumulate mtDNA deletions leading to decreased OXPHOS capacity therefore showing that mtDNA deletions can have detrimental effects when present at high relative heteroplasmy levels. A fly model expressing mitochondrial targeted restriction enzymes has also been used to introduce mtDNA deletions in postmitotic tissues (Kandul et al., 2016).

A special subclass of deleted mtDNA molecules is linear molecules with deletions. Linear mtDNA with deletions have been detected in the mtDNA mutator mouse (Bailey et al., 2009, Trifunovic et al., 2004) in addition to patients with mutations in the mitochondrial genome maintenance exonuclease 1 (MGME1). In the mtDNA mutator mouse, these deletions were detectable already in the embryo, the amount of deletions did not change with time and was similar in all tissues (Kukat and Trifunovic, 2009). In addition, it has been shown in other models that linear mtDNA is quickly degraded within mitochondria (Moretton et al., 2017, Bayona-Bafaluy et al., 2005) suggesting that the mtDNA mutator mouse deletions represent failed and transient replication products. A model was recently put forward suggesting how these linear deletions are formed in both mtDNA mutator mice and MGME1 patients (Uhler et al., 2016, Macao et al., 2015). These linear deletions are unlikely to have an effect on ageing due to their relatively low abundance and the much more significant effect of the inherited mtDNA point mutations (Ross et al., 2013, Edgar et al., 2009). Linear deletions have not been detected in the ageing tissues suggesting they are not contributing to ageing and are specific for the above-mentioned genetic defects.

1.4.3 MtDNA inheritance and purifying selection

In most metazoans, mtDNA is transmitted exclusively down the maternal line. Paternal leakage has been described in flies, mice and in a single male patient (Carelli, 2015) but these are more exceptions that prove the rule. Indeed, there is an array of species-specific mechanisms ensuring the exclusion of paternal mtDNA from oocytes. For instance, in nematodes, paternal mitochondria are engulfed by autophagosomes whereas in fruit flies paternal mtDNA is degraded by EndoG endonuclease during spermatogenesis (Sato and Sato, 2013). In mammals, mtDNA copy number is strongly downregulated during spermatogenesis (Rantanen et al., 2001, Larsson et al., 1997, Larsson et al., 1996) and paternal mitochondria are ubiquitinylated and targeted for degradation (Sato and Sato, 2013). In addition to these active mechanisms the oocyte contains several orders of magnitude more mtDNA than the sperm and therefore the paternal mtDNA can be diluted out. This does not still explain what is the advantage of uniparental inheritance of mtDNA. One benefit of excluding the paternal mtDNA is that DNA mutations are known to accumulate much faster in the male than female germline due to the constant production of male gametes (Kong et al., 2012). Another benefit could be to retain mtDNA in homoplasmic state as uniparental inheritance combined with random segregation of mitochondria seems to help in avoiding mutational meltdown (Radzvilavicius et al., 2017). As described below, there are mechanisms in the female germline returning mtDNA into the homoplasmic state and mice carrying two different mtDNAs show negative physiological effects (Sharpley et al., 2012, Hirose et al., 2017).

Although POLγ has a tendency to introduce preferably certain mutations and this mutagenesis might be affected by the surrounding sequence context, from a functional point-of-view mtDNA mutations introduced by POLγ are random. It is only afterwards that selection can limit the propagation of mtDNA molecules carrying harmful mutations. Indeed it has been observed that in somatic tissues mtDNA mutations show neutral drift (Baines et al., 2014, Greaves et al., 2012, Pereira et al., 2012, Elson et al., 2001) unless they affect regions essential for mtDNA maintenance (Wanrooij et al., 2012). Therefore the levels of individual mtDNA mutations can increase or decrease stochastically within the lifetime of a cell and individual. If the level of a pathogenic mutation surpasses a certain mutation and cell type specific threshold, usually 60% to 90%, it can lead to OXPHOS dysfunction (Rossignol et al., 2003, Boulet et al., 1992, Larsson et al., 1992). This random drift of mtDNA mutations is rather slow process as supported both computational models and human studies (Taylor et al., 2003, Elson et al., 2001, Brierley et al., 1998, Muller-Hocker, 1990, Muller-Hocker, 1989). Although there does seem to be any negative selection against pathogenic mutations in somatic tissues, it is widely known that in populations mtDNA protein coding genes show selection against amino acid changing mutations (Rand, 2008), a sign of purifying selection.

MtDNA mutations exist in a “hierarchy of populations” (Rand, 2011) meaning that there are multiple mtDNAs within a mitochondrion, multiple mitochondria in each cell, multiple oocytes in each

reproducing female and multiple females in a population. The (presumably) random sampling of mtDNAs for replication, random segregation of mitochondria in cytokinesis (for a contradictive result see (Katajisto et al., 2015)) and random sampling of oocytes combined with bottlenecks provide raw material for natural selection to work on. Indeed there has been a considerable interest in understanding purifying selection taking place in females (Stewart and Larsson, 2014). Several not mutually exclusive mechanisms have been proposed to explain this phenomenon. First, among the 10^5 copies of mtDNA, only a subset is set aside for the germline development creating essentially a bottleneck. Secondly, there seems to be a negative selection against amino acid changing mutations (Stewart et al., 2008). Thirdly, high heteroplasmy level tRNA mutations show negative selection in developing embryo (Freyer et al., 2012). Lastly, abundant mtDNA mutations have been shown to decrease female fertility (Ross et al., 2013). Most studies focusing on elucidating the mechanisms of purifying selection have focused on mammals. However, fruit flies have been also suggested to possess purifying selection. It was shown using two complementing mtDNAs that there is some selection against mtDNA carrying a temperature sensitive mutation in the late germarium stage (Ma et al., 2014) and that this selection might take place through selective replication of mtDNA molecules (Hill et al., 2014). Whether this selection is only specific to this temperature sensitive mutant is not known. There might also be selection in later stages of fly development as it was described recently how only a subset of mitochondria are selected to form fly primordial germ cells (PGCs) during cytoplasmic streaming (Hurd et al., 2016) but further studies are needed to show whether this affects the purifying selection of mtDNA. Because flies are ectotherms, temperature of the environment can greatly affect their physiology. Indeed, fluctuations in environmental temperature can have as far reaching consequences as affecting the speed of segregation of mtDNA molecules (De Stordeur, 1997, Matsuura et al., 1993).

In the context of purifying selection and mtDNA deletions, there are some interesting differences between humans and model organisms. In humans, mtDNA deletions are almost never inherited (Chinnery et al., 2004), whereas there are several populations of nematodes and flies carrying large mtDNA deletions for multiple generations (Tsang and Lemire, 2002, Beziat et al., 1997). Some results suggest that the limited population size of model organisms in laboratory conditions is limiting negative selection of deleted molecules (Phillips et al., 2015). In addition, some of these deletions are linked to unidentified nuclear traits suggesting constant formation of deletions (Farge et al., 2002, Le Goff et al., 2002). The modest negative selection against mtDNA deletions in the model organism could also stem from the differences in oocyte development in these organisms (Strome and Updike, 2015). For instance, fruit fly oocytes possess substantially more mtDNA in comparison with humans (Wolff et al., 2013), which might limit the strength of negative selection.

1.4.4 Mitochondria and ageing

Ageing can be defined as the slow decline in organismal fitness with advancing age. These chances are not limited to humans and animals in captivity, but instead most metazoans are known to present with some signs of ageing (Nussey et al., 2013). To some extent this decline in fitness with age is expected as evolutionary selection is minimal against traits that are harmful after reproduction age. Last decades have provided us with valuable insights into the changes that take place at the cellular level with age. These characteristics include, but are not limited to, shortening of the telomeres, genome instability, stem cell exhaustion, loss of proteostasis and mitochondrial dysfunction (Lopez-Otin et al., 2013).

Denham Harman hypothesized already in the 50's that ROS might be a major contributor to the natural ageing process as ROS can damage all cellular macromolecules (Harman, 1956). When it was later discovered that mitochondria are the major source of cellular ROS, this theory was refined into mitochondrial theory of ageing (Alexeyev, 2009, Harman, 1972). Originally ROS was considered to be only a harmful side product of cellular respiration and it took years to understand that ROS can also function as a signaling molecule (Scialo et al., 2017, Lagouge and Larsson, 2013). There is considerable evidence now that cells accumulate oxidative damage with age (Bokov et al., 2004). However, it has not been shown that this damage would contribute to ageing and in some models increasing ROS can have even lifespan extending effects (Scialo et al., 2016). Current mitochondrial ageing research is slowly moving past the idea that ROS damage is the cause for age-associated mitochondrial dysfunction. Instead, ROS should be handled as signaling molecules that can affect for instance stem cell maintenance (Kauppila et al., 2017).

Studying cellular changes in ageing tissues is challenging because the relative levels of different cell types and tissue composition change with age due to inflammations, increasing fibrosis, regeneration and changing environment (Vaitkus et al., 2015, Gram et al., 2015, Kular et al., 2014, Velarde, 2014). Nevertheless, multiple studies have tried to identify consistent changes in mitochondria with age. Some of the more robust changes include changes in mitochondrial morphology, abundance and OXPHOS activity (Shigenaga et al., 1994) whereas others, such as changes in mtDNA copy number, have provided mixed results (Kazachkova et al., 2013). One of the most consistent age-associated changes in mitochondria is the accumulation of mtDNA mutations and deletions which segregate randomly within each cell resulting in mosaic tissue OXPHOS defects (Greaves et al., 2014, Kennedy et al., 2013, Larsson, 2010, Bua et al., 2006). For example, based on the serial cross-sectioning of skeletal muscles of rats, monkeys and humans show that in older individuals 5%-15%, 28%-60% and 31% of fibers have biochemical defects at some point along their length, respectively (Bua et al., 2006, Wanagat et al., 2001, Lopez et al., 2000). Alone, however, these studies only show a positive correlation between mtDNA mutation load and ageing. More sophisticated studies using coupled mtDNA mutation load analysis and tissues histology have shown that individual cells with high

mtDNA mutation load correlate with impaired OXPHOS function and cell death (Herbst et al., 2007, Cao et al., 2001) and increasing the rate of mutation segregation by inducing mitochondrial biogenesis worsens the ageing effects (Herbst et al., 2016).

This random drift of mtDNA mutations within a cell is a relatively slow process as OXPHOS dysfunction is rarely seen in individuals below the age of 30. It has been therefore suggested that only inherited mtDNA mutations or mutations introduced early in life have sufficient amount of time to expand clonally and lead to OXPHOS dysfunction. It is interesting to note that in all metazoans studied so far mtDNA mutations seem to be introduced early in life, including zebra fish, mice and humans (Otten et al., 2016, Greaves et al., 2014, Ameur et al., 2011). However, it seems that in comparison to humans, some shorter lived organisms, like mice and flies, show limited age-associated clonal expansion of mtDNA mutations probably due to the shorter lifespan of these organism (Itsara et al., 2014, Greaves et al., 2011, Yui and Matsuura, 2006, Yui et al., 2003). It has been therefore questioned whether mtDNA mutations, especially somatic, can limit the lifespan of short-lived species such as fruit flies (Kowald and Kirkwood, 2013).

It was only the engineering of proofreading deficient *PolgA*$^{mut/mut}$ mouse (mtDNA mutator mouse) that brought mtDNA mutations to the limelight of ageing research (Kujoth et al., 2005, Trifunovic et al., 2004). These knock-in mice have a single aspartate to alanine mutation (MmD257A) in the exonuclease domain of *PolgA* and this residue is critical in controlling catalytic magnesium ions needed for exonuclease activity from yeast to humans (Longley et al., 2001, Vanderstraeten et al., 1998). At the phenotype level these mice show several signs of ageing, including progressive hair loss, curvature of the spine, reduced body size and increased mortality. At the molecular level the mtDNA of these mice carry mtDNA point mutations, linear deletions between the origins of replication and control region multimeres (Williams et al., 2010, Vermulst et al., 2008, Trifunovic et al., 2004). It was later shown that the premature ageing phenotype is largely caused by the inherited and clonally expanded mtDNA mutations (Ross et al., 2013) and even nuclear WT progeny of heterozygous mtDNA mutator females, which inherit on average two mutations per mtDNA, have shortened lifespan (Ross et al., 2014). An elegant biochemical characterization of the mtDNA mutator mice showed that the premature ageing phenotype is driven by decreased OXPHOS stability due to increased turnover of OXPHOS subunits (Edgar et al., 2009).

All in all, there is now correlative evidence showing that the level of mtDNA mutations increases with age in various organisms and that experimentally increasing mtDNA mutation load leads to a premature ageing phenotype. To unequivocally show that mtDNA mutations are contributing to the natural ageing process one would have to experimentally decrease mtDNA mutation load and test whether this would lead to lifespan extension. Decrease in mtDNA mutation load could be, at least in theory, achieved by increasing the absolute number of WT mtDNA copies, improving mtDNA repair

mechanisms or increasing the accuracy of POLγ, which is responsible for the introduction of most, if not all, mtDNA mutations.

1.5 ANTIMUTATOR DNA POLYMERASES

During evolution polymerases have been optimized for accuracy and speed. The error rates of DNA polymerases seem to reflect the amount of DNA synthesized per generation (Lynch, 2011). This feels intuitive because when DNA polymerase has more chances to introduce errors (replicating a substantial proportion of cellular DNA) there will be also higher selection pressure to increase the accuracy of the polymerase and vice versa. In this light it is interesting to note that despite the miniscule size of the mtDNA in comparison with nuclear chromosomes, POLγ is the most accurate mammalian DNA polymerases (Lynch, 2011). This could stem from the fact that there are thousands of mtDNA copies per cell (~1% of all cellular DNA) and that mtDNA replication is relaxed, i.e. it is cell cycle independent. Nevertheless, from flies to humans mtDNA has been shown to accumulate mutations several times faster than the nuclear DNA (Haag-Liautard et al., 2008, Howell et al., 2003). These mutations are probably caused by constant mtDNA turnover and inadequate repair mechanisms in mitochondria (Kazak et al., 2012). In addition, due to the multicopy nature of mtDNA even harmful mutations can drift at low level without any significant fitness cost and would not therefore be under strong negative selection in contrast to mutations in nDNA.

DNA replication is an astonishingly precise process and composed of several steps. First, polymerases have a tight nucleotide selection in the catalytic core to ensure proper Watson-Crick geometry with the accuracy of 10^{-4}-10^{-5} mut/bp for accurate polymerases like POLγ (Johnson and Johnson, 2001). The replication error rate is further decreased by exonucleolytic proofreading of misincoporated bases which in the case of POLγA decreases error rate up to 20 to 1500-fold depending on the system (Longley et al., 2001, Vanderstraeten et al., 1998). The last mechanism to increase replication accuracy is post-replicative mismatch repair (MMR). However, MMR has not been reported to exist in mitochondria to date (Kazak et al., 2012).

Targeted and random mutagenesis have been used extensively in the past to better understand the function of DNA polymerases (Herr et al., 2011). Although most mutations have negative effects such as decreased DNA binding or lack of polymerase activity, some mutations have been shown to increase proofreading efficiency of DNA polymerases. The “antimutator” phenotype of these mutated polymerases could stem from increased nucleotide selection, decreased polymerase activity or higher propensity to dissociate upon mismatch insertion (Reha-Krantz, 2010). The latter two both increase the proofreading/polymerase ratio giving the polymerase more opportunities to proofread. Engineering antimutator DNA polymerases is further complicated by the observations that amino acid changes

increasing the proofreading/polymerase ratio of certain DNA mutations might even decrease the ratio of other mutations (Reha-Krantz, 2010).

Several single amino acid mutations in *E. coli* Pol I have been shown to produce an antimutator phenotype (Minnick et al., 1999). Two of these Pol I mutations, Q849A and H881A, are of special interest as they are conserved in family A DNA polymerases, including POLγA. These residues are both on the catalytic core of the polymerase and interact with the incoming nucleotide or with the template base. Mutating one of these residues into alanine makes the catalytic core more specious and decreased the fidelity on the mutants. In the case of both mutations it seems that they increase the propensity of the polymerase to drop off upon mismatch insertion (Minnick et al., 1999) thereby giving the polymerase more opportunities to proofread. Although these mutations in the polymerase domain make polymerases more accurate, it is yet to be investigated whether the decreased fidelity would be sufficient to maintain adequate DNA replication speed *in vivo*.

Recently, an elegant genetic screening accompanied by thorough biochemical characterization was carried out by Foury and Szczepanowska to discover antimutator variants of yeast mitochondrial DNA polymerase MIP1 (Foury and Szczepanowska, 2011). Screening 3000 yeast transformants in a mismatch-deficient background resulted in 8 potential antimutator clones. As expected, all mutants showed changed exonuclease/polymerase ratios and many of them presented with decreased DNA synthesis resulting in mtDNA depletion *in vivo*. Interestingly, all but one candidate antimutator lost their antimutator phenotype in WT background suggesting that any mutation changing the exonuclease/polymerase ratio has to be extremely subtle not to limit DNA replication speed with detrimental effects.

1.6 MITOCHONDRIA AND STEM CELLS

During development some of the somatic stem cells are sequestered into so called stem cell niches where these cells are known to acquire quiescence (Li and Bhatia, 2011). Staying in quiescence is essential for maintaining stem cell self-renewal potential and loss of quiescence can lead to stem cell depletion, which would limit tissue regeneration upon damage (Cheung and Rando, 2013). The quiescent state correlates often with the presence of an extrinsically hypoxic niche, their dependency on glycolytic metabolism and presence of only a few mitochondria (Shyh-Chang and Ng, 2017). It has been hypothesized that this low dependency on OXPHOS would exist to minimize the production of ROS, which as a signaling molecule is known to promote stem cell differentiation (Morimoto et al., 2013).

MtDNA mutations are known to accumulate with age in somatic tissues, including stem cells (Fellous et al., 2009, McDonald et al., 2008, Taylor et al., 2003). Mutations could be expected to accumulate

even faster in somatic stem cells in comparison with post-mitotic cells because in the former mtDNA has to be replicated during meiosis in addition to constant mtDNA turnover. Indeed, the number of COX-deficient cells in colonic crypts increases with age (Taylor et al., 2003) and the colonic crypts of mtDNA mutator mice resembles that of old humans (Baines et al., 2014).

Both mouse and fly models have provided us with notable insights into stem cell function in the presence of mitochondrial dysfunction. In the mtDNA mutator mouse several stem cell pools seem to be affected by mtDNA mutations. For instance, small intestine-derived organoids cannot fully develop (Fox et al., 2012), hematopoietic stem cells present with abnormal differentiation (Ahlqvist et al., 2012), the number of quiescent neuronal stem cells is decreased accompanied by decreased self-renewal potential (Ahlqvist et al., 2012) and early erythroid development cannot proceed properly (Ahlqvist et al., 2015a, Li-Harms et al., 2015, Norddahl et al., 2011) leading to severe progressive anemia (Chen et al., 2009, Trifunovic et al., 2004). Some of these changes can be rescued by n-acetyl-l-cysteine (NAC) supplementation, which functions as a precursor for glutathione and as a direct ROS scavenger, suggesting that altered ROS signaling in stem cells might drive some of the observed phenotypes (Hämäläinen et al., 2015, Ahlqvist et al., 2012). Together these studies show how increased mtDNA mutation load can affect cell signaling and promote cell proliferation over stemness with detrimental effects.

The effects of mtDNA mutations on fruit fly stem cells have not been addressed to date due to the lack of proper genetic models. However, models with mutations in nuclear encoded mitochondrial proteins have been used to study the effects of impaired mitochondrial function on fruit fly stem cells. These studies have shown that stem cell reliance on glycolysis is important in maintaining stemness (Schell et al., 2017). Additionally, a screen trying to identify genes important for fruit fly follicle stem cell (FSC) maintenance discovered several mitochondrial proteins, including *tam* (DmPOLγA) that were needed for FSC maintenance (Wang et al., 2012). Some of the evidence suggested that this mitochondrial dysfunction would impair ROS signaling in FSCs leading to loss of stem cells. Ethyl methanesulfonate (EMS) screens to find cell cycle regulators discovered that proper mitochondrial function is also needed for cell cycle progression (Liao et al., 2006, Mandal et al., 2005). Follow-up studies were able to distinguish two signaling pathways by which mitochondrial dysfunction can block cell cycle progression and stem cell maintenance (Owusu-Ansah and Banerjee, 2009, Owusu-Ansah et al., 2008). In the first model, complex IV deficiency caused ATP depletion mediated activation of AMPK and p53 leading to loss of cyclin E. In the second model, complex I deficiency led to increased ROS which activated JNK-FOXO and JNK-Polycomb pathways resulting in the upregulation of cyclin E-CDK2 inhibitor Dacapo. These studies also showed that similar to mammals, lowering ROS levels decreases the differentiation of multipotent hematopoietic progenitor stem cells whereas increasing ROS levels has the opposite effect.

1.7 TREATING MITOCHONDRIAL DISEASES

There are currently no treatments for mitochondrial diseases (El-Hattab et al., 2017b, Pfeffer et al., 2012). Several approaches have been tested in model organisms and even in humans to rescue mitochondrial dysfunction (Viscomi, 2016) and can be categorized into four groups. Firstly, there are unspecific methods that could be potentially applied to variety of diseases. These methods are based on e.g. increasing mitochondrial biogenesis, affecting mitochondrial shape, modifying redox balance and using transgenic models to bypass OXPHOS blockages. Secondly, some promising gene therapy approaches have been developed to specifically eliminate mtDNA copies carrying a pathogenic mutation. Thirdly, a mitochondrial replacement therapy can be used to move nucleus into a cell with functional mitochondria and most importantly mtDNA (Richardson et al., 2015). Lastly, instead on focusing on the primary defect, several studies have tried to mitigate harmful secondary effects originating from mitochondrial dysfunction, such as removing accumulating toxic metabolites. It is noteworthy that in many cases approaches that improve mitochondrial function in flies also work in vertebrate models suggesting that fly can be used to find possible therapeutic targets for mitochondrial diseases.

1.7.1 Mitochondrial nucleotide metabolism

As discussed above, changes in dNTP pools can lead to loss of mtDNA maintenance in the form of mtDNA deletions, point mutations and depletion. Nucleotides are supplied both through *de novo* synthesis in cytosol followed by transport into mitochondria and through nucleotide salvage pathway within mitochondria. In the salvage pathway two enzymes, mitochondrial thymidine kinase 2 (TK2) and deoxyguanosine kinase (DGUOK), are needed to phosphorylate nucleosides. Expectedly, mutations in both *TK2* and *DGUOK* are known to lead to dNTP pool imbalances. A mouse knockout for *TK2* dies within the first few weeks of life due to severe mtDNA depletion and sequential OXPHOS dysfunction (Zhou et al., 2008). Interestingly, the phenotype of this mouse model can be partially rescued by transgenetic expression of a fruit fly nucleoside kinase (*dNK*), which is able to phosphorylate all four nucleosides (Krishnan et al., 2014, Krishnan et al., 2013). Similar to the *TK2* knockout, a mouse model carrying a pathogenic TK2 mutation H126N (HsH121N) shows short lifespan, impaired neuromuscular function, severe histological changes and mtDNA depletion (Garone et al., 2014). Surprisingly, many of these phenotypes can be alleviated by oral supplementation of dNTPs (Garone et al., 2014). Nucleoside injections can also improve the imbalanced dNTP pools of a third mouse model double knockout for thymidine phosphorylase (TP) and uridine phosphorylase (UP)(Cámara et al., 2014). In flies, *PINK1* knockout caused mitochondrial dysfunction can be partially rescued by overexpressing dNK or supplementing fly food with either nucleosides or folic acid (Tufi et al., 2014). Although still unpublished, nucleotide supplementation has been claimed to help also patients with TK2 deficiency (Gorman et al., 2016). Therefore dNTP supplementation seems as a very

promising approach to treat specific mitochondrial diseases caused by changes in nucleotide synthesis and salvage pathways.

A few other approaches in addition to supplementation have been experimented in a small number of patients. These include allogenic hematopoietic stem cell transplantation (AHSCT), carrier erythrocyte-entrapped deoxythymidine phosphorylase therapy (CEETP) and liver transplantation (De Giorgio et al., 2016, Di Meo et al., 2015). In the case of AHSCT and liver transplantation, performing large scale surgical operations to already weak patient and organ rejections are major challenges. Some less invasive approaches such as lentivirus and adeno-associated virus based gene replacements have provided promising results in mouse models but are yet to be tested in humans (Di Meo et al., 2015).

1.7.2 Mitochondrial biogenesis

One of the most studied mechanisms to improve mitochondrial function is to increase mitochondrial biogenesis. The reasoning behind this approach is that a higher number of mitochondria, even if defective, could compensate for the defect. The existence of mechanism to control mitochondrial biogenesis is supported by the fact that controlled changes in mitochondrial biogenesis seem to happen already in nature (Monnot et al., 2013, Tsang and Lemire, 2002). For instance, it is well known that dormant somatic stem cells have only a few mitochondria but increase mitochondrial biogenesis upon differentiation. Also various external stimuli such as hormones, exercise and even dietary restriction have been shown to increase mitochondrial mass. In addition, the muscles of some patients with mutations in mitochondrial tRNA genes present with ragged-red-fibers (RRF) due to the loss of OXPHOS activity leading to compensatory increase in mitochondrial mass (Boulet et al., 1992). Increasing the mitochondrial mass in cells with mtDNA mutations is a double-edged sword due to the random drift of mtDNA mutations (Kauppila et al., 2017). In the ideal case the relative levels of mtDNA mutations decreases or remains the same resulting in improved cellular function. In the worst case the levels of the mutated mtDNA increases leading to cell death.

Perhaps the most heavily targeted pathway to increase mitochondrial biogenesis is the AMP-dependent kinase (AMPK)-peroxisome proliferator-activated receptor-γ1α (PGC1α)-peroxisomal proliferator activator receptors α/β/γ (PPARs α/β/γ) pathway. AMPK can be activated by AICAR, an adenosine monophosphate analog, which increased mitochondrial biogenesis and improved the phenotype of two mouse models with COX deficiency. Similarly, overexpressing PGC-1α in mice, leads to muscle fiber-type switch towards more oxidative fibers with more mitochondria (Kauppila et al., 2017). Although PGC-1α –mediated biogenesis might be limited to muscle, it has been shown improve the exercise capacity of COX deficient mice (Viscomi et al., 2011). A pan-PPAR agonist bezafibrate does not, however, improve the phenotype of mice with COX deficiency (Yatsuga and Suomalainen, 2012, Viscomi et al., 2011) although the original, now retracted, study reported

otherwise. Another way to increase mitochondrial biogenesis is based on creatine depletion (Schmidt et al., 2004) or inhibiting creatine transporter using beta-guanidinopropionic acid (GPA), a creatine analogue (Herbst et al., 2013, Wiesner et al., 1999). This decrease in creatine levels is thought to decrease also the levels of ATP leading to AMPK activation (Yang et al., 2015). However, increasing overall mitochondrial biogenesis in ageing muscle using GPA leads to accumulation of mtDNA deletions probably due to increased clonal expansion of low level deletions (Herbst et al., 2013, Herbst et al., 2016). Therefore increasing mitochondrial biogenesis can have negative consequences in the long run.

Similar to mammals, AMPK in flies is working on the same pathway involving the fly PGC1α ortholog *spargel* (Ng et al., 2017). Studies trying to characterize the function of *spargel* have provided conflicting results. It was originally reported that neither the decreased expression of *spargel* nor the ectopic expression of *spargel* have any effect on mitochondrial mass (Tiefenböck et al., 2010). A later study using a different *spargel* overexpression line did however show an increase in mitochondrial mass (Rera et al., 2011). Nevertheless, ectopic *spargel* overexpression and its pharmacological activation by pyrroloquinoline quinone (PQQ) are able to improve neuromuscular function of several fly models with mitochondrial dysfunction (Ng et al., 2017). Overexpression of *spargel* can also partially rescue the mechanical stress sensitivity of *sesB*^{1} flies with mutation in the mitochondrial ADP,ATP carrier protein and *tko(25t)* flies carrying a mutation in mitochondrial ribosomal protein (Vartiainen et al., 2014, Chen et al., 2012). All in all, affecting mitochondria through PGC1α seems promising both in invertebrates and vertebrates. However, it is not fully understood how PGC1α actually affects mitochondrial function and it has multitude of other functions (Mukherjee and Duttaroy, 2013) making it a rather unspecific target to affect mitochondrial function.

Although studies often focus quantifying the relative WT:mutant mtDNA ratio, some studies have suggested this to be too simplistic. Indeed it might be the absolute number of WT molecules that determines disease penetrance (Durham et al., 2007). To test this "maintenance of wild type" hypothesis (Stewart and Chinnery, 2015) it would be sufficient to increase specifically mtDNA copy number without affecting mitochondrial mass. As described above, specific increase in mtDNA copy number could be obtained by overexpressing TFAM or TWINKLE. Improving mitochondrial dysfunction originating from mtDNA mutations through this approach can be seen as highly beneficial as it would avoid possible complications caused by the activation of major cellular signaling pathways.

1.7.3 Nicotinamide adenine dinucleotide

Nicotinamide adenine dinucleotide (NAD^+) has also received attention as a regulator of mitochondrial biogenesis (Gong et al., 2013). NAD^+ affects the AMPK-PGC1α-PPARα/β/γ pathway by being a substrate to Sirtuin 1 (SIRT1), which in turn activates PGC1α by deacetylation. SIRT1 activity is

sensitive to changing cellular NAD^+ levels and therefore studies have tried to increase the levels of NAD^+ in various ways. An increase in NAD^+ levels can be achieved by either increasing the amount of NAD^+ precursors or inhibiting/removing other NAD^+ utilizing enzymes, such as poly [ADP-ribose] polymerase 1 (PARP1). *In vivo,* feeding COX deficient $Sco2^{KOKI}$ mice and mtDNA deletor mice a NAD^+ precursor nicotinamide riboside (NR) showed marked improvement in mitochondrial function (Khan et al., 2014, Cerutti et al., 2014) as did the pharmacological inhibition or genetic removal of PARP1 (Cerutti et al., 2014). The phenotype of flies carrying mutations in mitochondrial *Pink1* or *park* can be also improved by supplementing food with NAD^+ precursor nicotinamide or by mutating the NAD^+ consumer *Parp* (Lehmann et al., 2017, Lehmann et al., 2016). Mootha et al. developed recently an interesting tool to specifically increase mitochondrial NADH levels (Titov et al., 2016). This can be achieved by transgenic expression of mitochondrial targeted NADH oxidase from *Lactobacillus brevis* (*Lb*NOX). This resulted in increased NAD^+ recycling which was beneficial in rescuing cell proliferation when OXPHOS, mitochondrial translation or mitochondrial replication were chemically inhibited. It will be interesting to see in the future whether this approach to increase NADH cycling will be also beneficial in animal models with mitochondrial defects.

It should be noted that NAD^+ is a cofactor in numerous cellular reactions involved in glycolysis, lactate formation, pyruvate-to-acetyl-CoA conversion, β-oxidation, citric acid cycle, OXPHOS and protein acetylations (Katsyuba and Auwerx, 2017). Additionally, its phosphorylated form NADP+ is involved in ROS generation and fatty acid, cholesterol and DNA synthesis (Ying, 2008). On the one hand, increasing NAD^+ is a rather unspecific method to improve mitochondrial function and could potentially have several undesired side effects. On the other hand, to date animal models and humans have not shown any adverse effects (Katsyuba and Auwerx, 2017).

1.7.4 Dietary supplements

Dietary supplements are desirable ways to manage patients with mitochondrial diseases due to their low cost and availability. Some supplements often used by patients include antioxidants, modulators of mitochondrial electron flux, nitric acid precursors, energy buffers and promoters of fatty acid oxidation (Gorman et al., 2016). However, dietary supplements have several problems, including lack of efficacy and safety studies, limited post-marketing control and products come usually as multi-ingredient products causing potentially undesirable interactions (Camp et al., 2016). Apart from treating CoQ deficiency with CoQ supplements, there is currently little evidence to support the efficacy of dietary supplements in treating mitochondrial diseases (Avula et al., 2014).

It has been also hypothesized that high-fat or ketogenic diets would be beneficial in bypassing complex I defects by feeding electrons to CoQ through electron-transferring-flavoprotein dehydrogenase (ETF). Results using a fly model with a pathogenic mutation in the mitochondrial ATP6 gene have shown that this fly is more dependent on ketogenesis and glycolysis (Celotto et al.,

2011). Both time-restricted feeding and ketogenic diet were later shown to improve the neuromuscular dysfunction of this fly model (Fogle et al., 2016). Ketogenic diet has positive effects in the mtDNA deletor mouse model (Ahola-Erkkilä et al., 2010) and in the Harlequin mouse with complex I deficiency (Schiff et al., 2011). One study showed that ketogenic diet has even some efficacy in children with various OXPHOS defects (Kang et al., 2007). However, a recent pilot study reported that high-fat diet had severe negative effects on patients with PEO including progressive muscle pain and fiber damage (Ahola et al., 2016). Therefore although high-fat diet might have efficacy in some mitochondrial disorders, more studies are needed to understand why it has such negative effects on others.

1.7.5 Other pharmacological approaches

Several other pharmacological agents have been tested to treat mitochondrial diseases with little success. They usually aim to modulate mitochondrial electron flux, inhibit nucleotide catabolism, protect cardiolipin or remove accumulating hydrogen sulfide (El-Hattab et al., 2017b, Cámara et al., 2014). Some of these pharmacological agents have been tested also in fruit flies, especially in the fly model of Friedreich's ataxia (FRDA), a mitochondrial disease caused by mutations in Frataxin (FXN) (Tricoire et al., 2014, Soriano et al., 2013). Furthermore, flies have been used even as a drug-screening platform for novel drugs against FRDA (Seguin et al., 2015).

Two recent reports showed that rapamycin, a small molecule inhibitor of mechanistic target of rapamycin complex 1 (mTORC1), can have positive effects in the FRDA fly model and in flies with homoplasmic ND2 mutation (Wang et al., 2016, Calap-Quintana et al., 2015). In line with the results from flies, rapamycin can also alleviate the phenotypes of *NDUFS4* knockout mice, *TK2* knockout mouse and the mtDNA deletor mice (Siegmund et al., 2017, Khan et al., 2017, Johnson et al., 2013). Because mTORC1 is known to affect a plethora of cellular functions ranging from immune function and adipogenesis to autophagy and glucose homeostasis (Saxton and Sabatini, 2017), great care should be taken when administering rapamycin. Indeed, in mice rapamycin is known to cause glucose intolerance, insulin resistance, testicular atrophy and nephrotoxicity (Neff et al., 2013, Lamming et al., 2012, Wilkinson et al., 2012). A major challenge in rapamycin treatments is to find a dose and a duration of the treatment that would have a desired effect while minimizing the side effects (Johnson and Kaeberlein, 2016).

1.7.6 Hypoxia

Mitochondria are major oxygen sinks in respiring eukaryotic cells. Therefore it came as a surprise when a genome-wide Cas9 screen identified hypoxia response to be protective against mitochondrial OXPHOS defects (Jain et al., 2016). This finding was even extended to *in vivo* as the *NDUFS4* knockout mice showed marked increase in survival under hypoxia (Ferrari et al., 2017, Jain et al., 2016). Whether this hypoxia-mediated improvement is specific to this mouse model is yet unclear. It

is probable, however, that hypoxia would not improve all mitochondrial defects. For example, mtDNA mutator mice suffer already from anemia due to impaired erythroid maturation (Ahlqvist et al., 2015a). In fruit flies, hypoxia has been shown to aggravate sensitivity to mechanical stress of several mitochondrial mutants, including *ND2*, mRpS12 (*tko*) and ethanolamine kinase (*eas*) (Burman et al., 2014, Whelan et al., 2010) showing that hypoxia is actually harmful in these fly models.

1.8 AIMS OF THE THESIS

Assessing the effects of mtDNA mutations on fruit fly physiology

Homoplasmic mtDNA mutations are known to decrease the physiology and lifespan of fruit flies. However, it is not known whether somatic mtDNA mutations are limiting the natural lifespan of flies and especially whether it would be possible to decrease mtDNA mutation load by increasing the accuracy of POLγA. To this end we are going to genomically engineer the *tamas* (DmPOLγA) locus and use this genetic tool to replace the endogenous gene with DmPOLγA variants having higher and lower proofreading/polymerase activity ratio and assess the effects of mtDNA mutations of fly physiology and ageing.

Testing the "maintenance of wild type" hypothesis in the mtDNA mutator mouse

There are currently no treatments for diseases caused by mtDNA mutations affecting mitochondrial translation. To test the theory that in mtDNA diseases the absolute number of WT mtDNA copies determines the disease penetrance, we are going to take advantage of the observation that TFAM overexpression can increase mtDNA copy number. To this end we are going to establish a mouse model overexpressing TFAM and investigate whether it would be sufficient to alleviate male sterility in the mtDNA mutator mouse model.

MATERIALS AND METHODS

2.1 *IN VITRO* EXPERIMENTS

2.1.1 Expression and purification of the recombinant HsPOLγA

Variant HsPOLγA constructs were engineered using QuickChange Lightning Site-directed mutagenesis kit (Agilent) according to manufacturer's protocol. The presence of HsPOLγA mutations was verified by sequencing (Eurofins). HsPOLγA, HsPOLγB, TWINKLE and mtSSB were purified as described previously (Korhonen et al., 2004, Korhonen et al., 2003, Falkenberg et al., 2002). Briefly, HsPOLγA, HsPOLγAB, TWINKLE and mtSSB were amplified from human cDNA without the leader peptide and cloned into pBacPAK9 vector (Clontech) with an N-terminal His_6 tag except for mtSSB. These constructs were used prepare *A. californica* nuclear polyhedrosis virus particles. *S. frugiperda* (Sf9) cells were maintained and propagated in suspension in SFM 900 medium (Invitrogen), containing 5% fetal calf serum, at 27°C. For purification of HsPOLγ, Sf9 cells were co-infected with recombinant baculoviruses encoding HsPOLγA and HsPOLγB. Sf9 cells were grown in suspension and collected 60-72 h post infection. Cells were frozen in liquid nitrogen and thawed in lysis buffer (Table 2.1) for 20 min. Cells were disrupted using Dounce homogenizer (20 strokes) and NaCl was added to the final concentration of 0.8 M. The homogenate was swirled for 40 min at 4°C and cleared by centrifugating 45 min at 45 000 rpm (Beckman TLA 100.3).

POLγ was first purified from whole cell extracts by supplementing it with 10 mM imidazole. 2 ml of Ni^{2+}-NTA matrix superflow (APBiotech) was pre-equilibrated with buffer A (Table 2.1) and supplemented with 10 mM imidazole and 1.0 M NaCl for 60 min. The matrix was collected by centrifugation 1500 g for 10 min and resuspended in buffer A supplemented with 40 mM imidazole and 1.0 M NaCl. The matrix was poured into a column and washed with 10 column volumes using the same buffer. POLγ was eluted with buffer A supplemented with 250 mM imidazole and 1.0 M NaCl. The peaks were dialyzed in buffer B (Table 2.1) supplemented with 0.1 M NaCl. For second round of purification, 1 ml HiTrap Heparin column (Amersham Biosciences) was equilibrated using buffer B supplemented with 0.1 M NaCl. The column was washed three column volumes using the same buffer and a linear gradient (10 ml) of buffer B (0.1-1.0 M NaCl) was used to elute POLγ which was eluted around 0.8 M NaCl.

TWINKLE was purified from whole cell extracts by diluting it with equal volume of buffer C (Table 2.1) supplemented with 20 mM imidazole. This was added to 1 ml of Ni^{2+}-agarose Superflow beads (Qiagen) and incubated for 60 min. Beads were collected by centrifugation 2500 rpm for 10 min (JA-17), washed once with 15 ml of buffer C supplemented with 40 mM imidazole and centrifuged as before. The column was washed with 10 column volumes of buffer C supplemented with 40 mM imidazole and the sample was loaded on top. TWINKLE was eluted with 15 column volumes of buffer

A supplemented with 250 mM imidazole. The peak fractions were pooled and diluted to equal volume of buffer D (Table 2.1). For second round of purification 2ml hydroxyapatite column (Bio-Rad) was equilibrated in buffer D. The column was washed with 3 volumes of buffer D and TWINKLE was eluted using a 20 ml linear gradient of buffer D to buffer D with 400 mM KPO_4 pH 7.2. The peak fractions were diluted in 2 volumes of buffer E (Table 2.1) and loaded on a 1 ml HiTrap SP column (Amersham Biosciences), which was equilibrated with buffer E supplemented with 150 mM NaCl. The column was washed with 3 column volumes of buffer E supplemented with 0.3 M NaCl. TWINKLE was eluted and the peak fractions were pooled using a 6 ml linear gradient of buffer E with 0.3-1.0 M NaCl. The pooled fractions were dialyzed against buffer E using 0.2 M NaCl.

Table 2.1: Buffers for protein purification

Lysis buffer	
Tris-HCl pH 8.0	25 mM
2-mercaptoethanol	20 mM
Protease inhibitors	1x
Buffer A	
Tris-HCl pH 8.0	25 mM
Glycerol	10%
Protease inhibitors	1x
2-mercaptoethanol	20 mM
Buffer C	
Tris-HCl pH 8.0	50 mM
NaCl	0.6 M
Glycerol	20%
2-mercaptoethanol	20 mM
Protease inhibitors	1x
Dithiothreitol (DTT)	1 mM
EDTA	0.5 mM
Buffer E	
Tris-HCl pH 7.7	20 mM
Glycerol	10%
Dithiothreitol (DTT)	1 mM
EDTA	0.5 mM

Protease inhibitors (100x)	
Phenylmethylsulfonylfluoride (PMSF)	1 mM
Pepstatin A	2 mM
Leupeptin	0.6 mM
Benzamidine	2 mM
Buffer B	
Tris-HCl pH 8.0	20 mM
Glycerol	10%
Protease inhibitors	1x
Dithiothreitol (DTT)	1 mM
EDTA	0.5 mM
Buffer D	
KPO_4 pH 7.2	10 mM
Glycerol	10%
Dithiothreitol (DTT)	1 mM
NaCl	0.1 M
Protease inhibitors	1x

MtSSB whole cell lysate was clarified using buffer E supplemented with 0.1 M NaCl and loaded onto a 5 ml CM Superose column (Amersham Biosciences) equilibrated with buffer E supplemented with 0.1 M NaCl. MtSSB was collected from the flow-through and loaded onto 5 ml HiTrap heparin-Sepharose column (Amersham Biosciences) equilibrated with buffer E supplemented with 0.1 M NaCl. The peak fractions were collected and loaded onto 1 ml hydroxyapatite column equilibrated with buffer D and washed with 3 column volumes using the same buffer. MtSSB was eluted using a 10 ml linear gradient of buffer D to buffer D with 400 mM KPO_4. Peak fractions were pooled and dialyzed against the buffer using 0.2 M NaCl.

The estimated purity of all proteins was at least 95% by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie blue staining. In the text, the holoenzyme containing both POLγA and POLγB will be referred to POLγ.

2.1.2 EMSA and coupled exonuclease/polymerase assays

The ability of POLγ to bind DNA was assessed by EMSA as previously described (Farge et al., 2007). To prepare a primed template with a 15 bp single-stranded 5'-tail, a 20 bp oligonucleotide (5′-CGGTCGAGTCTAGAGGAGCC-3′) was annealed to a ^{32}P 5'-labelled 35 bp oligonucleotide (5′-TTTTTTTTTTATCCGGGCTCCTCTAGACTCGACCG-3′) or to the same oligonucleotide without poly-dT stretch (5′-GACAACCAGCAGCCGGGCTCCTCTAGACTCGACCG-3). Each reaction contained 10 fmol of primed template, 300 μM ddGTP, 3 mM dCTP and the shown POLγ holoenzyme concentrations. The reactions were run on 6% native polyacrylamide gels (0.5x TBE) for 35 min at 180 V and visualized using autoradiography. To obtain the K_d values the band intensities were quantified using Fujifilm Multi Gauge V3.1 software. The fraction of bound DNA was determined as bound/(bound+unbound) after subtracting the background signal. The fraction of DNA bound in each reaction was plotted against the corresponding POLγ concentration. A non-linear regression using the equation (Fraction bound = (MaxB × [POLγ])/(MaxB+[POLγ]) from EXCELs add-in "Solver" was used to obtain K_d values (the midpoint of MaxB) with the MaxB set to 1. Coupled exonuclease/polymerase assay was done as described previously with minor modifications (Roos et al., 2013). Briefly, EMSA substrate with poly-dT stretch was used in the reaction mixture (Table 2.2). Fixed amount (150 fmol) of POLγA variants and 600 fmol of POLγB was used. Reactions were stopped using 10 μl TBE-Urea sample buffer (Bio-Rad), incubated for 3 min at 90°C and run on a 15% denaturing polyacrylamide gel electrophoresis (PAGE) in 1xTBE and visualized using autoradiography.

2.1.3 Second strand DNA synthesis

A 5'-end ^{32}P-labelled short primer (5′- CTATCTCAGCGATCTGTCTATTTCGTTCATCC -3′) was annealed to single stranded pBlueScript SK(+). For the reactions 150 fmol of POLγA with or without 300 fmol of POLγB was added to the second strand DNA synthesis reaction mixture (Table 2.2). The reactions were incubated at 37°C and stopped by adding 10 μl TBE-Urea sample buffer (Bio-Rad) followed by 3 min incubation at 90°C. The products were analyzed on a 0.9% agarose gel and visualized by autoradiography.

Table 2.2: Second strand DNA synthesis reaction mixture	
DNA template	10 fmol
Tris-HCl pH 7.8	25 mM
Dithiothreitol (DTT)	1 mM
$MgCl_2$	10 mM
BSA	0.1 mg/ml
Glycerol	10%
dNTP	0.2 μM

2.1.4 *In vitro* rolling circle replication

A 70 bp long primer (5′-42[T]-ATCTCAGCGATCTGTCTATTTCGTTCAT -3′) was annealed to a single-stranded pBlueScript SK(+). One cycle of DNA synthesis was done using KOD polymerase (Novagen) to produce a double-stranded template with a preformed replication fork. The rolling circle reaction mixtures (Table 2.3, 20 μl) were incubated at 37°C and stopped by adding 6 μl of stop alkaline gel loading buffer. Products were run on a 0.8% denaturing agarose gel electrophoresis and visualized by autoradiography.

Table 2.3: Rolling circle reaction mixture			
Tris-HCl pH 8.0	25 mM	dTTP	100 μM
$MgCl_2$	10.5 mM	dCTP	10 μM
Dithiothreitol (DTT)	1 mM	α-^{32}P-dCTP	2 μCi
BSA	0.1 mg/ml	RNase inhibitor	4 U
ATP	400 μM	DNA template	10 fmol
CTP	150 μM	TWINKLE	100 fmol
GTP	150 μM	POLγA	250 fmol
UTP	150 μM	POLγB	375 fmol
dATP	100 μM	mtSSB	5 pmol
dGTP	100 μM	POLRMT	250 fmol

2.1.5 3'-5' exonuclease assay

A ^{32}P 5'-labelled primer (5′- CTATCTCAGCGATCTGTCTATTTCGTTCATCG -3′) with a one-nucleotide mismatch at the 3′-end was annealed to single stranded pBlueScript SK(+). The reactions were performed as in the second strand DNA synthesis assay but in the absence or presence of dNTPs. Reactions were incubated at 37°C for the times indicated and samples were analyzed by electrophoresis using a 20% denaturing PAGE and visualized by autoradiography.

2.1.6 Processivity assay

Processivity assay was done by mixing 10 μl of assay mixture A (Table 2.4) with 10 μl of assay mixture B (Table 2.4) followed by incubation at 37°C for 10 min. Reactions were stopped with 20 μl of stop solution (Table 2.4). Samples were analyzed on 12% denaturing PAGE.

Table 2.4: Solutions for processivity assay					
Processivity assay mixture A		**Processivity assay mixture B**		**Stop solution**	
Tris-HCl pH 7.8	25 mM	$MgCl_2$	10 mM	Formamide	95%
Dithiothreitol (DTT)	1 mM	dNTP	100 μM	EDTA	20 mM
BSA	0.1 mg/ml	Heparin	0.001 mg/ml	Bromophenol blue	0.1%
DNA template	25 fmol				
POLγA	50 fmol				
POLγB	200 fmol				

2.2 GENERATION AND MAINTENANCE OF TRANSGENIC FLY LINES

2.2.1 Fly maintenance and stocks

Unless specified otherwise all flies were maintained at 25°C in 65% humidity with 12:12 h light/dark (LD) cycle. Flies were fed on a sucrose/yeast/agar food (Table 2.6, 1xSYA) (5% w/v sucrose, 10% w/v brewer's yeast, 1.5% w/v agar) (Bass et al., 2007) unless stated otherwise.

Table 2.6: 1xSugar/Yeast/Agar (1xSYA) food		
Ingredient	For 1l	Final
Sugar	50 g	5% w/v
Brewer's yeast	100 g	10% w/v
Agar	15 g	1.5% w/v
Nipagin	30 ml	3% v/v
Propionic acid	3 ml	0.3% v/v
Water	896 ml	

Table 2.7: Fly lines used			
Fly line	**Chromosome**	**From**	**Flybase ID**
w,vas-int;;TM6B,Tb/MKRS,Sb	1	Huang et al. 2009	
w,vas-int;tamKO;;	1,2	In house	
w;tam KO/+	2	In house	FBal0323340
w,Cre;Sco/CyO;+	1,2	Huang et al. 2009	
w; Rescue/+;	2	In house	FBal0323341
w; DmQ1009A/+;	2	In house	FBal0323342
w; DmH1038A/+;	2	In house	FBal0323343
w; DmD263A/+;	2	In house	FBal0323344
w;Df(2L)Exel7059;+	2	Bloomington	FBab0037923
w;Df(2L)BSC252;+	2	Bloomington	FBab0045023
w;Df(2L)BSC694;+	2	Bloomington	FBab0045759
w;tam3;+	2	Bloomington	FBst0003410
w;tam4;+	2	Bloomington	FBst0025145
w;TFAM RNAi #1;+	2	VDRC	FBst0479013
w;;TFAM RNAi #2;+	3	VDRC	FBst0462182
w;;daGAL4	3	Bloomington	FBal0290424
w;CyO;+	2	Bloomington	
w;CyO GFP;+	2	Bloomington	

All lines (Table 2.7) were backcrossed into Wolbachia-free white Dahomey (wDahT) wild type outbred genetic background for at least 6 generations with the exception of genomic deficiency lines and *tam*^{3} and *tam*^{4} mutants. For all experiments adult flies were allowed to mate for two days and once

mated flies were allocated to new vials with 1x SYA food under light CO_2 anesthesia. The following transgenic fly lines were either made in house or ordered from the shown stock centers.

2.2.2 Genomic engineering of DmPOLγA flies

Genetically engineered flies were made as described by Huang et al. (Huang et al., 2009). In this two-step process, the endogenous *tamas* gene is first replaced by a site-specific recombination site making a founder knockout line (Fig. 2.1). This knockout line is further used to introduce wild type (rescue control) and mutant variants of *tamas* to the endogenous locus (Fig. 2.2).

To replace the *tamas* gene with a site-specific recombination site the 3' and 5' flanking regions (~4 kb each) of the *tamas* locus were first amplified from the *tamas* BAC clone (RP98–30I21, BACPAC Resource Center, Oakland, California). For ET cloning EL250 bacteria were transformed with the BAC clone and pBlueScript II SK(+) phagemid vector (pBS, Stratagene) was amplified with primers overlapping either with the 3' or 5' flanking regions (Table S1). The full length flanking regions were introduced to the pBS vector by ET cloning (Zhang et al., 2000). Following verification of the sequences the flanking regions were cloned into the pGX-attP targeting vector (Huang et al., 2009). The pGX-attP targeting vector was introduced to the third chromosome by P-element mediated germ line transformation (BestGene Inc) using the 3'P and 5'P sites. The targeting vector was mobilized using heat shock inducible expression of Flippase (FLP) and linearized using heat shock inducible expression of I-SceI as described by Huang et al. (Huang et al., 2008). Following this, homologous recombinants were screened by PCR using primers PCR3 and PCR4 (Table S2). A single founder line was established which was further reduced using Cre-recombinase to remove the *white*^{hs} marker. The absence of *tamas* in the corresponding knockout (KO) line was verified by PCR using PCR1 primers and PCR2 as a positive control (Table S2). To re-introduce any *tamas* variants to the endogenous *tamas* locus, the KO line was first crossed with the Φ31-integrase and the resulting line was used for injections.

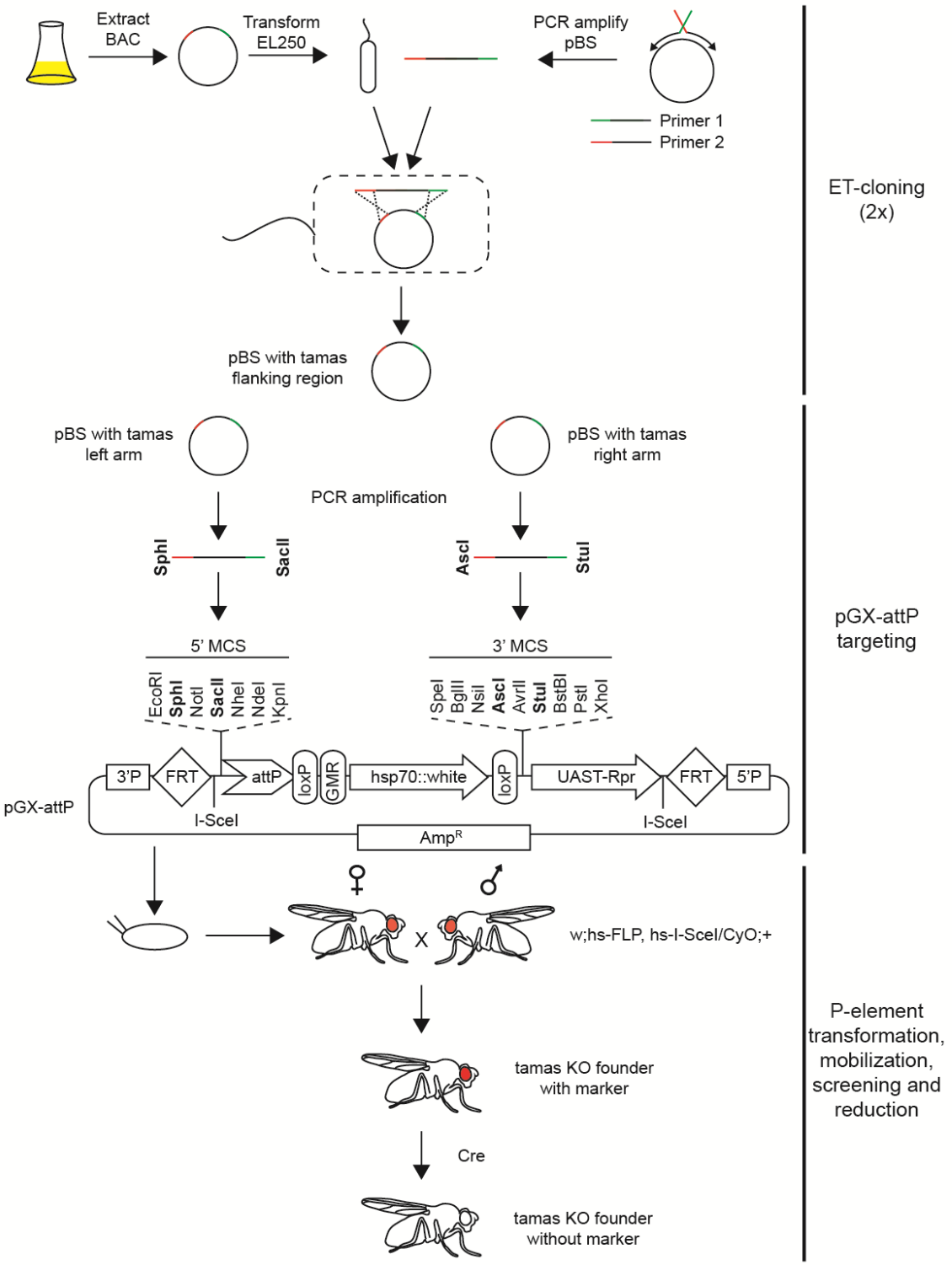


Figure 2.1: Genomic engineering of the *tamas* locus

To reintroduce WT *tamas* to the *tamas* locus (rescue control), the *tamas* construct was first amplified by PCR using primers PCR8 (Table S2). Following the sequence verification of the construct it was cloned into the pGEattB^{GMR} vector. The resulting pGEattB^{GMR}-Rescue construct was used for mutagenesis to make *tamas* variants using the QuickChange Lightning Site-directed mutagenesis kit according to manufacturer's instructions and the listed primers (Table S3). After the sequence verification of all the constructs, the rescue and mutant constructs were injected into the Φ31-integrase

tamas KO lines by in-house *Drosophila* transgenic core facility. A single site-specific integration was verified by PCR using primers PCR5 and PCR6 (Table S2) and Southern blot analyses.

To verify that the *white*^{hs} marker of the genomically engineered flies did not interfere with *DmPOLγA* function, all *DmPOLγA* mutants were crossed with Cre-recombinase to remove the marker. Precise removal of the marked was verified by PCR using primers PCR5 and PCR7 (Table S2). *DmPOLγA* mutants behaved similarly with or without the marker and therefore all experiments were performed using flies carrying the *white*^{hs} marker.

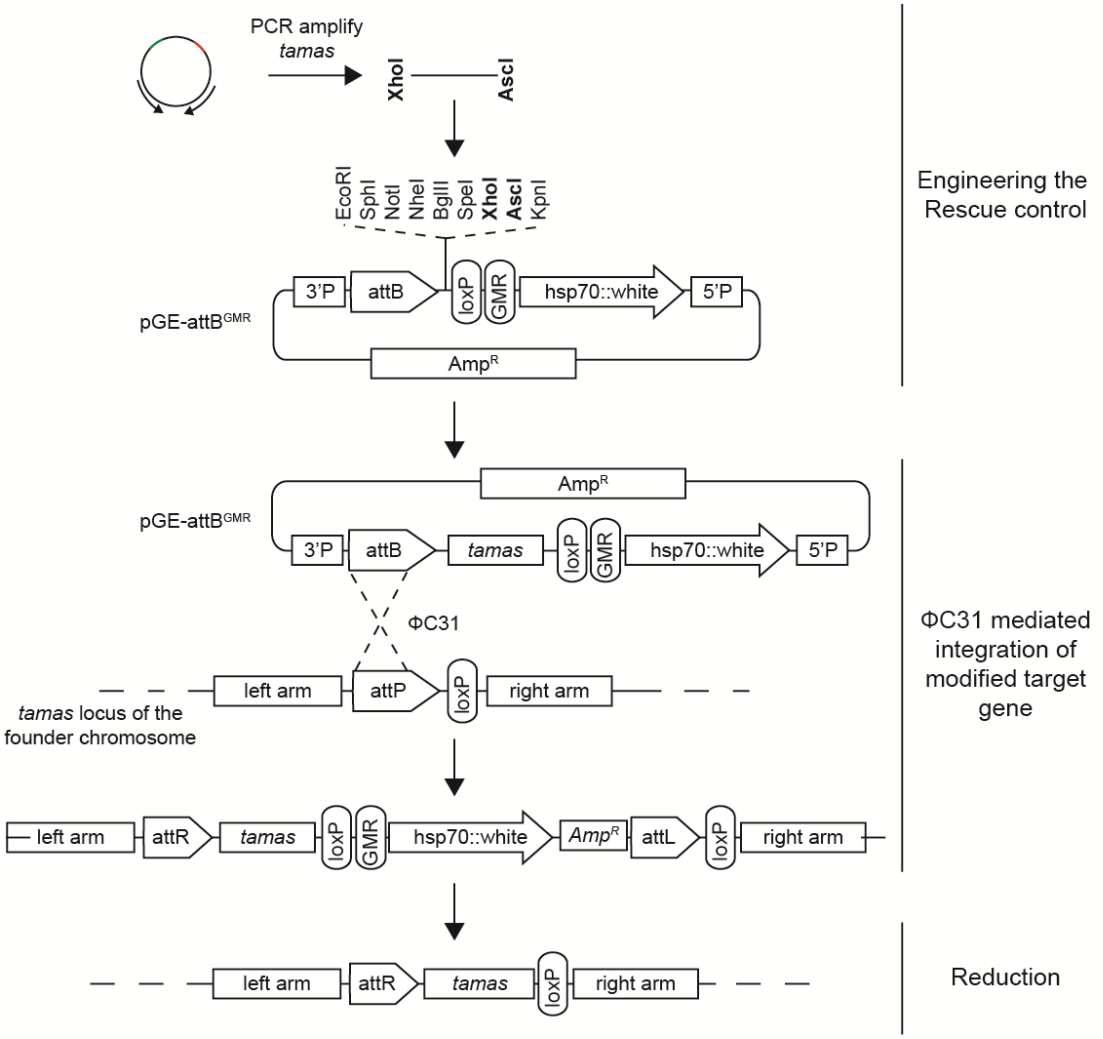


Figure 2.2: Genomic engineering to introduce *DmPOLγA* alleles to the *tamas* locus

2.3 MOLECULAR CHARACTERIZATION OF TRANSGENIC FLIES

2.3.1 Isolation of total DNA and mtDNA for qRT-PCR and Southern blot analyses

To quantify relative mtDNA copy number total DNA extractions were prepared from L3 larvae using DNeasy Blood and Tissue kit (Qiagen). 10 larvae per biological replicate and 5 biological replicates per genotype were prepared. MtDNA gene CytB and nuclear gene RpL32 were amplified using SYBR-Green qPCR using primers listed in Table S5 and 7900HT Fast Real Time PCR System (Applied Biosystems). All data were normalized against the WT genotype.

For Southern blot analysis total DNA extractions were prepared from 20-30 L3 larvae. Samples were homogenized in 400 µl of buffer A (Table 2.8) and incubated at 65°C for 30 min before adding 800 µl freshly made buffer B (Table 2.8) and left on ice for 60 min. Samples were centrifuged 12000g for 15 min. The supernatant was transferred into a new tube and 540 µl isopropanol was added and centrifuged as before. Resulting pellet was washed with 70% ethanol, dried and resuspended in 100 µl nuclease free water supplemented with 20 µg/ml RNase A. Samples were incubated for 1 h at 37°C and stored at 4°C. 1-3 µg of total DNA was restriction digested either with EcoRV, PstI, NdeI, NsiI or StyI (Table 2.8). Digestions were run on 0.8% agarose gels and blotted to Hybond-N+ membrane (Amersham Bioscience). ^{32}P-labeled ND2, COXI and 12S rRNA probes were prepared using primers in Table S4 according to manufacturer's instructions (Prime-IT II Random Primer Labeling kit, Agilent). To detect reintroduced *tamas* alleles a probe was prepared by amplifying the *tamas* cDNA.

Table 2.8: Buffers for total DNA isolation and RE analysis

Buffer A		**Buffer B**		**Restriction enzymes (New England Biolabs)**	
Tris-HCl pH 7.5	100 mM	Potassium acetate (KOAc)	5 M	EcoRV	# R0195L
EDTA	100 mM	LiCl	6 M	PstI	# R0140L
NaCl	100 mM			NdeI	# R0111L
SDS	0.5%			NsiI	# R0127L
				StyI	# R0500L

2.3.2 Isolation of RNA for qPCR analysis

5 d larvae were used for total RNA extraction using ToTALLY RNA isolation kit (Ambion). For RT-qPCR extracted RNA was DNase treated (Turbo DNA-free kit, Ambion) and reverse transcribed (High capacity cDNA Reverse Transcription kit, Applied Biosystems). Gene expression was quantified using TaqMan probes for the below listed genes and nuclear gene RpL32 was used for normalization (Table 2.9).

Table 2.9: TaqMan probes for RT-qPCR

Gene	**Probe**
tamas	Dm01841857_g1
CG8978	Dm01807408_g1
CG7833	Dm01842615_g1
CG7811	Dm01841741_g1
CG33650	Not available anymore

2.3.3 Detection of fly mtDNA point mutations

MtDNA mutation load was quantified by post-PCR cloning and sequencing (Wanrooij et al., 2012) adapted to flies. Briefly, total DNA was extracted whole embryo or larvae or from adult thorax using DNeasy Blood and Tissue kit (Qiagen). The target region (2194-3382, GenBank U37541.1) covering partially COXI, tRNA Leu and COXII was amplified using a high-fidelity polymerase (New England Biolabs #M0530L) with primers in Table S6. PCR products were cloned using the Zero Blunt TOPO PCR Cloning kit (Invitrogen). For each fly 96 clones were cultured and sequenced using the M13 primers (Table S6). Sequences were aligned using SeqScape 2.7 (Applied Biosystems).

For only detecting heteroplasmic mutations present at high levels, total DNA extractions were done as above. The mtDNA coding region was amplified in two overlapping fragments (Table S7: 36 For/6864 Rev and 6376 For/14899 Rev) by long-range PCR (TaKaRa LA Taq DNA polymerase #RR002M). PCR products were sequenced using primers listed in Table S7 and sequences were assembled using SeqScape 2.7 (Applied Biosystems). Heteroplasmy detection levels was set to 33% to categorize all detected mtDNA mutations to low (<33%), middle (33-66%) and high (>66%) heteroplasmy categories.

2.3.4 Oxygen consumption rate measurements

5 d larvae were collected and the functionality of OXPHOS was assessed as described elsewhere (Wredenberg et al., 2013). Briefly, 5 larvae were dissected just before measurements in 100 µl of respiration buffer (Table 2.10). Oxygen consumption was monitored at 27°C using oxygraph (OROBOROS). State 3 respiration was measured in the presence of proline (10 mM), pyruvate (10 mM), malate (5 mM), glutamate (5 mM) and ADP (1 mM). This was followed by state 4 respiration by blocking complex V with oligomycin (250 ng/ml). Uncoupled state was reached by adding 1 µM CCCP. Oxygen consumption rates were normalized to total protein content (Bradford, Sigma).

Table 2.10: Respiration buffer	
Tris-HCl pH 7.2	20 mM
KH_2PO_4	4 mM
$MgCl_2$	2 mM
EGTA	1 mM
Sucrose	120 mM
KCl	50 mM
Digitonin	0.01%

2.3.5 Lipid quantification from adult flies

Lipid quantification was performed essentially as described before (Grönke et al., 2003). Flies were collected before and after starvation and stored at -80°C. 4 flies per each biological replicate were homogenized in 1 ml 1xPBS with 0.05% Tween-20 using lysing matrix (MP Biomedicals #116913500). Triacylglycerol (TAG) levels were quantified using the Infinity Triglyceride kit

(Thermo Fisher Scientific #TR22421) and a triglyceride standard (Cayman Chemicals #10010509). Lipid levels were normalized to total protein content of the homogenate determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific #23225).

2.3.6 Blue-Native PAGE

Blue-Native polyacrylamide gel electrophoresis (BN-PAGE) was done as described before (Bratic et al., 2011, Schägger et al., 1994, Schägger and von Jagow, 1991). Mitochondria were isolated by homogenizing 2 ml of adult flies in the mitochondrial isolation buffer (Table 2.11, MIB) supplemented with 1% mM fatty acid free BSA. Nuclei debris was removed by centrifugation 10000g for 10 min and filtering the supernatant through cell strainer (Falcon #352350). Mitochondria were pelleted by centrifugating 3000 g for 10 min and washed once with 5 ml of MIB+BSA. Mitochondria were resuspended in MIB, frozen in liquid nitrogen and stored at -80°C.

For BN-PAGE, 75µg of mitochondria were lysed in 50 µl of ice-cold solubilization buffer (Table 2.11) and incubated for 15 min on ice. Unsolubilized material was removed by centrifugation 16100 g for 10 min and supernatant was transferred into new tube supplemented with 5 µl 10x loading dye (Table 2.11). Samples were loaded to BN-PAGE using 3-13% gradient polyacrylamide gel.

Table 2.11: Solutions for mitochondrial isolation and BN-PAGE

Mitochondrial isolation buffer (MIB)		**Solubilization buffer**	
Tris-HCl pH 7.4	5 mM	Tris-HCl pH 7.4	20 mM
EGTA	2 mM	EDTA	0.1 mM
Sucrose	250 mM	NaCl	50 mM
		Glycerol	10%
		Digitonin	1%
10x Loading dye		**Complex I reagent**	
Bis-Tris pH 7.0	100 mM	Tris-HCl pH 7.4	2 mM
6-aminocaproic acid	500 mM	NADH	0.1 mg/ml
Coomassie Brilliant Blue G-250	5% w/v	Iodonitrotetrazolium	2.5 mg/ml
		Complex IV reagent	
		PBS pH 7.4	0.05 mM
		3,3'-diaminobenzidine tetrahydrochloride (DAB)	25 mg
		Cytochrome C	50 mg
		Sucrose	3.75 g
		Catalase	1 mg

For complex I in-gel activity assays, the BN-PAGE gels were incubated for 5 min at RT in complex I reagent (Table 2.11) and the reaction was stopped with 10% acetic acid/10% methanol. For complex IV in-gel activity assays, the BN-PAGE gels were incubated for 1 h at RT in 50 ml complex IV reagent (Table 2.11).

2.3.7 Percoll gradients and LC-MS

Flies were paralyzed on ice and homogenized in 1xMIB+BSA as above using 7 ml buffer/1 ml of flies. Cell debris was removed by centrifugating twice 800g for 5 min. Mitochondria were pelleted 6000 g for 15 min and washed once in 7 ml of 1xMIB+BSA. Two-step Percoll gradient was prepared using a 2 ml of 40% Percoll/1xMIB cushion under 8 ml of 20% Percoll/1xMIB. Mitochondrial pellet was resuspended in 1xMIB and layered on top of the gradient followed by centrifugation 40 000g for 35 min. Mitochondrial fraction was collected and washed twice with 1xMIB and stored at -80°C.

LC-MS analysis was done as described previously (Li and Franz, 2014). Mitochondrial pellets were lysed and in-solution digested with trypsin (Promega # V5280). Resulting peptides were cleaned with StageTips before applying to LC-MS. An Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific), coupled with an EASY-nLC 1000 UHPLC (Thermo Fisher Scientific) was used for peptide analysis. Peptides were separated using a 25 cm long reversed-phase C18 column with 75 μm inner diameter (PicoFrit, LC Packings). The gradient was from 5% to 25% of acetonitrile in 0.1% of formic acid over 120 min and further to 40% over 10 min. The column was subsequently washed and re-equilibrated. The flow rate was set to 200 nl/min. MS spectra were acquired in a data-dependent manner with a top speed method. For MS, the mass range was set to 300−1500 m/z and resolution to 60 K at 200 m/z. The AGC target of MS was set to 5e5, and the maximum injection time was 60 ms. Peptides were fragmented with HCD with collision energy of 27. The resolution was set to 30K. The AGC target of MSMS was 5e5 and the maximum injection time was 60 ms.

For analyzing the LC-MS raw data MaxQuant version 1.5.3.8 (Cox and Mann, 2008) with integrated Andromeda search engine (Cox et al., 2011) was used. The raw data were searched against the *D. melanogaster* proteome from UniProt (knowledgebase 2016_04). The following parameters were used for data analysis: for ‘’digestion’’ specific with Trypsin, Max. missed cleavages 2; for label-free quantification, match between runs is selected. Other parameters were set as default. Protein quantification significant analysis was performed with the Perseus statistical framework (http://www.perseus-framework.org/) version 1.5.2.4. After removing the contaminants and reverse identifications, the intensities were transformed to log2. The replicates of each genotype were grouped and filtered with at least 3 validate values in at least one group. The missing values were imputed using normal distribution with width of 0.3 and down shift of 1.8 (Cox et al., 2014). Multi-sample comparison (ANOVA analysis) was performed to identify the significantly different proteins within 3 different genotypes followed by Z-score normalization. Benjamini-Hochberg FDR 0.05 was used for significant truncation.

2.3.8 Intestinal stem cell staining and stress assay

Adult female guts were dissected in 1xPBS, incubated in fixative (Table 2.12) for 45 min at RT and washed using the wash buffer (Table 2.12) for 1 h at 4°C. Both primary (rabbit anti-PH3, Millipore;

rat anti-Delta, M.Rand) and secondary antibodies (Jackson Immunoresearch) were incubated in washing buffer over night at 4°C with 1 h washing between. Hoechst was used to stain DNA.

Table 2.12: Gut staining buffers			
Fixative		**Washing buffer**	
Glutamic acid	100 mM	PBS	1x
KCl	25 mM	BSA	0.5%
$MgSO_4$	20 mM	Triton X-100	0.1%
Sodium phosphate	4 mM		
$MgCl_2$	1 mM		
Formaldehyde	4%		

2.4 PHENOTYPICAL CHARACTERIZATION OF TRANSGENIC LINES

2.4.1 Body weight

To measure the larval body mass flies carrying different *DmPOLγA* alleles were first crossed with CyO GFP balancer/marker. Crosses were allowed to lay eggs on grape juice agar plates for one day followed by the collection of eggs. 20 µl squirts were transferred to bottles and the 6 day old homozygous larvae were collected based on the absence of GFP. For each genotype 10 biological replicates each with two larvae were weighted. In the case of adult flies 7-10 days aged mated females were collected. For each genotype 5-6 biological replicates each with 5 flies were weighted. For adult flies 4 adults/biological replicate were weighted simultaneously and total of 10 biological replicates per genotype were weighted.

2.4.2 Developmental analysis

The measure the developmental time from egg lay to eclosion, crosses were allowed to lay eggs on grape juice agar plates for three hours. For each cross, 5 biological replicates each with 100 eggs were collected and transferred into wide plastic vials with 1xSYA food. The eclosed flies were scored every 12 hrs.

To count the proportion of larvae reaching late L3 stage, eggs were first collected as for the developmental time assay followed by counting the number of larvae at 6 d AEL. At least six biological replicates were done per genotype. To quantify the percentage of flies reaching the pupal and adult stage 50 L3 larvae were collected and transferred into fresh vials. The number of pupae and adult flies was scored every 12 h with minimum of three replicates per genotype.

2.4.3 Lifespan, dietary restriction and starvation assays

For lifespan experiments 150-200 female flies were distributed equally in 10 vials with 1.0x SYA medium and transferred into new vials every 2-3 days. Dead flies were scored during each transfer. Dietary restriction (DR) experiments were done according to an optimized protocol (Bass et al., 2007)

in which flies are kept on 0.1x, 0.5x, 1.0x or 2.0x SYA food. For starvation assay 100 females were distributed to 5 vials with 1.0x SYA for 10 days. Flies were moved to starvation medium (1% agarose) and dead flies were scored three times per day and frozen at -80°C for lipid quantification.

Log-rank test was used for statistical analysis to compare lifespan differences between genotypes. Due to the high number of flies per genotype only $p<0.0001$ was considered significant. Results from all assays are expressed as the proportion of survivors ± 95% confidence interval. To compare whether all genotypes respond similarly to DR parametric survival analysis with logistic distribution (lowest Akaike information criterion) was used.

2.4.4 Locomotor activity

Drosophila locomotor activity assay was performed using the locomotor activity monitor system (DAM2, TriKinetics). To avoid erroneous activity counts caused by crawling larvae, virgin females were collected and aged for 7-10 days before the experiment. Food (1x SYA) was added into the 5 mm x 65 mm polycarbonate tubes on the morning of the experiment and sealed with paraffin. Flies were anesthetized with carbon dioxide and transferred into the activity tubes which were plugged with cotton. The tubes were inserted into the activity monitors located in a temperature (25°C), humidity (65 %) and light (12:12 LD) controlled incubator. The flies were monitored for four days using 5 min bins. Data was processed using R Studio (Version 3.4.0) and the activity during morning bout (8:00-12:00) was used for statistical analysis. All flies dying during the experiment were completely removed from analysis.

2.4.5 Mechanical stimulation “bang sensitivity” assay

For each genotype 50 female flies were distributed in the density of 5 flies per vial. Sensitivity to mechanical stress was assessed by vortexing vials upside down for 10 s at maximum speed and the time it took for each fly to regain the ability to stand upright was recorded. All flies were analyzed every 7-10 days.

2.4.6 Feeding assay

The feeding activity of adult flies was quantified as described elsewhere (Wong et al., 2009). For each genotype 5 females per biological replicate (10 replicates per genotype) was sorted into vials. Recording of the feeding behavior was done by scoring the number of proboscis extensions (PE) for each vial within a 3 s time window. Each vial was recorded every 5 min total of 9 times. The measurement was repeated every second day total of 7 rounds. The feeding index was obtained by averaging the daily PE averages during the length of the experiment.

2.5 MOUSE MODELS

2.5.1 Mouse husbandry

All mice (Table 2.13) were backcrossed to inbred C57BL/6NCrl background. Mice were kept under 12 h light/dark cycle at 21°C and fed ad libitum on standard mouse food (ssniff RM-H Low-Phytoestrogen) except during breeding and newly weaned mice were fed enhanced diet (ssniff M-Z Low-Phytoestrogen). All experiments were approved and permitted by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany. Animal work was carried out in accordance with the recommendations and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Table 2.13: Mouse lines used		
Mouse line	**From**	**MGI**
C57BL/6NCrl	Charles River	N/A
PolgA$^{mut/mut}$*: PolgA*$^{D257A/D257A}$	(Trifunovic et al., 2004)	3046825
Tfam$^{+/KO}$	(Larsson et al., 1998)	1860956
Tfam$^{+/OE}$	This paper	N/A

2.5.2 Generation of transgenic mice

Bacterial artificial chromosome (BAC) clone containing the whole mouse *Tfam* gene was identified using the Clone Finder from the National Center for Biotechnology Information database. BAC clone RP23-145J8 covering 73 kb and 117 kb regions upstream and downstream of *Tfam*, respectively, was acquired from Children's Hospital Oakland Research Institute BACPAC Resources Center. A synonymous PvuI cut site was introduced to the clone by ET recombination to discriminate between the endogenous and transgenic *Tfam* genes. Modified BAC was purified by cesium chloride gradient and injected into the pronucleus of fertilized oocytes. Founders were identified by PCR and PvuI-restriction analysis. Other mouse models used have been published elsewhere (Table 2.13).

2.5.3 Testis histology

For light microscopy testes and epididymis were fixed in Bouin's fixative (Table 2.14), dehydrated and paraffin-embedded. 5 µm sections were mounted on glass slides, deparaffinized using xylene and stained with hematoxylin and eosin.

Table 2.14: Bouin's fixative	
Acetic acid	5%
Formaldehyde	9%
Picric acid	0.9%

2.5.4 Electron microscopy

For visualizing mitochondrial ultrastructure testis samples were fixed using fixing solution (Table 2.15) at room temperature for 30 min followed by 24 h at 4°C. Testes were postfixed using 2% OsO_4 and embedded in Araldite. 70 nm sections were stained with uranyl acetate and lead citrate and analyzed by electron microscopy (Zeiss). Visualized mitochondria in round spermatids were subcategorized into three groups based on cristae shape and matrix structure: condensed, intermediate and long type. Condensed mitochondria have flat cristae and large matrix volume, intermediate mitochondria are small and have oval shape and long type mitochondria are tubular or dumbbell shape. Double blind quantification was performed by two independent researchers using ImageJ.

Table 2.15: Electron microscopy fixing solution	
Glutaraldehyde	2%
Paraformaldehyde	0.5%
Sodium cacodylate	0.1 M
$CaCl_2$	3 mM

2.5.5 Immunohistochemistry

Tissue sections prepared as in testis histology were immunostained for immunohistochemistry. Endogenous peroxidase activity was quenched by incubating tissue sections in methanol with 3% H_2O_2 for 10 min at 37°C. Antigen retrieval was done using antigen retrieval buffer (Dako Target Retrieval Solution, Citrate pH 6) followed by blocking using BSA. Primary antibody against COXI (1:500, Abcam, cat # 1470, RRID: AB_2084810) was used to detect mitochondrial encoded COXI. HRP-conjugated secondary antibody and the HRP substrate diaminobenzidine was used to produce brown precipitate. Slides were stained using hematoxylin, dehydrated and mounted for bright-field microscopy. For semiquantitative assessment of COX signal, cells were categorized into high, intermediate or low expressive based on COXI staining intensity. Double blind quantification was performed by two independent researchers using ImageJ.

2.5.6 COX/SDH Double-labeling Enzyme Histochemistry

Fresh testes were embedded in optimal cutting temperature (OCT) compound and cut into 10 µm sections using cryomicrotome and air dried. 1 ml of freshly prepare buffer A (Table 2.16) was to the slides and incubated for 25 min at 37°C. Slides were washed three times in 0.1 M PBS pH 7.0 and 1 ml of buffer B (Table 2.16) was added again for 25 min at 37°C. Slides were washed as above, dehydrated and mounted for bright-field microscopy.

Table 2.16: COX/SDS solutions			
Buffer A		**Buffer B**	
3,3′-diaminobenzidine tetrahydrochloride (DAB)	4 mM	Nitroblue tetrazolium	1.5 mM
Cytochrome C	100 μM	Sodium succinate	130 mM
Catalase	20-50 μg	Phenazine methosulfate	0.2 mM
		Sodium azide	1 mM

2.5.7 Sperm motility analysis

For sperm motility analysis the cauda region of right epididymis was clamped proximally and distally, excised and rinsed with pre-warmed PBS. Tissue was placed in 1.5 ml tube with pre-warmed M2 medium (Sigma), unclamped and pierced to allow sperm to diffuse into the medium for 10 min at 37°C. Medium was diluted with fresh medium and 10 μl of suspension was used for computer-assisted semen analysis (CASA, Hamilton Thorne Research Beverly).

2.5.8 Sperm morphology analysis

Spermatozoa were collected from cauda epididymis, spread on glass slides and fixed using 4% paraformaldehyde followed by hematoxylin and eosin (HE) staining. Spermatozoa deformities were classified categorized as described by Wyrobek and Bruce (Wyrobek and Bruce, 1975).

2.5.9 Fluorescence-activated cell sorting (FACS)

FACS was done essentially as described elsewhere (Biswas et al., 2013). Testes were digested using FACS digestion buffer (Table 2.17) for 1 h at 32°C shaking. Cell suspension was filtered through a 40 μm cell strainer and resuspended in FACS incubation buffer (Table 2.17) at the density of 2 million cells/ml. Hoechst 33342 (5 mg/ml, Hoechst) was added and cells were incubated for 1 h at 32°C. 7-AAD (eBioscience) was added before FACS analysis to exclude dead cells. FACSAria Fusion cell sorter (BD Biosciences) with FACSDIVA software (BD Biosciences) was used to analyze cells. Hoechst was excited using 355 nm UV laser and detected using two different emission filters, 379/28 nm and 675/50 nm with a 670 nm longpass filter between them, to separate the haploid spermatids, diploid spermatogonia and somatic cells, and tetraploid spermatocytes according to DNA content. 7-AAD was excited with a 561 nm yellow laser and detected using a 675/20 nm emission filter. $PolgA^{mut}Tfam^{WT}$ sample was always paired with a WT (Control), $PolgA^{mut}Tfam^{KO}$ or $PolgA^{mut}Tfam^{OE}$ sample.

Table 2.17: FACS buffers			
Digestion buffer		**Incubation buffer**	
Hanks' balanced salt solution (HBSS)	1x	HBSS	1x
HEPES pH 7.2	20 mM	HEPES pH 7.2	20 mM
$MgSO_4$	1.2 mM	$MgSO_4$	1.2 mM
$CaCl_2$	1.3 mM	$CaCl_2$	1.3 mM
Sodium pyruvate	6.6 mM	Sodium pyruvate	6.6 mM
Lactate	0.05%	Lactate	0.05%
DNase	4 μg/ml	Glutamine	0.05%
Collagenase	0.4 mg/ml	Fetal bovine serum	1%

2.5.10 Relative mtDNA copy number analysis

Total DNA extraction from FACS-sorted spermatocytes and spermatids was done using DNeasy Blood & Tissue kit (Qiagen). TaqMan Universal PCR master mix (Thermo Fisher Scientific) in combination with TaqMan probes detecting mtDNA (ATP6, ND2, 16S) or nuclear DNA (18S)(Table 2.18).

Table 2.18: TaqMan probes for mouse mtDNA copy number analysis	
Gene	**Probe**
ATP6	Mm03649417-g1
ND2	Mm04225288_s1
16S	Mm03975671_s1
18S	Hs99999901_s1

2.5.11 Mouse mtDNA point mutation load analysis

MtDNA mutation load from mice was quantified as in flies with the following modifications. Total DNA extractions were used to amplify the WANCY region (4921-5953, GenBank JF286601.1) of mouse mtDNA (Table S8), cloned and sequenced using M13 primers as described above for the fly. Data were filtered against known NUMTs, two of which are known to overlap with the analyzed region and differ by 19-20 sites.

2.5.12 Label-free quantitative proteomics

FACS sorted spermatocytes were used for total proteome analysis as described for the proteome analysis of fly mitochondria with the following modifications. The gradient was from 5% to 25% of acetonitrile in 0.1% of formic acid over 155 min and further to 40% over 20 min. The column was subsequently washed and re-equilibrated. The flow rate was set to 250 nl/min. MS spectra were acquired in a data-dependent manner with a top speed method. For MS maximum injection time of 80 ms was used. The raw data were searched against the mouse proteome from UniProt (knowledgebase 2016_04).

ASSESSING THE EFFECTS OF mtDNA MUTATIONS ON FRUIT FLY PHYSIOLOGY USING "MUTATOR" AND "ANTIMUTATOR" DmPOLγA VARIANTS

3.1 Introduction

POLγA is the main mitochondrial DNA-dependent DNA polymerase responsible for mtDNA replication and repair. An increasing number of studies suggest that mtDNA mutations originate from endogenous replication errors. Therefore, engineering POLγA to either decrease or increase its proofreading activity would be an attractive model to study the effects of increased or decreased levels of mtDNA mutations, respectively, on organismal physiology and ageing. Here, I first verified the functionality of HsPOLγA variants that are predicted to have decreased exonuclease activity (proofreading-deficient, exo$^-$) or increased proofreading/polymerization ratio (polymerase-deficient, pol$^-$). Following this I used genomic engineering to modify the *tamas* (*DmPOLγA* gene) locus enabling us to study the effects of absence of DmPOLγA and the presence of different DmPOLγA variants in the context of the endogenous gene locus. These fly models carrying mutant variants of DmPOLγA were further used to assess the effects of mtDNA mutations of fruit fly development, physiology and lifespan.

3.2 Results

3.2.1 Verifying exo$^-$ and pol$^-$ variants of HsPOLγA *in vitro*

On the one hand, two single amino acid mutations in *E. coli* DNA Pol I (Q849A and H881A) known to increase its proofreading activity are conserved in family A DNA polymerases, including POLγA. The corresponding mutations in fly and human POLγA are DmQ1009A, DmH1038A and HsQ1102A, HsH1134A, respectively (Fig. 3.1A and B). On the other hand, the exonuclease activity of POLγA depends on catalytic magnesium ions coordinated by three aspartate residues. Mutating one of these aspartate residues to alanine (Fig. 3.1A and B, DmD263A and HsD274A) is sufficient to lose the coordination of the catalytic magnesium ions and thus reduce the exonuclease activity without impairing polymerase activity (Trifunovic et al., 2004).

To characterize the polymerase and exonuclease activities of WT and variant polymerases *in vitro*, these fly (DmPOLγA) and human (HsPOLγA) recombinant polymerases were first expressed in insect Sf9 cells and purified to high purity (Fig. 3.2). Recombinant DmPOLγA is known to be an extremely unstable protein and can be only purified when co-expressed with the accessory subunit DmPOLγB (Farr and Kaguni, 2002). Despite several attempts we were not able to obtain enzymatically active DmPOLγA even when co-expressed with DmPOLγB. Therefore all *in vitro* experiments were performed using only the HsPOLγ.

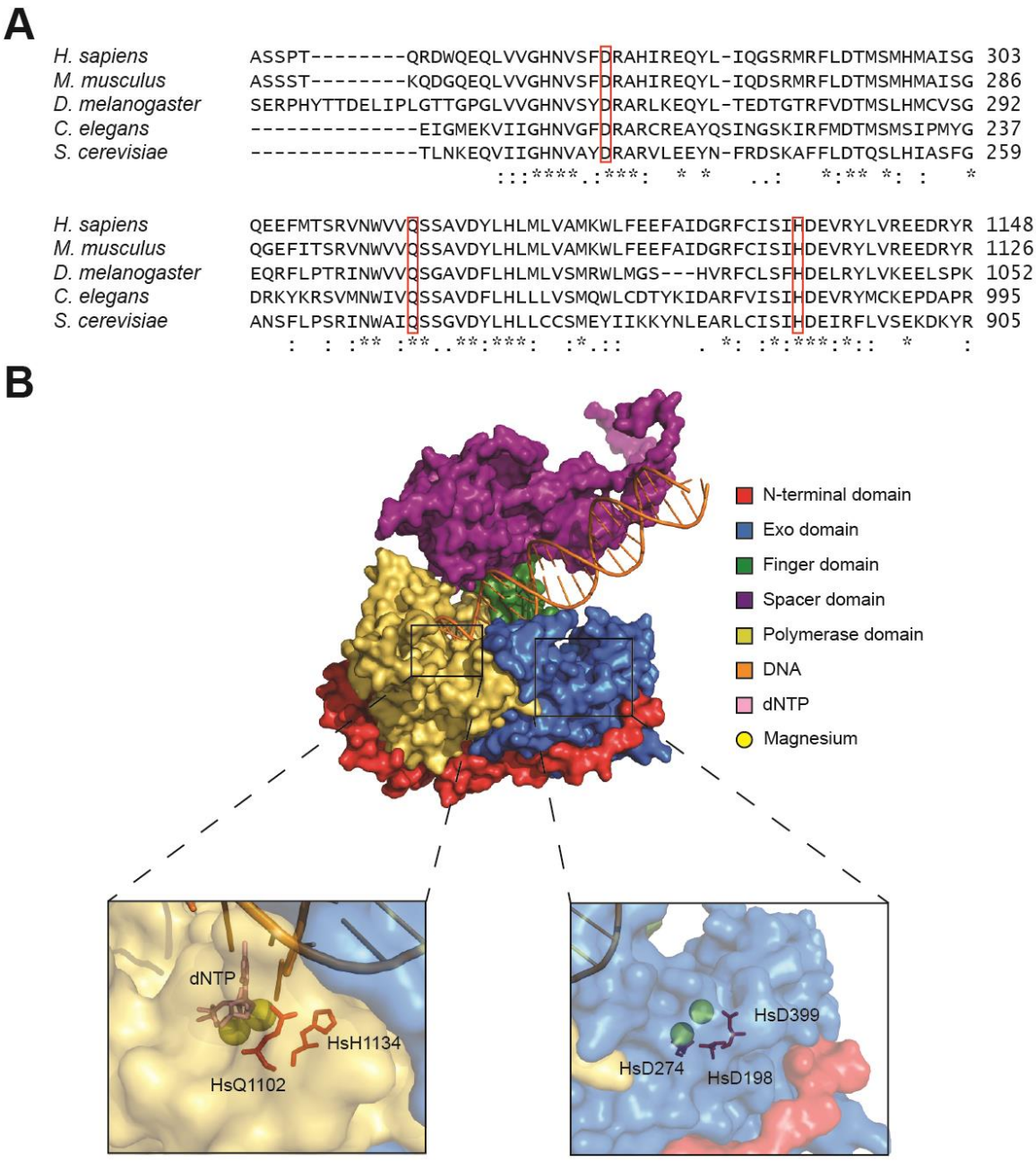
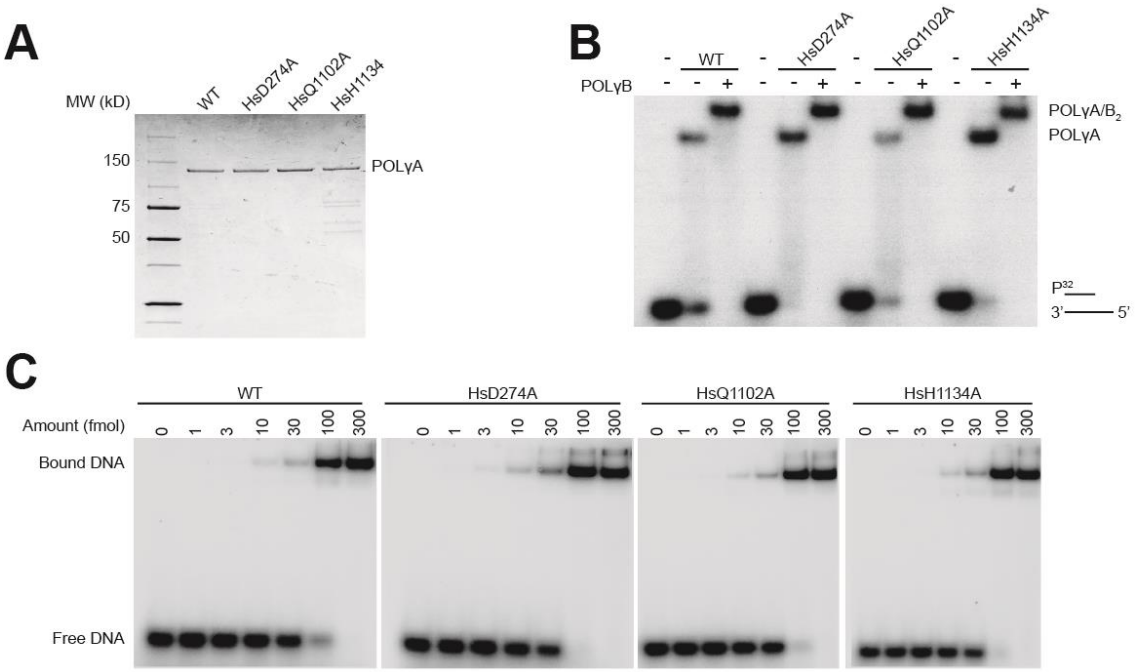


Figure 3.1: POLγA sequence alignment and structure
A) Sequence alignment of human (NP_002684.1), mouse (NP_059490.2), fruit fly (NP_476821.1), nematode (NP_496592.1) and yeast (NP_014975.2) POLγA sequences near the mutated residues (HsD274/DmD263, HsQ1102/DmQ1009, HsH1134/DmH1038) indicated by red boxes. Alignment was done using Clustal Omega (Sievers et al., 2011). **B**) Human POLγA structure (PDB:4ZTZ) showing the five POLγA domains and positioning of the mutated residues in the polymerase domain (HsQ1102, HsH1134) and exonuclease domain (HsD274) relative to catalytic magnesium ions and incoming nucleotide. Two catalytic magnesium ions in the exonuclease domain were positioned by hand.

Several pathogenic POLγA mutations are known to decrease DNA binding affinity of this polymerase. Therefore we first investigated whether the WT, exo$^-$ and pol$^-$ variants of HsPOLγA are able to bind DNA in the presence and absence of HsPOLγB. In electrophoretic mobility shift assay (EMSA) all HsPOLγA variants showed indistinguishable ability to bind DNA and interact with HsPOLγB in comparison with WT polymerase (Fig. 3.2A). Further quantification of binding affinities showed similar values for all polymerases (Fig. 3.2C and Table 3.1) suggesting the mutations do not impair DNA binding ability of the polymerase or interactions with the accessory subunit.

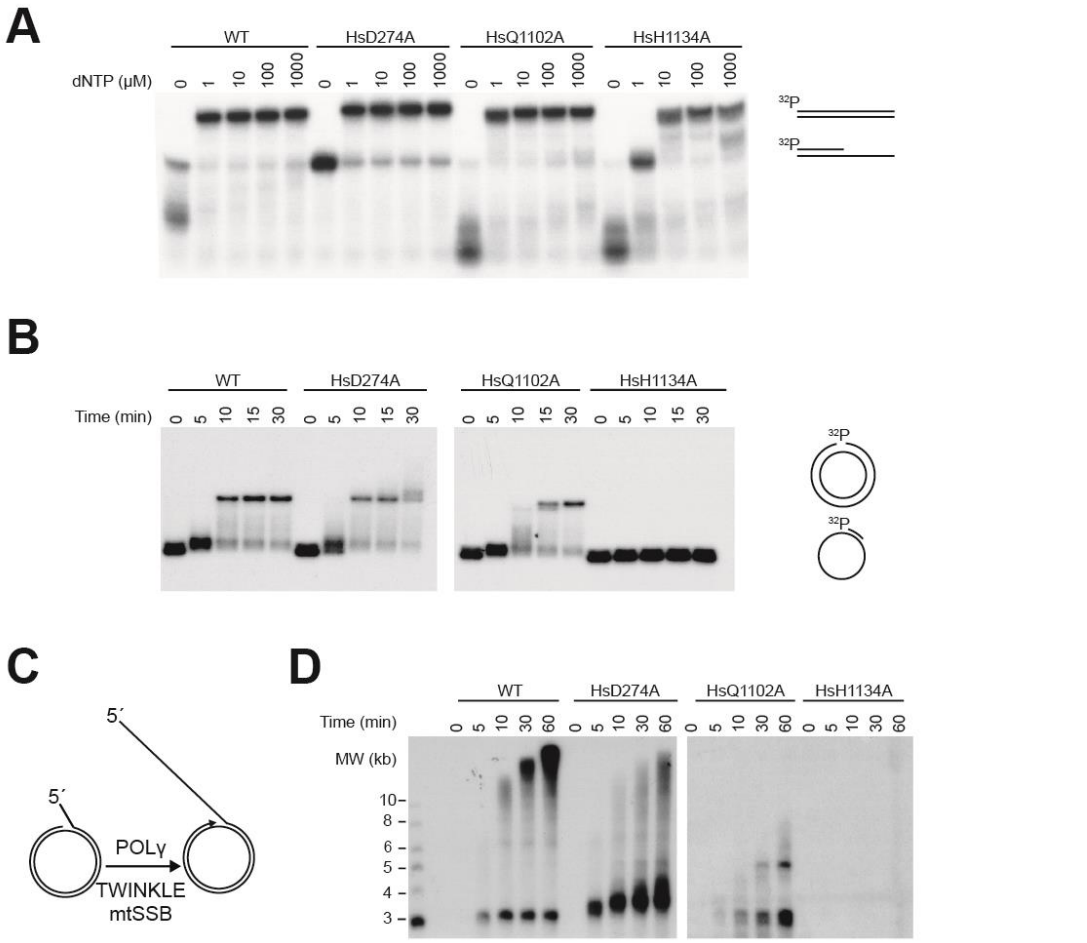


Adapted from Bratic et al., 2015

Figure 3.2: HsPOLγA purification and DNA binding
A) Coomassie Brilliant Blue stained 4-20% SDS-PAGE using purified HsPOLγA (~140 kDa). **B**) EMSA to assess the DNA binding ability of WT and HsPOLγA variants in the absence and presence of HsPOLγB. **C**) EMSA using WT and mutant HsPOLγA to quantify DNA binding affinities. Each line contains 10 fmol of DNA substrate and the indicated amount of polymerase. Quantification shown in Table 3.1.

Table 3.1: DNA binding affinities of WT HsPOLγ and HsPOLγ variants

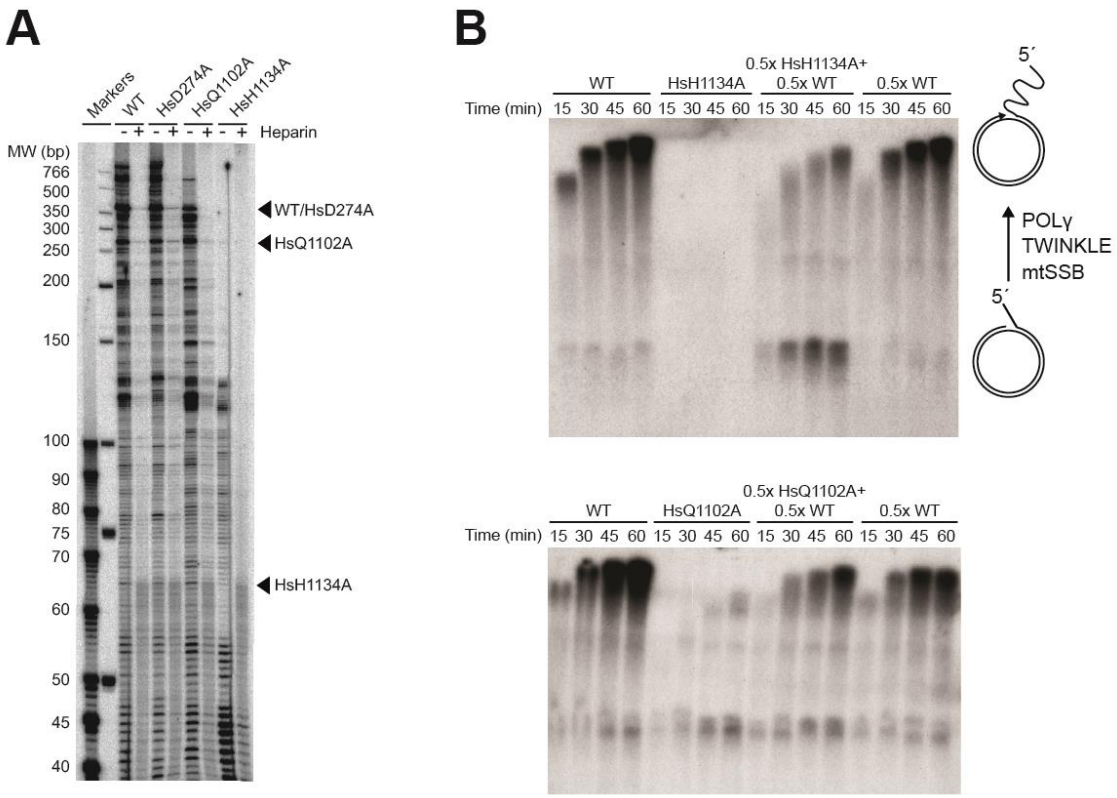
HsPOLγ	K_d (nM)
WT	5.5±1.4
HsD274A	2.8±0.1
HsQ1102A	3.8±0.2
HsH1134A	3.5±0.5



Adapted from Bratic et al., 2015

Figure 3.3: *In vitro* characterization of the WT, exo$^-$ (HsD274A), and pol$^-$ (HsQ1102A, HsH1134A) HsPOLγA polymerases.
A) Short primer degradation and extension assay in the absence and presence of increasing amount of dNTPs, respectively. **B**) Longer primer extension assay using a 32 bp primer hybridized to a single-stranded pBS plasmid. **C**) Schematic view of the rolling circle replication assay in which the functionality of different HsPOLγA variants is assessed together with mtSSB and TWINKLE (minimal replisome). **D**) Rolling circle replication assay as shown in (C) using all HsPOLγA variants. The rolling circle replication was stopped at the showed time points.

HsPOLγA has both 5' to 3' polymerase and 3' to 5' exonuclease activities. In the absence of dNTPs the exonuclease activity of HsPOLγA will predominate over the polymerase activity, whereas at high dNTP concentration the exonuclease activity is minimal (Johnson and Johnson, 2001) and the polymerase will engage in DNA replication. We investigated how different POLγA variants degrade or synthesize DNA in the absence and presence of dNTPs, respectively, using a short 35 bp filling assay (Fig. 3.3A). As expected, the exo$^-$ HsD274A showed no primer degradation in the absence of nucleotides but was able to synthesize DNA in the presence of nucleotides (Fig. 3.3A). In contrast, in the absence of nucleotides the pol$^-$ variants HsQ1102A and HsH1134A degraded the short primer even faster in comparison with WT HsPOLγA showing a higher exonuclease activity (Fig. 3.3A). On this short template the HsQ1102A variant was able to synthesize a full length product whereas the HsH1134A variant presented with decreased ability to synthesize DNA even in high dNTP concentrations (Fig. 3.3A).



Adapted from Bratic et al., 2015

Figure 3.4: HsPOLγA processivity and competition assay
A) Single-turnover conditions to assess the processivity WT HsPOLγA and HsPOLγA variants. Excess heparin traps any dissociated polymerase making sure that each extension product originates from a single turnover. **B**) *In vitro* competition assay for WT and HsPOLγA variants during rolling circle replication in the presence of mtSSB and TWINKLE.

To further assess the DNA replication ability of these POLγA variants we used a longer circular ~3 kb ssDNA template (Fig. 3.3B). Both HsD274A and HsQ1102A variants were slightly less efficient in synthesizing long stretches of DNA, whereas the HsH1134A showed barely any replication product under these conditions in comparison with the WT polymerase (Fig. 3.3B). To investigate how these HsPOLγA variants are able to synthesize even longer stretches of DNA we studied them in the context of the minimal replisome, where TWINKLE unwinds the already replicated DNA resulting in rolling circle replication (Fig. 3.3C). Both WT and HsD274A POLγA were able to engage in rolling circle replication in the presence of mtSSB and TWINKLE, although the exo$^-$ mutant was somewhat less efficient (Fig. 3.3D). Both pol$^-$ polymerases showed a drastic decrease or even absence of replication products (Fig. 3.3D). A processivity assay in the presence and absence of heparin showed that while WT and HsD274A have similar processivity, both pol$^-$ mutants had decreased processivity and that among these two variants the phenotype of the HsH1134A variant was more severe (Fig. 3.4A). Additional competition assay showed that the HsH1134A mutant was slightly dominant negative partially explaining its poor ability to synthesize DNA whereas the HsQ1102A did not inhibit the WT polymerase and was therefore recessive (Fig. 3.4B). In overall, the *in vitro* characterization of HsPOLγA mutants shows that the exo$^-$ (HsD274A) variant has strongly reduced exonuclease activity but is still able to synthesize DNA with almost same extent than the WT enzyme, whereas the two pol$^-$

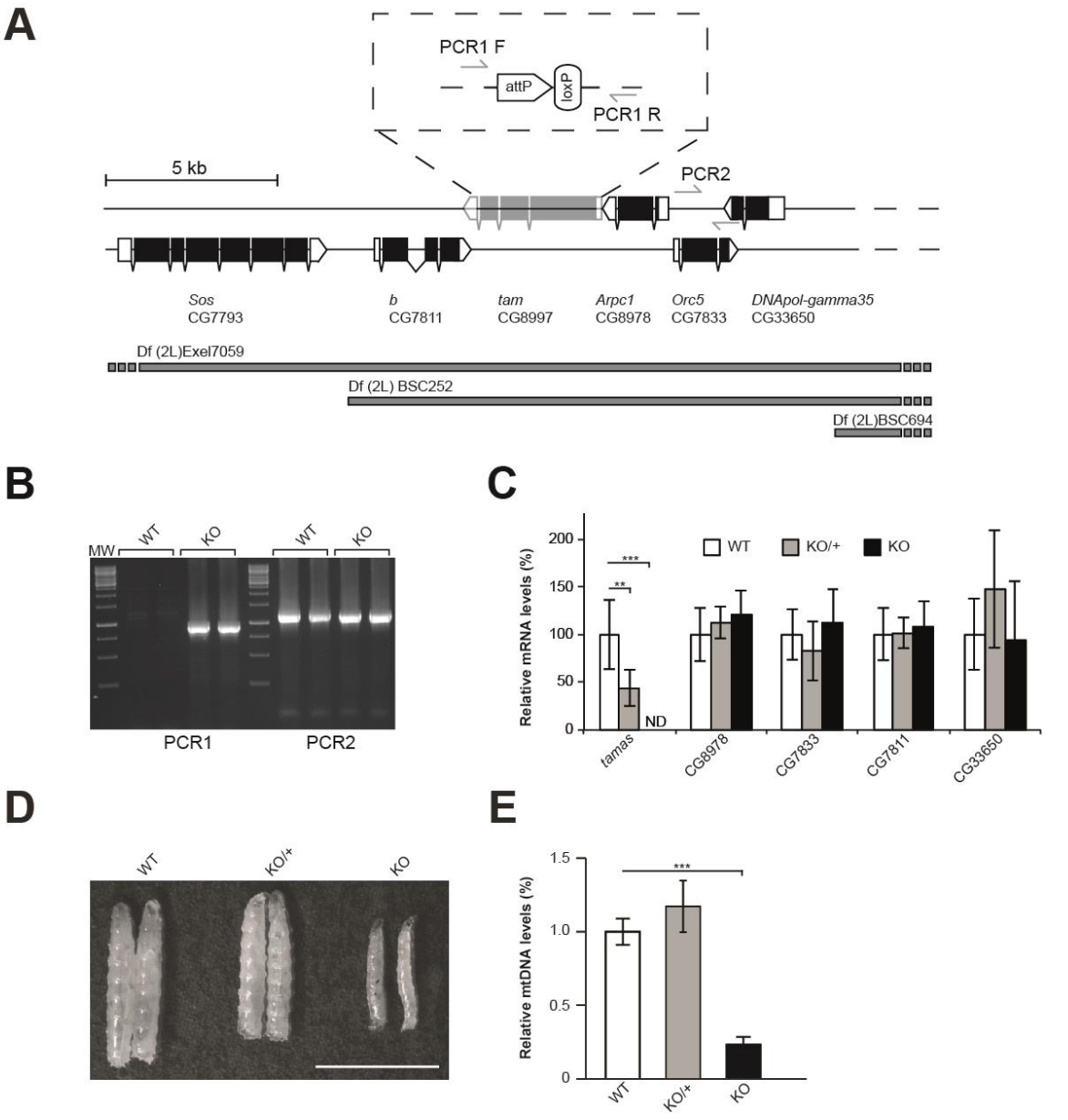
variants (HsQ1102A, HsH1134A) have increased exonuclease activity and strongly reduced polymerase activity in comparison with the WT polymerase.

3.2.2 Genomic engineering of the *tamas* (*DmPOLγA*) gene locus and generation of parental knockout lines

In order to establish *DmPOLγA* knockout founder line and fly lines carrying different variants of DmPOLγA we took advantage of the recently developed genomic engineering technique (Huang et al., 2009). This method has several advantages over more conventional techniques. First, using this technique one can establish pure knockout fly lines by removing the target gene of interest. Secondly, by reintroducing the target gene back to the endogenous locus it is possible to detect any aberrations caused by the genetic manipulation of the genomic locus. Thirdly, because all variants of interest are introduced to the endogenous locus the expression of the re-introduced gene will be under the control of the endogenous promoter. The last advantage is especially relevant in the case of DmPOLγA, because overexpression of catalytic subunit alone has been shown to cause mtDNA depletion and larval lethality (Lefai et al., 2000a).

To establish a *DmPOLγA* knockout founder line we first introduced the 3' and 5' flanking regions of the *DmPOLγA* (*tamas*) gene to the pGX-attP targeting vector (Fig. 2.1). This vector was further used for ends-out recombination to remove the *DmPOLγA* gene and replace it with site-specific recombination site at the cytological position 34D1 (Fig. 2.1 and 3.5A). The precise replacement of the *DmPOLγA* gene with a site-specific recombination site was verified by PCR, sequencing and complementation crosses (Fig. 3.5B and Table 3.2). In hemizygous *DmPOLγA* the mRNA steady-state levels were decreased by 50% of the WT levels and could not be detected in the knockout (Fig. 3.5C) showing an efficient removal of the *DmPOLγA*. Importantly, excision of *DmPOLγA* or introduction of the site-specific recombination site did not disturb the expression of other genes in the *DmPOLγA* locus (Fig. 3.5C). The hemizygous *DmPOLγA* developed normally into adulthood whereas the knockout larvae presented with decreased body size (Fig. 3.5D and 3.8A) and lethality before pupariation (Table 3.3) showing that DmPOLγA is essential for fly survival. To verify that the lethality of the knockout larvae is caused by the absence of *DmPOLγA* , hemizygous founders were crossed with genomic deficiencies covering or adjacent to *DmPOLγA* locus. Deficiencies covering the *DmPOLγA* locus (Df(2L)Exel7059, Df(2L)BSC252) could not complement the hemizygous *DmPOLγA* larvae whereas a deficiency adjacent to *DmPOLγA* (Df(2L)BSC694) showed full complementation (Fig. 3.5A and Table 3.2). None of the used deficiencies could complement each other (Table 3.2). The hemizygous *DmPOLγA* had no changes in relative mtDNA copy number whereas the homozygous knockout presented with strong mtDNA depletion (Fig. 3.5E) showing that DmPOLγA is the main replicative DNA polymerase in fly mitochondria. These results resemble the ones observed in the hemizygous *PolγA* mouse, where the MmPolγA mRNA levels were decreased by 50% without affecting mtDNA copy number (Hance et al., 2005). The results from both flies and mice

show that POLγA expression is not under tight regulation as the expression of the remaining allele does not show compensatory upregulation and that the POLγA levels are not limiting mtDNA copy number.



Adapted from Bratic et al., 2015

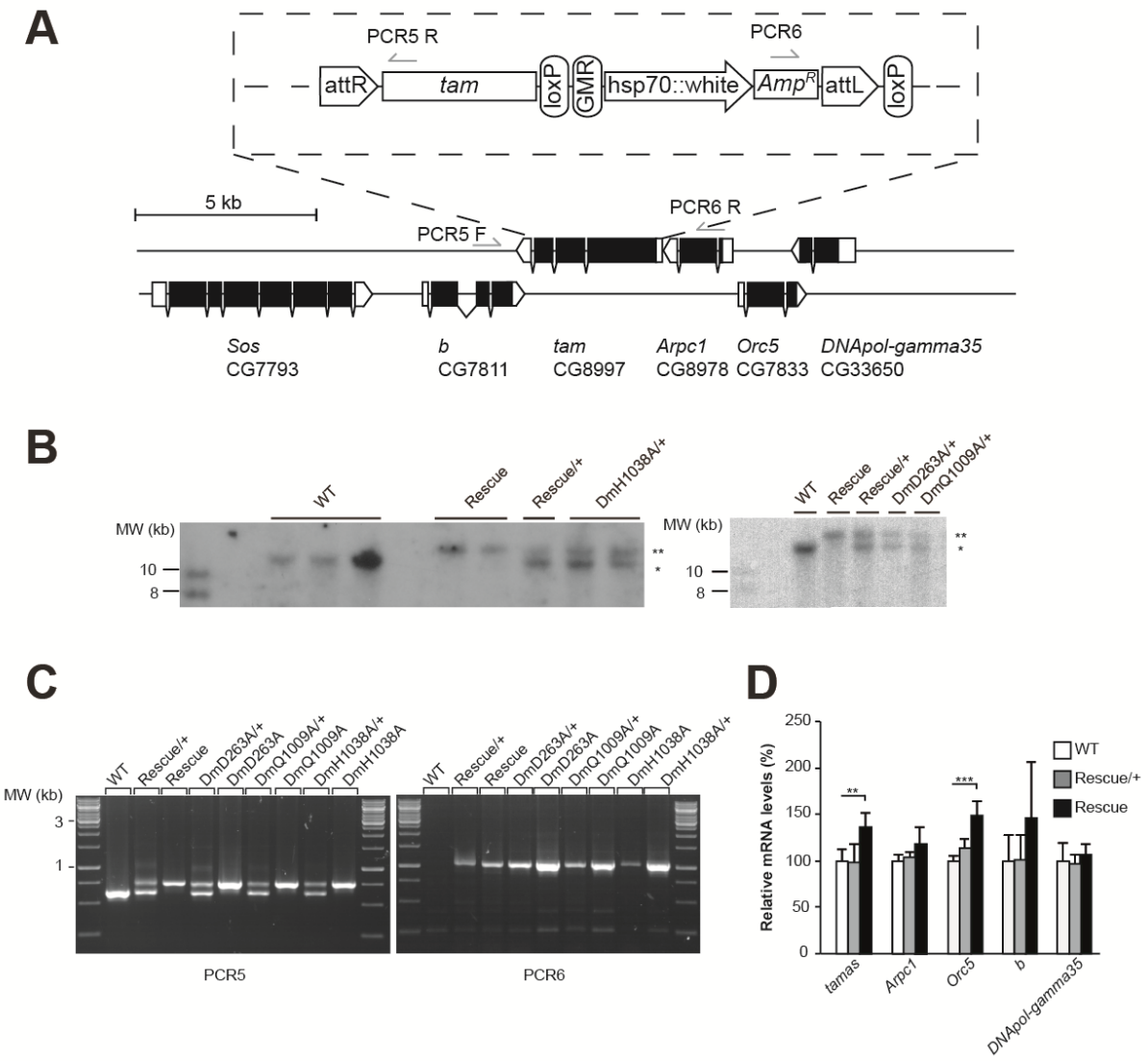
Figure 3.5: Genomic engineering of the *DmPOLγA* (*tamas*) locus to establish a knockout founder line
A) Schematic view of the *DmPOLγA* (*tamas*) locus, primer positions and locations of the genomic deficiencies used to verify the absence of *DmPOLγA* in the knockout founder line. PCR1 was used to verify the excision of the *DmPOLγA* and PCR2 was used as a positive control. **B**) PCR1 and PCR2 to verify *DmPOLγA* excision shown in (A). **C**) The steady-state mRNA levels of all the genes in the *DmPOLγA* locus were quantified by qRT-PCR in 5-day-old WT (white bar), hemizygous *DmPOLγA* ((KO/+, grey bar) and homozygous knockout *DmPOLγA* (KO, black bar) larvae. ND = not detected. One-way ANOVA with Dunnett's post hoc test. ***p<0.001, **p<0.01. Data are represented as mean ± SD. n=5. **D**) Representative image of the 5-day-old WT, hemizygous (KO/+) and homozygous knockout (KO) *DmPOLγA* larvae. Scale bar = 5 mm. **E**) Relative mtDNA copy number was quantified by qPCR in 5-day-old WT (white bar), hemizygous *DmPOLγA* ((KO/+, grey bar) and homozygous knockout *DmPOLγA* (KO, black bar) larvae. One-way ANOVA with Dunnett's post hoc test. ***p<0.001. Data are represented as mean ± SD. n=4–6.

Table 3.2: Complementation crosses with genomic deficiencies and founder line ([#] Expected eclosion rate 50 %. [*] Expected eclosion rate 25%)			
Genotype	**Df(2L)Exel7059**	**Df(2L)BSC252**	**Df(2L)BSC694**
WT	50% (213/425)[#]	49% (196/404)[#]	52% (215/413)[#]
KO	0% (0/219)[*]	0% (0/211)[*]	39% (73/185)[*]
Df(2L)BSC694	0% (0/68)[*]	0% (0/277)[*]	0% (0/277)[*]
Df(2L)BSC252	0% (0/387)[*]		

3.2.3 Reintroduction of WT *DmPOLγA* and mutants to the native locus

DmPOLγA resides in a dense 11 kb gene cluster with 4 other genes (Fig. 3.5A)(Lefai et al., 2000b). Therefore to make sure our genetic engineering approach does not affect the expression of the neighboring genes we re-introduced the WT *DmPOLγA* (Rescue) allele to the endogenous locus (Fig. 3.6A). The correct re-introduction of the Rescue allele was verified by Southern blotting and PCR (Fig. 3.6A-C). Homozygous Rescue flies were viable demonstrating that the absence of the *DmPOLγA* caused the lethality of the knockout founder line. Rescue flies presented with no or mild increases in the expression levels of the neighboring genes (Fig. 3.6D) showing that genetic engineering does not have major effects on other genes in the *DmPOLγA* locus.

The knockout founder line was further used to introduce the three *DmPOLγA* mutant alleles similar to the Rescue allele. In a strike contrast to the Rescue flies, flies homozygous for the exo$^-$ (DmD263A) or pol$^-$ (DmQ1009A, DmH1038A) alleles died mostly during the late larval stages (Table 3.3) and reducing genomically engineered flies, i.e. removing the mini white marker (Fig. 3.6A), had no effect of viability. The phenotype of the pol$^-$ flies was more severe as these larvae were significantly smaller in comparison with WT, Rescue or exo$^-$ larvae (Fig. 3.7A and B). Relative mtDNA copy number of genomically engineered larvae was determined to better understand the reason for developmental lethality. As heterozygous none of the larvae had changes in relative mtDNA copy number, but as homozygous the pol$^-$ larvae presented with severe mtDNA depletion (Fig. 3.7C). This mtDNA depletion is in line with the *in vitro* experiments showing that in HsPOLγA these variants have difficulties in replicating long products (Fig. 3.3D). In addition, a patient carrying mutation HsH1134R *in trans* with HsY831C were previously showed to have mtDNA depletion (Taanman et al., 2009) suggesting that mutating the conserved HsH1134 can impair polymerase function. To see whether this mtDNA depletion in pol$^-$ larvae leads to impaired OXPHOS capacity, oxygen consumption rates were measured from permeabilized 5-day-old larvae. Only pol$^-$ larvae showed significant reduction in both state 3 and maximal (uncoupled) respiration (Fig. 3.7D) suggesting impaired mitochondrial function in these larvae probably caused by the decreased mtDNA copy number (Fig. 3.7C).

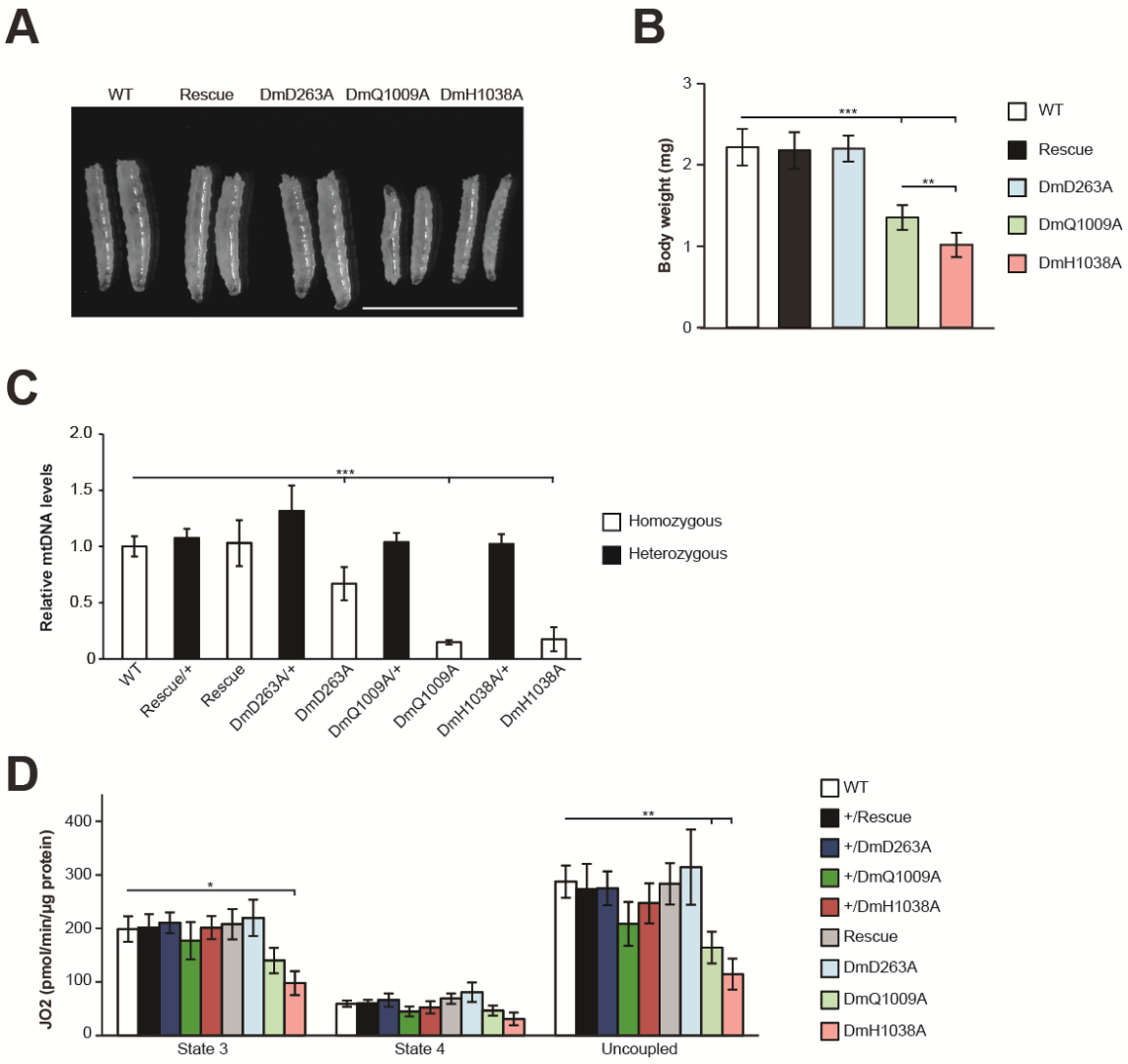


Adapted from Bratic et al., 2015

Figure 3.6: Reintroduction of Rescue, exo$^-$ and pol$^-$ alleles
A) Precise integration of the *DmPOLγA* alleles was verified by Southern blot. Presence of the mini white gene in genomically engineered flies results in shift in the locus size. ^{32}P-labeled *DmPOLγA* cDNA was used as a probe. **B**) Scheme to verify specific integration of *DmPOLγA* alleles by (C) PCR and positioning of the neighboring genes used for RT-qPCR (D). **C**) PCR to verify specific integration of *DmPOLγA* alleles as shown in (B). **D**) RT-qPCR to quantify the expression level of all genes in the *DmPOLγA* locus shown in (B). RNA was extracted from 5-day-old adult flies. One-way ANOVA with Dunnett's post hoc test. ***p<0.001, **p<0.01. Data are represented as mean ± SD. n=5.

To better understand the nature of these alleles we crossed all genomically engineered flies with the knockout founder line, genomic deficiencies covering or adjacent to *DmPOLγA*, (Fig. 3.5A) in addition to *tam3* and *tam4* hypomorphic *DmPOLγA* alleles (Iyengar et al., 1999). Only the Rescue allele was able to complement hemizygous *DmPOLγA*, genomic deficiencies covering *DmPOLγA* and the *DmPOLγA* hypomorphic alleles (Table 3.4 and 3.5). Interestingly, hemizygous pol$^-$ larvae developed further when the alleles were inherited paternally than maternally (Fig. 3.3) suggesting a negative maternal effect. The homozygous exo$^-$ (DmD263A) larvae died before pupariation, but hemizygous exo$^-$ larvae produced sick escapers (Table 3.3) suggesting that the exo$^-$ allele might have some dominant negative effects. It has been shown before for instance that mtDNA mutator mice do not have supercoiled mtDNA (Kolesar et al., 2013) suggestive of extensive nicking of mtDNA

although the reason for this is unclear. Further quantification of the body mass and mtDNA copy number of hemizygous larvae correlated well with the developmental phenotypes (Fig. 3.8).



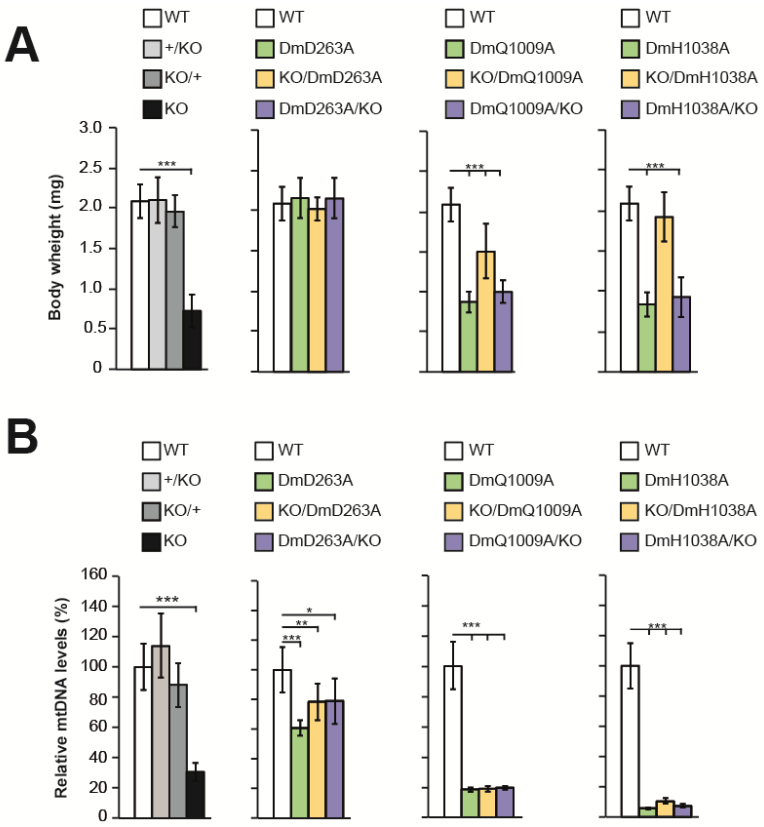
Adapted from Bratic et al., 2015

Figure 3.7: Homozygous exo$^-$ and pol$^-$ flies are developmentally lethal
A) Representative image of WT and homozygous genomically engineered larvae. Scale bar = 5 mm. **B**) Body mass quantification of WT and homozygous genomically engineered 5-day-old larvae. Tukey's Multiple Comparison Test. ***p<0.001, **p<0.01. Data are represented as mean ± SD. *n*=20. **C**) Relative MtDNA copy number was determined by qPCR from heterozygous (black bar) and homozygous (white bar) genomically engineered 5-day-old-larvae. Kruskal–Wallis test with Dunnett's *post hoc* test. ***p<0.001. Data are represented as mean ± SD. *n*=4–6. **D**) 5-day-old larvae were used to assess oxygen consumption rates at phosphorylating (state 3), non-phosphorylating (state 4) and uncoupled conditions. All measurements were normalized to total protein content. Mann–Whitney test, two-tailed. **p<0.01, *p<0.05. Data are represented as mean ± SD. n=4.

Table 3.3: Developmental analysis of DmPOLγA mutant flies ([#] Expected eclosion rate 50 %. [*] Expected eclosion rate 25%)			
Genotype	**3rd instar**	**Pupae**	**Adult**
WT	91±5%	95±1%	95±5%
♀+/KO♂	85±6%	78±6%	76±3%
♀KO/+♂	90±11%	69±9%	0%
♀KO♂	13±5%	0%	0%
♀DmD263A♂	79±16%	49%	0%
♀DmQ1009A♂	43±15%	2±1%	0%
♀DmH1038A♂	44±17%	0%	0%
♀KO/DmD263A♂	92±23%	77±24%	19±3%
♀KO/DmQ1009A♂	51±19%	35±18%	0%
♀KO/DmH1038A♂	91±12%	38±14%	0%
♀DmD263A/KO♂	70±9%	80±14%	24%
♀DmQ1009A/KO♂	58±18%	11±5%	0%
♀DmH1038A/KO♂	62±25%	0.7±1%	0%

Table 3.4: Complementation crosses with genomic deficiencies and knock-in lines ([#] Expected eclosion rate 50 %. [*] Expected eclosion rate 25%)			
Genotype	**Df(2L)Exel7059**	**Df(2L)BSC252**	**Df(2L)BSC694**
Rescue	52% (368/707)[#]	49% (232/474)[#]	51% (299/522)[#]
DmD263A	0% (0/139)[*]	0% (0/110)[*]	34% (156/466)[*]
DmQ1009A	0% (0/485)[*]	0% (0/125)[*]	36% (204/568)[*]
DmH1038A	0% (0/513)[*]	0% (0/145)[*]	36% (113/318)[*]

Table 3.5: Complementation crosses with *tam*^{3} and *tam*^{4} hypomorphs ([#] Expected eclosion rate 50 %. [*] Expected eclosion rate 25%)		
Genotype	***tam*^{3}**	***tam*^{4}**
Rescue	62% (47/76)[#]	56% (27/48)[#]
DmD263A	0% (0/140)[*]	0% (0/93)[*]
DmQ1009A	0% (0/91)[*]	0% (0/43)[*]
DmH1038A	0% (0/110)[*]	0% (0/59)[*]
Df(2L)Exel7059	0% (0/100)[*]	0% (0/64)[*]
Df(2L)BSC252	0% (0/95)[*]	ND
Df(2L)BSC694	0% (51/148)[*]	21% (12/56)[*]
***tam*^{4}**	0% (0/71)[*]	ND

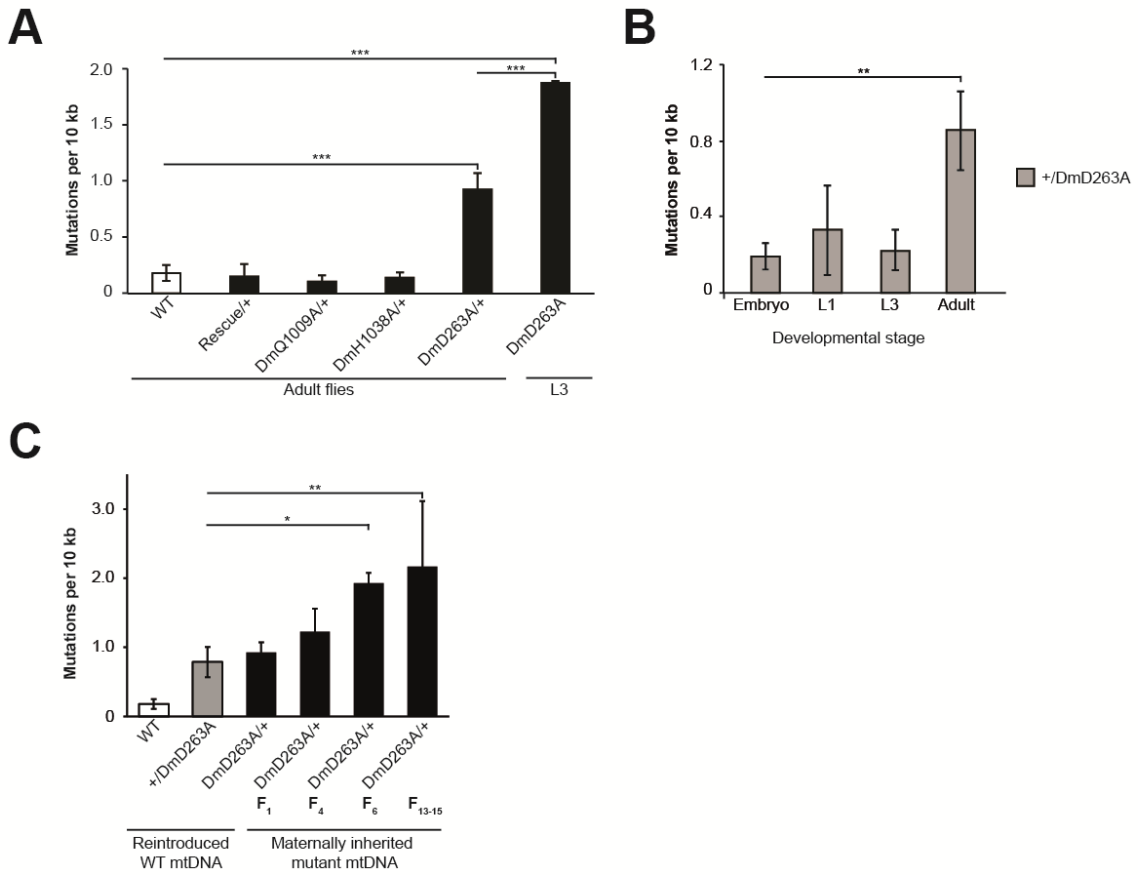


Adapted from Bratic et al., Nat. Comm. 2015

Figure 3.8: Body weight and relative mtDNA copy number of hemizygous and homozygous genomically engineered flies
A) Body weight of genomically engineered 5-day-old larvae. Genotypes are written as "maternal allele/paternal allele". One-way ANOVA with Dunnett's post hoc test. ***p<0.001, **p<0.01, *p<0.05. Data are represented as mean ± SD. n=20. **B**) Relative mtDNA quantification from hemizygous and homozygous genomically engineered 5-day-old larvae. Genotypes are written as "maternal allele/paternal allele". One-way ANOVA with Dunnett's post hoc test. ***p<0.001, **p<0.01, *p<0.05. Data are represented as mean ± SD. n=5.

3.2.4 MtDNA mutations accumulate during development in exo$^-$ flies

Because the homozygous exo$^-$ and pol$^-$ flies showed developmental lethality, the heterozygous adults were used for extensive post-PCR cloning and sequencing to quantify mtDNA mutation loads in these flies. The mtDNA mutation load of the WT flies was low ($\sim 10^{-5}$ mutations per bp) and was similar in Rescue and pol$^-$ flies (Fig. 3.9A). In contrast, flies having one exo$^-$ allele showed significantly increased mtDNA mutation load (Fig. 3.9A). MtDNA mutations have been shown to accumulate mostly in early development in fish, mice and humans (Otten et al., 2016, Greaves et al., 2014, Ameur et al., 2011). To investigate whether this is the case also in flies mtDNA mutation load was quantified from heterozygous exo$^-$ embryos, L1 larvae, L3 larvae and adult flies (Fig. 3.9B). Interestingly, increase in mtDNA mutation load could be observed only in adult flies (Fig. 3.9B) suggesting a major mtDNA mutation burst during morphogenesis. In this light it is interesting to note that the homozygous exo$^-$ larvae had even more mutations that the heterozygous adult flies suggesting a high mtDNA mutagenesis in these larvae (Fig. 3.9A).



Adapted from Bratic et al., 2015

Figure 3.9: MtDNA mutation load analysis of heterozygous flies, during development and intercrossed flies
A) MtDNA mutation load was quantified from heterozygous genomically engineered flies and homozygous exo$^-$ larvae. One-way ANOVA with Dunnett's post hoc test. ***p<0.001. Data are represented as mean ± SD **B**) MtDNA mutation load quantified from heterozygous exo$^-$ eggs, L1 larvae, L3 larvae, young adults and old adults. One-way ANOVA with Tukey's test. **p<0.01. Data are represented as mean ± SD. *n*=3–4. **C**) MtDNA mutation load quantified from WT flies (white bar), heterozygous exo$^-$ flies with only somatic mutations (+/D263A, grey bar) and 1, 4, 6 and 13-15 generations intercrossed heterozygous exo$^-$ flies. All genotypes were compared against the +/D263A genotype. One-way ANOVA with Dunnett's post hoc test. **p<0.01, *p<0.05. Data are represented as mean ± SD. n=3–6.

MtDNA mutations can be inherited when the mutagenesis takes place in the female germline. To find out how mtDNA mutations accumulate in the female germline, a crossing was prepare where the exo$^-$ allele is inherited paternally so that all the detected mtDNA mutations are somatic (+/DmD263A, Fig. 3.9C). These flies were further intercrossed for 1, 4, 6 and 13-15 generations and used for mtDNA mutation load analysis. The accumulation of mtDNA mutation in each generation was slow as significant differences could be detected only after 6 generations in comparison with the flies with only somatic mutations (Fig. 3.9C). This result is in high contrast to mammals were rapid shifts in heteroplasmy levels have been detected between generations (Stewart and Larsson, 2014).

DNA polymerases make more transition (purine>purine or pyrimidine>pyrimidine) than transversion (purine>pyrimidine or pyrimidine>purine) errors during DNA replication. In line with this most mutations observed in the heterozygous exo$^-$ flies were transitions (Table 3.6) and all transitions were equally abundant. Among the transversion mutations observed T>A transversions were by far the most common (Table 3.6). A surprising finding was that up to 24% of all mutations were short insertions and deletions (indels) (Table 3.6). Whether this high frequency of indels is something intrinsic to the

fly DmPOLγA is not known but it could be also related to the AT-richness and abundant homopolymeric stretches of fly mtDNA.

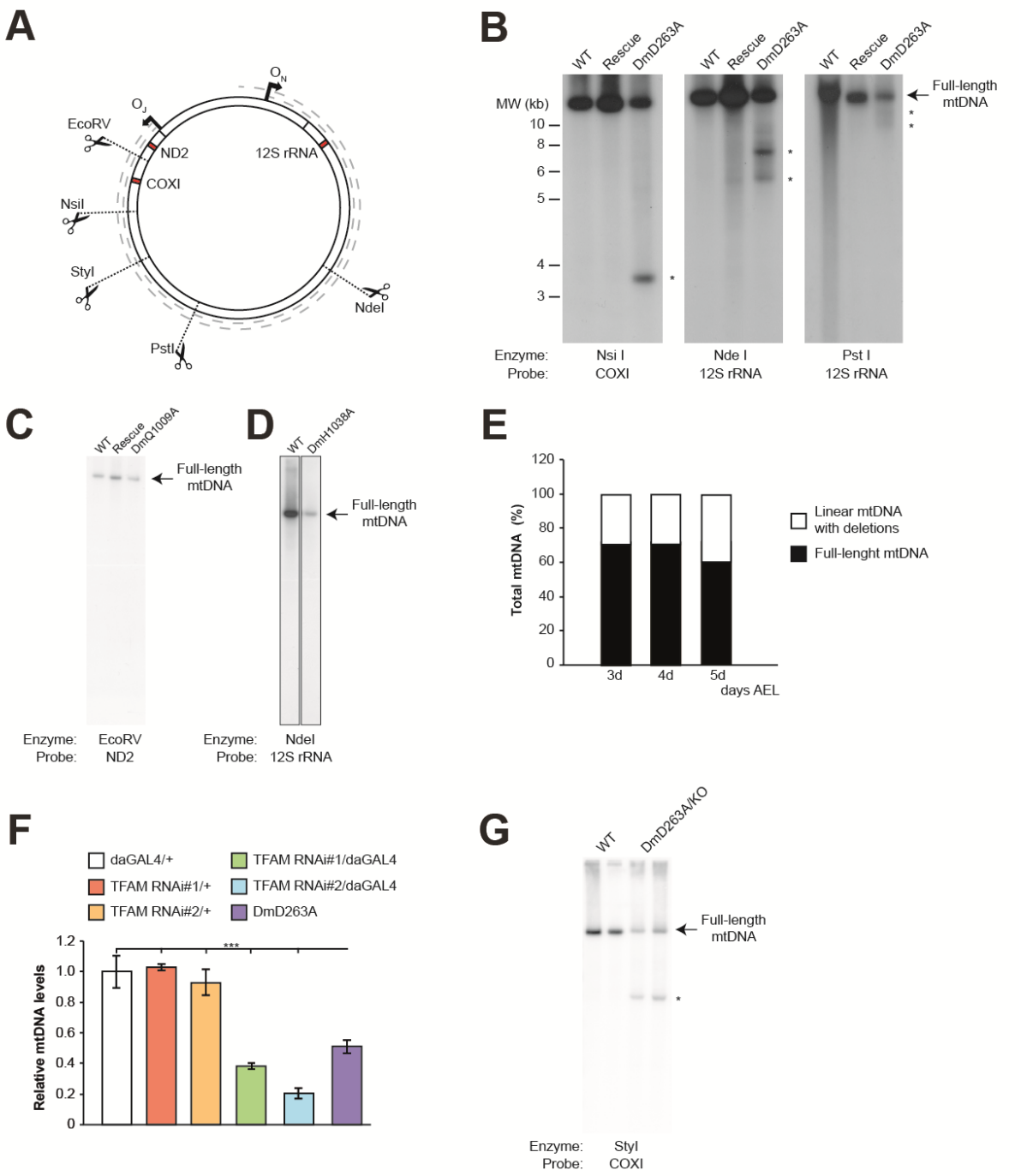
Table 3.6: MtDNA mutation pattern in exo⁻ flies				
Mutation		**Fraction**		
Transition	**A>G**	0.13	0.25	0.51
	T>C	0.12		
	C>T	0.15	0.26	
	G>A	0.12		
Transversion	**A>C**	0.00	0.02	0.25
	T>G	0.02		
	A>T	0.04	0.18	
	T>A	0.14		
	C>A	0.01	0.05	
	G>T	0.04		
	C>G	0.00	0.00	
	G>C	0.00		
Insertion/deletion		0.24		

Adapted from Bratic et al., 2015

Table 3.6: MtDNA mutation pattern in heterozygous exo⁻ flies.
Mutational data was pooled from total of 18 flies and all the mutations are shown from the perspective of the heavy strand. Because it is not possible to identify the strand in which the mutagenesis took place. These indistinguishable mutations are combined in the second column and the third column compared the frequencies of transition mutations, transversion mutations and indels.

3.2.5 Homozygous exo⁻ larvae carry linear mtDNA with deletions between replication origins

The mtDNA mutator mouse has well described mtDNA with linear deletions between the replication origins (Bailey et al., 2009, Trifunovic et al., 2004). To investigate whether the mtDNA integrity is compromised also in exo⁻ larvae, an extensive deletion mapping was performed by restriction enzyme digestion and Southern (Fig. 3.10A). Only homozygous exo⁻ larvae carried two linear mtDNA molecules (Fig. 3.10A, B and C) and the ends of those fragments were at close proximity with the replication origins (Fig. 3.10A). To understand whether these linear molecules were accumulating in exo⁻ larvae the relative levels of linear fragments was quantified from 3d, 4d and 5d old larvae (Fig. 3.10E) but the amount of deletions remained relative stable during this short period. Linear mtDNA is known to be actively degraded in mitochondria (Moretton et al., 2017, Bayona-Bafaluy et al., 2005) suggesting that these fragments are produced constantly. Recently, a model was put forward to explain how impairing the exonuclease function of POLγA allows the polymerase to perform short strand displacement creating unligatable DNA ends during mtDNA replication (Uhler et al., 2016, Macao et al., 2015). Our fly model is in line with this model as exo⁻ larvae had similar linear mtDNA deletions as has been observed in the mtDNA mutator mouse.



Adapted from Bratic et al., 2015

Figure 3.10: Relative mtDNA copy number and mtDNA integrity analysis
A) Schematic map of fruit fly mtDNA presenting the restriction enzyme cut sites (EcoRV, NsiI, PstI, StyI, NdeI), probe positions (red bars: ND2, COXI, 12S) and replication origins (O_J and O_N). Dotted line shows the approximate size and positioning of the two deleted molecules in homozygous exo$^-$ larvae. **B/C/D**) Southern analysis of (B) homozygous exo$^-$ larvae, (C) homozygous rescue and DmQ1009A larvae and (D) homozygous DmH1038A larvae to detect linear mtDNA deletions using the shown restriction enzymes and probes. Stars designate deleted molecules **E**) Quantification of linear deletion levels in homozygous exo$^-$ larvae from 3 d, 4 d and 5 d old larvae. **F**) Relative mtDNA copy number determination by qPCR from two different TFAM knockdown lines and homozygous exo$^-$ larvae in addition to controls. All genotypes were compared against the homozygous exo$^-$ larvae. One-way ANOVA with Dunnett's *post hoc* test. ***p<0.001. Data are represented as mean ± SD. *n*=5. **G**) Southern analysis of hemizygous exo$^-$ escaper flies to detect linear mtDNA deletions.

At the age of 5 days, the linear deletions of the exo$^-$ larvae reached 40% of total mtDNA. To study whether this amount of mtDNA depletion is sufficient to cause developmental lethality, TFAM levels were downregulated by RNAi and thereby also mtDNA copy number (Fig. 3.10F). TFAM knockdown in flies produced significant mtDNA depletion (Fig. 3.10F) but importantly these flies were still able to enter pupariation and even produce sick escapers (Table 3.7) suggesting that the level of mtDNA

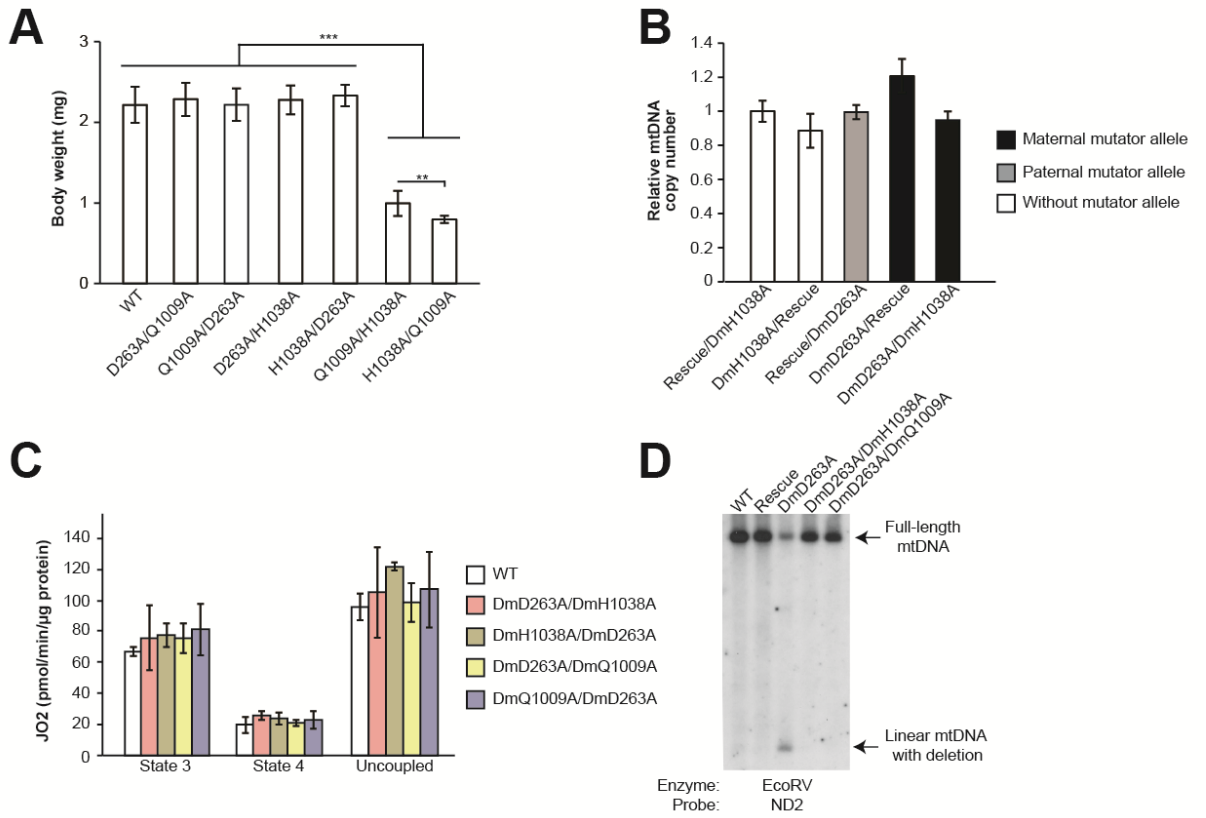
depletion present in exo$^-$ larvae is not sufficient alone to cause developmental lethality. Linear mtDNA deletions were also detectable in hemizygous exo$^-$ escaper flies (Fig. 3.10G) at somewhat higher level in comparison with the homozygous exo$^-$ larvae suggesting increased production of decreased turnover of the linear molecules. Unfortunately, it was not feasible to quantify the mtDNA copy number in hemizygous exo$^-$ escapers due to the low number of flies.

Table 3.7: Developmental analysis of TFAM RNAi flies
(Expected eclosion rate 100%)

Genotype	3rd instar	Pupae	Adult
daGAL4/+	78.4%	78.4%	77.8%
TFAM RNAi #1/+	80.8%	80.8%	80.4%
TFAM RNAi #2/+	94%	94%	94%
TFAM RNAi #1/daGAL4	87.8%	80.2%	11.2%
TFAM RNAi #2/daGAL4	79.6%	36.7%	0%

3.2.6 Complementation between exo$^-$ and pol$^-$ *DmPOLγA* alleles

The fact that heterozygous exo$^-$ flies have half of the mtDNA mutation load of the homozygous exo$^-$ larvae suggests that there is some level of complementation between the exo$^-$ and WT allele. In addition, it has been debated in the literature whether POLγA has to dissociate from the primer-template pair to switch between DNA synthesis and proofreading (intermolecular change) or whether the primer end can switch from polymerase site to exonuclease site within a molecule (intramolecular change) as *in vitro* studies have provided contradictive results (Johnson and Johnson, 2001, Olson and Kaguni, 1992). Using the pol$^-$ and exo$^-$ flies provides an interesting tool to address this question *in vivo* because the pol$^-$ alleles are poor polymerases but have higher than WT proofreading activity whereas the exo$^-$ allele has no proofreading activity but is a processive polymerase. Series of crosses were performed to obtain compound heterozygous flies and to detect genetic complementation. As expected, the two pol$^-$ mutants did not show complementation. In contrast, flies carrying one copy of the exo$^-$ allele and one copy either of the pol$^-$ alleles were viable and apparently WT-like as reflected by the body mass (Fig. 3.11A), mtDNA copy number (Fig. 3.11B) and oxygen consumption rates (Fig. 3.11C). The direction of inheritance of the alleles had no effect on these phenotypes. In addition, the linear deletions present in the homozygous exo$^-$ larvae were absent in compound heterozygous flies (Fig. 3.11D). All in all, we conclude that compound heterozygote flies carrying one functional polymerase allele and one functional proofreading allele have no phenotype due to genetic complementation.

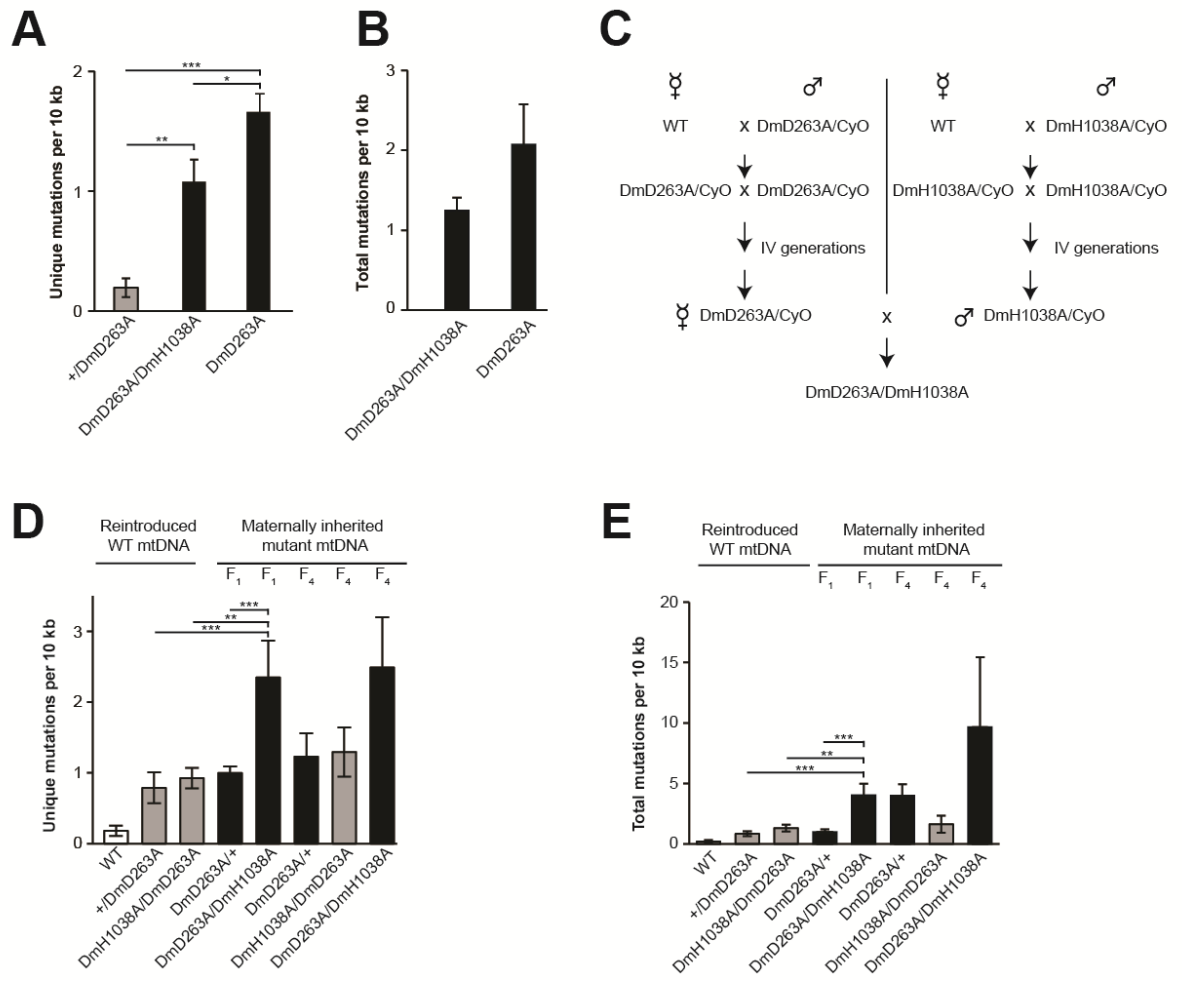


Adapted from Bratic et al., 2015

Figure 3.11: Genetic complementation at the *DmPOLγA* locus
A) Body weight comparison of compound heterozygote flies. In the name of each genotype the first and second alleles corresponds to maternally and paternally inherited alleles, respectively. Tukey's Multiple Comparison Test. ***p<0.001, **p<0.01. Data are represented as mean ± SD. n=20. **B**) Relative mtDNA copy number in compound heterozygous flies without mtDNA mutations (white bar), with only somatic mtDNA mutations (grey bar) or with both somatic and inherited mtDNA mutations (black bar). Kruskal-Wallis test with Dunnett's post hoc test. Data are represented as mean ± SD **C**) Oxygen consumption rate measurements using 5-day-old complementing larvae. Data are represented as mean ± SD **D**) Southern to detect linear mtDNA deletions in complementing 5-day-old larvae. Homozygous exo$^-$ larvae (DmD263A) were used as a positive control.

A comparison between mtDNA mutation loads in larvae showed that compound heterozygous flies have less unique mtDNA mutations in comparison with homozygous exo$^-$ larvae (Fig. 3.12D) although both larvae had similar amount of total mtDNA mutations (Fig. 3.12E). This suggests that homozygous exo$^-$ larvae have higher mtDNA mutagenesis than the compound heterozygous larvae, but the latter is able to reach similar levels of total mtDNA mutations probably due to faster clonal expansion of mtDNA mutations. To test this hypothesis a series of crosses were made to generate fly lines with higher mtDNA mutational background (Fig. 3.12A), which would enable the detection of clonal expansion of mtDNA mutations. When the exo$^-$ allele was inherited paternally, the heterozygous exo$^-$ flies (+/DmD263A) and compound heterozygous flies (DmH1038A/DmD263A) had similar levels of unique and total mtDNA mutations (Fig. 3.12B and C). In contrast, when heterozygous exo$^-$ flies or compound heterozygous flies inherited mtDNA mutations for one generation (F_1), the complementing flies had a marked increase in mtDNA mutation loads (Fig. 3.12B and C). This difference was even higher after intercrossing the exo$^-$ flies for four generations before made complementing (F_4) (Fig. 3.12A-C). These results suggest that the presence of these *DmPOLγA* alleles as compound heterozygous either increases mtDNA turnover resulting in higher clonal

expansion of mtDNA mutations, or that the size of the genetic bottleneck is decreased in these flies causing rapid shifts in mutation loads.



Adapted from Bratic et al., 2015

Figure 3.12: Quantification of mtDNA mutation load in compound heterozygous flies
A) Crossing scheme to obtain intercrossed compound heterozygous flies used in (B and C). **B**) Unique mtDNA mutation load quantification from WT flies (white bar), flies with only somatic mutations (grey bar), and flies with both somatic and inherited mutations (black bar). One-way ANOVA with Dunnett's post hoc test. ***P<0.001, **P<0.01. Data are represented as mean ± SD. n=3–6. **C**) Total mtDNA mutation load of flies shown in (B). Data are represented as mean ± SD **D**) Unique mtDNA mutation load quantification from heterozygous exo$^-$ larvae (+/D263A), compound heterozygous larvae (D263A/H1038A) and homozygous exo$^-$ larvae (D263A/D263A). Tukey's Multiple Comparison test. ***P<0.001, **P<0.01, *P<0.05. Data are represented as mean ± SD. n=3. **E**) Total mtDNA mutation load quantification from larvae shown in (D). Data are represented as mean ± SD

3.2.7 *In vitro* complementation between exo$^-$ and pol$^-$ HsPOLγA variants

To investigate whether the exo- and pol- complementation can be reconstituted *in vitro* the recombinant HsPOLγA function was studied in the context of a DNA primer containing a 3' mismatch (Fig. 3.13A).The ability of different HsPOLγA polymerases to remove a mismatch and continue DNA replication was first studied in the absence and presence of dNTPs (Fig. 3.13B). In the absence of dNTPs the recombinant WT and pol$^-$ HsPOLγA will remove the mismatch and degrade the primer (Fig. 3.13A) and as shown before this degradation is faster in the pol$^-$ HsPOLγA in comparison with WT polymerase (Fig. 3.13B). As expected, the exo$^-$ showed no primer degradation (Fig. 3.13B). In the presence of dNTPs the WT and pol$^-$ HsPOLγA were able to remove the mismatch and continue DNA replication although the HsH1134A was substantially slower in DNA replication relative to WT

enzyme (Fig. 3.13B). Exo$^-$ HsPOLγA had great difficulties in producing full length replication products probably due to its difficulties in bypassing 3' mismatch of the primer.

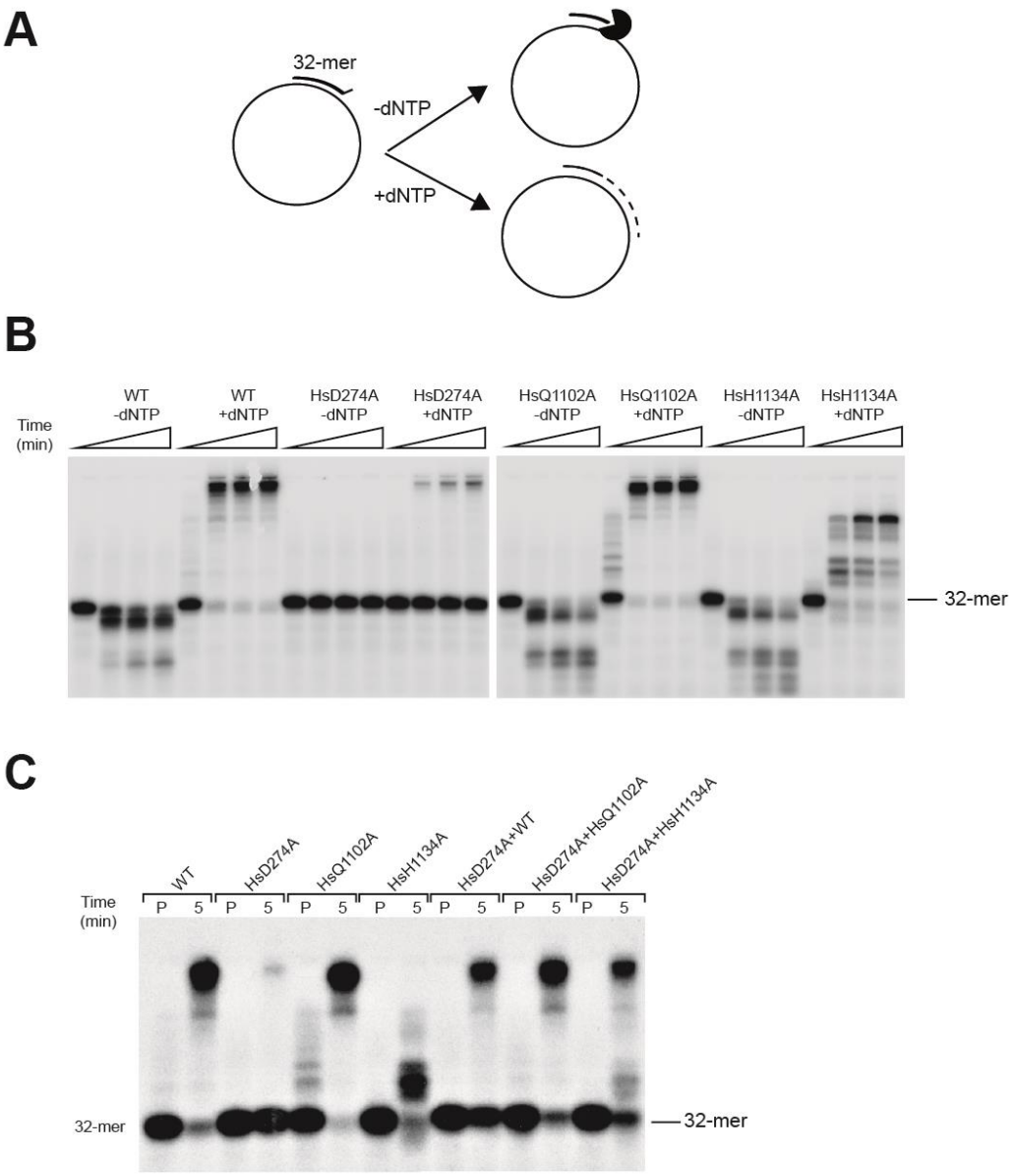


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Figure 3.13: Exo$^-$ and pol$^-$ recombinant HsPOLγA proteins complement each other *in vitro*
A) Schematic model of the *in vitro* replication and proofreading activity assay for recombinant HsPOLγA. The assay included a single-stranded pBS plasmid with 5' labeled 32 bp primer carrying a single 3' mismatch. In the absence of nucleotides a WT HsPOLγA will first remove the mismatch and further degrade the primer whereas in the presence of nucleotides HsPOLγA will first remove the mismatch and proceed with DNA replication. **B**) Polymerase and proofreading assays for WT, exo$^-$, and pol$^-$ variants of HsPOLγA as shown in A. **C**) Similar to reaction shown in B) but using a single time point and combination of different HsPOLγA variants. "P" means pre-incubation and "5" refers to the 5 min reaction time.

The ability of different HsPOLγA variants to complement each other in DNA replication was assessed with the same experimental setup as above using only a single time point (Fig. 3.13C). As before, both exo$^-$ HsD274A and pol$^-$ HsH1134A had difficulties in DNA replication using a primer carrying a 3' mismatch (Fig. 3.13C). In contrast, mixing these two mutants showed a synergistic effect resulting in full length replication products (Fig. 3.13C). The exonuclease and polymerase activities of POLγA reside in different domain of the polymerase. These results support the idea that polymerases carrying

defects in different functional domain of the polymerase can complement each other in DNA replication. For this to take place *in vitro* and *in vivo*, POLγA has to frequently dissociate and reassociate with the template-primer pair to switch between DNA replication and proofreading.

3.2.8 Accumulation of mtDNA mutation in flies with age

Proper mitochondrial function is essential for fly development as illustrated by the developmental lethality of the homozygous exo$^-$, pol$^-$ and TFAM knockdown flies (Table 3.3 and 3.7). Here established genomically engineered fly models provide an interesting tool to study the effects of mtDNA mutations on fly physiology. To first verify that mtDNA is replicated in adult flies, mtDNA of adult flies was labeled using pulse-chase 5-bromo-2′-deoxyuridine (BrdU) labeling. Feeding BrdU produced a strong labeling after four days of feeding (Fig. 3.14A and B). The signal was rapidly lost both in males and females although the latter showed somewhat faster loss signal (Fig. 3.14C and D) probably owing to the exceptionally high mtDNA replication taking place in female ovaries (Hurd et al., 2016). To see whether this turnover is sufficient to increase mtDNA mutation load with age, mtDNA mutation load was quantified from the thorax of young and old flies (Fig. 3.15A). Although there was an increasing trend, the mtDNA mutation load was not significantly different between young and old flies. These results show that even though mtDNA is turned over in adult flies, this turnover rate is not sufficient to cause a detectable increase in fly mtDNA mutation load with age.

Fly thorax is mostly composed of the post-mitotic flight muscle and would therefore only accumulate mtDNA mutations when mtDNA is turned over. In contrast, mtDNA would be expected to accumulate mutations faster in proliferative tissues, such as the female germline. To quantify whether mtDNA mutation load increases in the female germline, mtDNA mutations were detected from the progeny of young and old female flies (Fig. 3.15B). This avoids any biases caused by the changing ovarian structure with age. Almost no mutations could be detected in the progeny of young and old WT female flies (Fig. 3.15B). As expected, heterozygous exo$^-$ transmitted mtDNA mutations to the WT progeny (Fig. 3.15B) and although there were no differences in the amount of unique mtDNA mutations, the progeny of older females had more total mutations (Fig. 3.15B), suggesting clonal expansion of mtDNA mutation with age in the female germline. Of note, similar accumulation of mtDNA mutations does not take place in mammalian female germline (Boucret et al., 2017) probably due to the differences in the way oocytes are produced in these organisms.

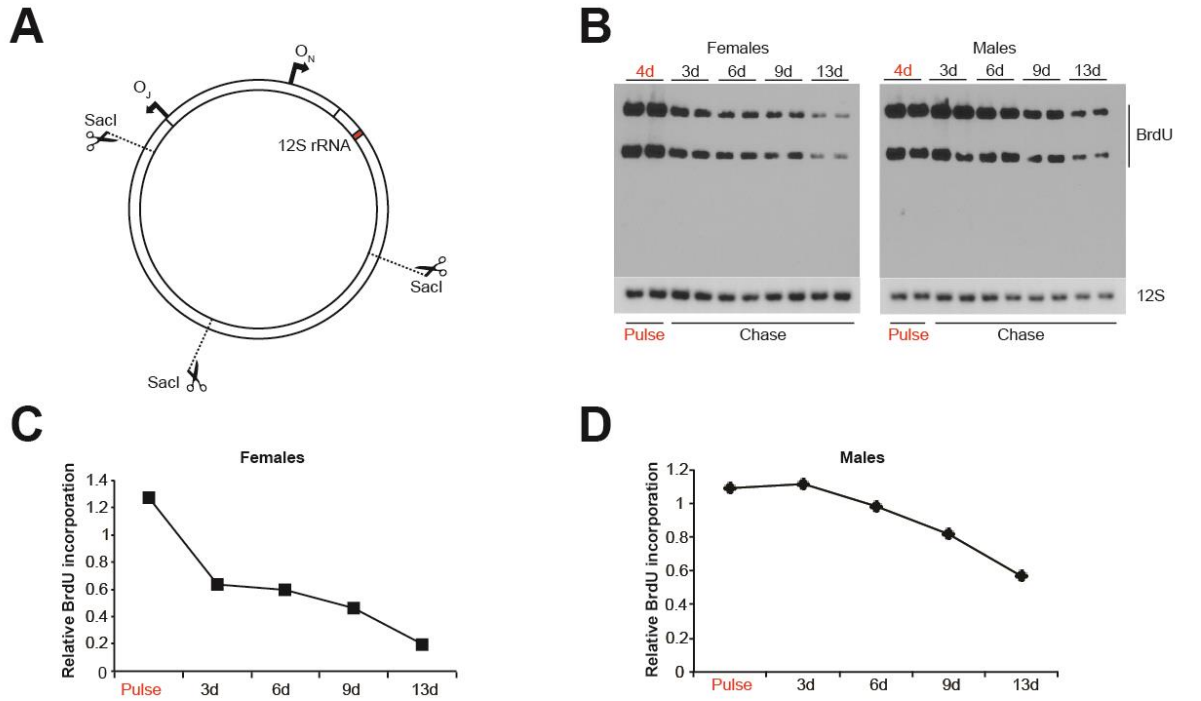

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Figure 3.14: MtDNA turnover in adult flies

A) Schematic view of mtDNA cut from three sites using SacI. **B**) A Southern and southwestern to detect mtDNA and mtDNA incorporated BrdU, respectively. Adult flies were fed BrdU on a pulse-chase experiment, mtDNA was extracted on the showed time points, restriction digested into three fragments as shown in (A) and run on agarose. **C**/**D**) Quantification of BrdU turnover in (C) female and (D) male flies.

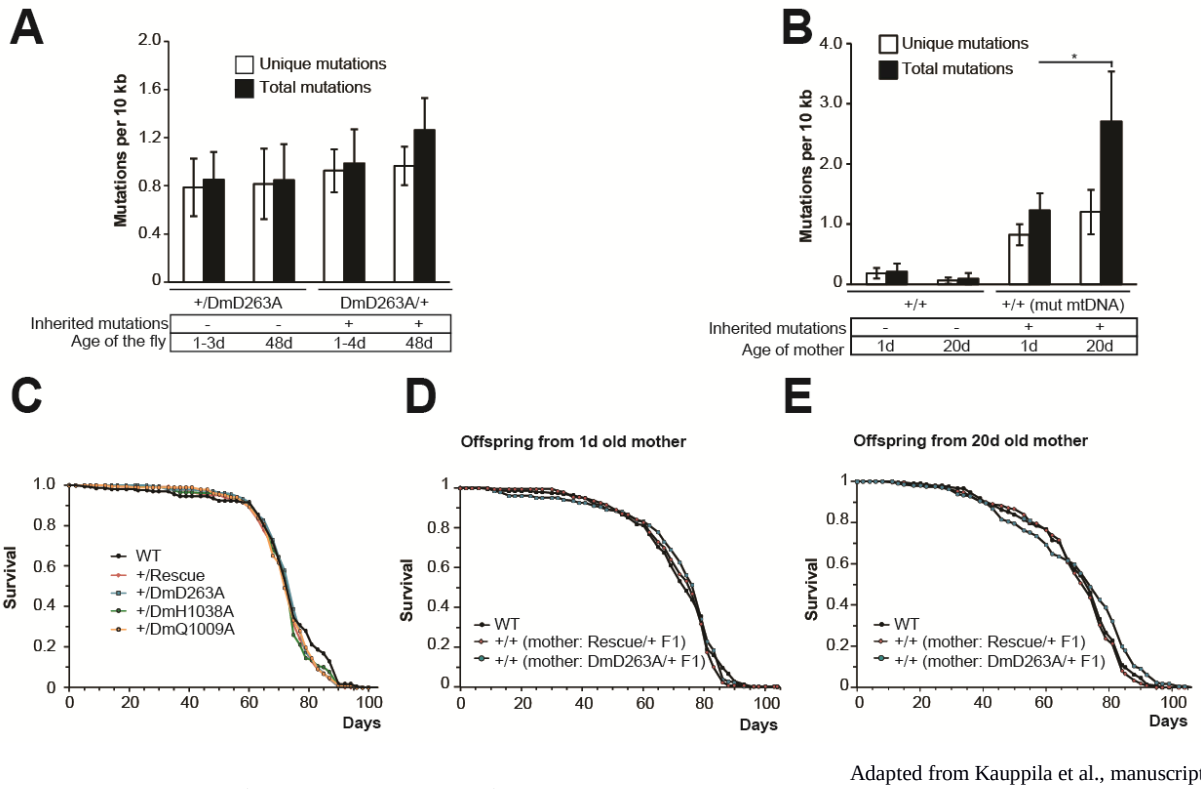

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Figure 3.15: Accumulation of mtDNA mutations in adult flies

A) Quantification of unique (white bar) and total (black bar) mtDNA mutation loads from young and old heterozygous exo$^-$ flies. Flies had only somatic (+/DmD263A) or both inherited and somatic (D263A/+) mtDNA mutations. Data are represented as mean ± SD. **B**) Quantification of unique (white bar) and total (black bar) mtDNA mutation loads from WT progeny of young and old WT and heterozygous exo$^-$ flies. Student's two-tailed t-test. *p<0.05. Data are represented as mean ± SD. **C**) Lifespan of flies inheriting genomically engineered *DmPOLγA* alleles paternally. Log-rank test. **D**/**E**) Lifespan of flies originating from (D) young or (E) old WT, heterozygous Rescue or heterozygous exo$^-$ flies. Only flies with WT nuclear background were used for the experiments. Log-rank test.

The mtDNA mutator mouse has been used extensively to study how mtDNA mutations affect ageing (Kujoth et al., 2005, Trifunovic et al., 2004) and the results have shown that inherited mtDNA mutations have stronger effect on mammalian lifespan in comparison with somatic mtDNA mutations (Ross et al., 2013). To date there has been no efforts to study whether mtDNA mutations limit the lifespan of short-lived organisms. Series of genetic crosses were done to assess the role of exo$^-$, pol$^-$ *DmPOLγA* alleles and somatic mtDNA mutations on fly lifespan. In the experiment all the alleles were inherited paternally to minimize any maternal effects. The lifespan of heterozygous exo$^-$ and pol$^-$ flies were similar to WT control (Fig. 3.15C, Table S9), even though the exo$^-$ flies have an increase in somatic mtDNA mutation load. This result suggests that although all of the exo$^-$ and pol$^-$ alleles are developmentally lethal as homozygous, as heterozygous they do not limit lifespan of flies.

Older exo$^-$ female flies transmit more clonally expanded mtDNA mutations to the progeny (Fig. 3.15B). These progenies of young and old exo$^-$ females were used to detect, whether inherited mtDNA mutations limit the lifespan of flies as they do in mice. Two nuclear WT strains were obtained from the same female population at day 1 and day 20. During 20 days each female has laid already hundreds of eggs and providing time for the germline cells to accumulate mtDNA mutations. The progeny from old females showed consistently poorer survival in comparison with progeny from young females (Fig. 3.15D and E). However, this effect was independent of fly genotype, and also from mtDNA mutations, as flies without inherited mtDNA mutations were indistinguishable for flies with inherited mtDNA mutations (Fig. 3.15D and E, Table S10, Table S11). The differences observed between the progeny from young and old females were likely contributed by the Lansing effect (Priest et al., 2002, Lansing, 1947). All in all, these results suggest the mtDNA mutation can clonally expand in the female germline but these mutations do not limit the lifespan of flies.

3.2.9 MtDNA mutations cause developmental delay

Mutations in several mitochondrial proteins are known to cause developmental delay in fruit flies, including *sesB, tko, Scsα* and *bonsai* (Quan et al., 2017, Galloni, 2003, Toivonen et al., 2001, Zhang et al., 1999) among others suggesting that mitochondrial function is essential during development. Developmental delay is also observable in flies with different mtDNA haplotypes (Salminen et al., 2017, Meiklejohn et al., 2013) showing that even milder differences in mitochondrial function can have developmental effects. To study how exo$^-$ and pol$^-$ alleles and compound heterozygosity affect fruit fly development, the developmental time from egg lay to eclosion was measured. Flies with only somatic mtDNA mutations and flies with one generation inherited mtDNA mutations eclosed mostly at day 11 with subpopulation of flies inheriting the exo$^-$ allele maternally showing mild developmental delay (Fig. 3.16A). To further increase mtDNA mutation load exo$^-$ flies were intercrossed for four generations before made compound heterozygous (Figure 3.12A). All fly lines with maternally accumulated mtDNA mutations (DmD263A/Rescue, DmD263A/DmH1038A and DmD263A/DmQ1009A) showed clear developmental delay (Fig. 3.16A). To investigate how much

mtDNA mutations contribute to the observed developmental delay independently from any nuclear background, all flies were outcrossed twice to WT background. MtDNA mutations were either kept or removed depending whether WT males of females were used for these outcrosses, respectively (Fig. 3.16B). Additionally, a heterozygous exo$^-$ fly line intercrossed for two years was included in the experiment and as above the mutated mtDNA was either maintained (Fig. 3.16B, green line) or removed (Fig. 3.16B, red line). Only flies inheriting mtDNA mutations maternally showed a strong developmental delay (Fig. 3.16B). This was even more clear in the case of heterozygous exo$^-$ flies intercrossed for two years showing how accumulating mtDNA mutations correlate with the severity of developmental delay.

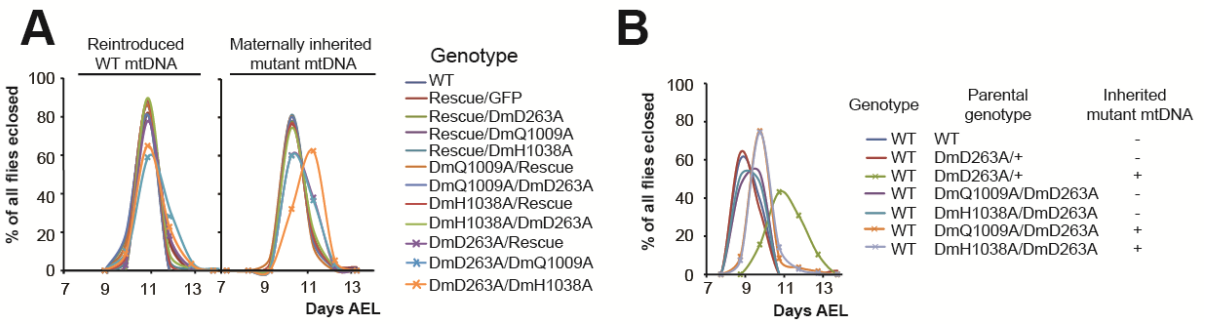

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Figure 3.16: Developmental time analysis
A) Developmental time analysis of flies with recently introduced WT mtDNA and flies with inherited mtDNA mutations. **B**) Developmental time analysis for flies with accumulated mtDNA mutations

The fact that flies with mtDNA mutations show developmental delay already at very low mtDNA mutation load levels could be caused by a combination of several factors. Firstly, the energetic demand during fruit fly development is exceptionally high so that even minor disturbances in mitochondrial ATP production could have negative effects on development. Secondly, functional mitochondrial Fe-S cluster synthesis is needed for steroidogenesis. In the case of fruit flies, impaired mitochondrial function results in failed 20-hydroxyecdysone (20E) synthesis (Llorens et al., 2015), a key hormone needed to enter pupariation. Thirdly, mitochondrial dysfunction can cause cell cycle delays (Xie and Dubrovsky, 2015) and limit cell growth (Frei et al., 2005) explaining why genetic screens trying to identify cell cycle regulators often discover mitochondrial proteins (Liao et al., 2006, Mandal et al., 2005, Frei et al., 2005). Combination of these negative effects could have interesting consequences at the population level as flies with clonally expanded pathogenic mtDNA mutations would have a competitive disadvantage. In would be interesting to investigate whether flies developing faster would have less pathogenic mutations in comparison with flies with strong developmental delay.

3.2.10 Slow clonal expansion of mtDNA mutations across generations

The observation that the exo$^-$ allele does not shorten the lifespan of flies in in stark contrast to the mouse models carrying the analogous mutation (Ross et al., 2014, Kujoth et al., 2005, Trifunovic et

al., 2004). The exo$^-$ flies have half of the mtDNA mutation load of the heterozygous mouse, $0.9*10^{-4}$ mut/bp and $2*10^{-4}$ mut/bp (Ross et al., 2013), respectively, which could explain some of the observed differences. This difference can be circumvented by intercrossing exo$^-$ flies for several consecutive generations (Fig. 3.9C) resulting in accumulation of mtDNA mutations. To this end exo$^-$ flies were intercrossed for 1, 6 and 15 generations to see whether this level of increase in mtDNA mutation load would be sufficient to limit fly lifespan. Surprisingly, none of the intercrossed flies showed any consistent changes in lifespan relative to WT and Rescue controls (Fig. 3.17A-C, Table S12-14). In a similar manner, exo$^-$ flies intercrossed for two years (~30 generations) and then outcrossed to obtain flies with WT nuclear background but carrying mtDNA mutations (+/+; mut mtDNA) were used for lifespan analysis but again there were no differences relative to control flies (Fig. 3.17D, Table S15). The above and published (Solignac et al., 1987) results suggest that the clonal expansion in flies between generations is minimal and it is only the clonally expanded mtDNA mutations that can cause biochemical defects. The compound heterozygous flies were shown to have increased mtDNA mutagenesis and rapid accumulation mtDNA between generations (Fig. 3.12B and C) and could be used to generate fly lines with even higher mtDNA mutation loads. Compound heterozygous flies with only somatic mutations had WT-like lifespan (Fig. 3.17E and F, Table S16) suggesting that the compound heterozygosity itself does not have negative effects on ageing. Also flies intercrossed for four generations before made compound heterozygous had normal lifespans (Fig. 3.17E and G, Table S17).

It is somewhat surprising that flies are so tolerant against mtDNA mutations. Previous studies have shown that flies carrying (near) homoplasmic mtDNA mutations have severe biochemical defects and shortened lifespan (Burman et al., 2014, Celotto et al., 2011, Xu et al., 2008). This could suggest that mtDNA mutations in intercrossed exo$^-$ flies or compound heterozygous flies have not clonally expanded to a certain level to cause biochemical defects. To detect high heteroplasmy level mtDNA mutations in intercrossed exo$^-$ flies or compound heterozygous flies, the full mtDNA coding region of these flies was sequenced and any detected heteroplasmic mutations were categorized into low (<33%), middle (33%-66%) and high (>66%) heteroplasmy level mutations. Using this approach no mutations could be detected in flies with only somatic mtDNA mutations (+/DmD263A) and even after 15 generations only a few mutations were categorized to be at high level (Fig. 3.17K) probably explaining the lack of phenotype in these flies.

To further increase mtDNA mutation load the compound heterozygous flies with increased mtDNA mutagenesis and faster clonal expansion of mtDNA mutations were intercrossed for 5 and more than 35 generations (Fig. 3.17H). Compound heterozygous flies showed reduced lifespan only after 35 generations of intercrossing (Fig. 3.17I and J, Table S18 and Table S19). In line with this result, these flies carry multiple high heteroplasmy level mutations (Fig. 3.17K). These results show that mtDNA mutations expand clonally across generations at a slow rate, but artificially increasing mtDNA

mutagenesis and clonal expansion can eventually result in flies with enough mtDNA mutations to shorten lifespan.

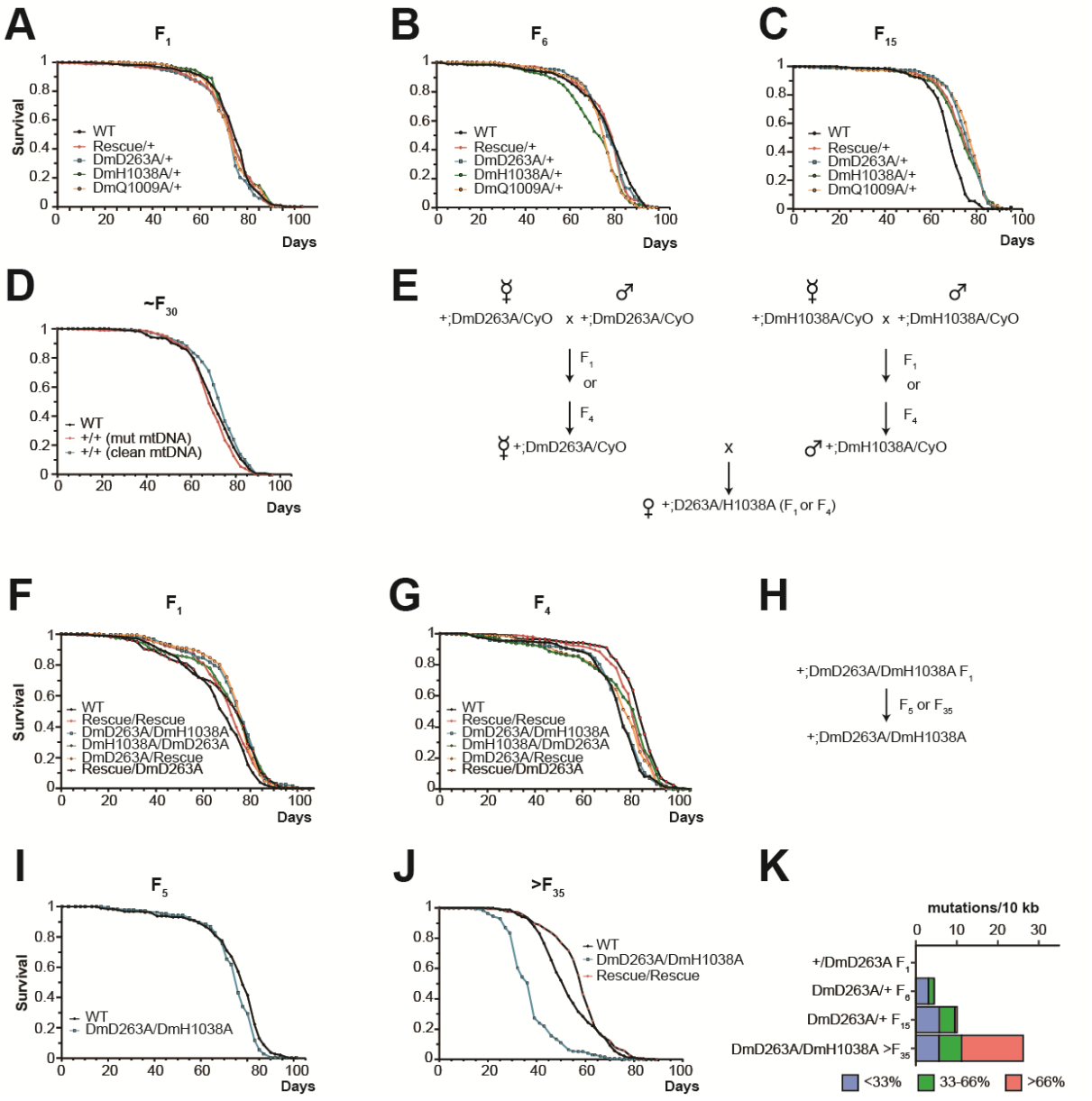

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Figure 3.17: Effects of mtDNA mutations on fly lifespan

A/B/C) Lifespans of flies transmitting genomically engineered DmPOLγA alleles for (A) one, (B) 6 and (C) 15 generations. **D**) Lifespan of flies accumulating mtDNA mutations for ~30 generations in the heterozygous exo- background followed by outcrossing of the allele. As a control WT mtDNA was re-introduced to the population (+/+ (clean mtDNA)). **E**) Crossing scheme to obtain flies intercrossed one or 4 generations prior to making flies compound heterozygous. **F/G**) Lifespans of compound heterozygous flies crossed for (F) one or (G) 4 generations before making compound heterozygous as shown in (E). **H**) Crossing scheme to generate 5 and >35 generations intercrossed compound heterozygous flies. **I/J**) Lifespans of compound heterozygous flies intercrossed for (I) 5 or (J) more than 35 generations as shown in (H). **K**) MtDNA mutations detected by Sanger sequencing in the shown fly genotypes. All detected mutations were categorized into low (<33%), middle (33-66%) or high (>66%) heteroplasmy level mutations.

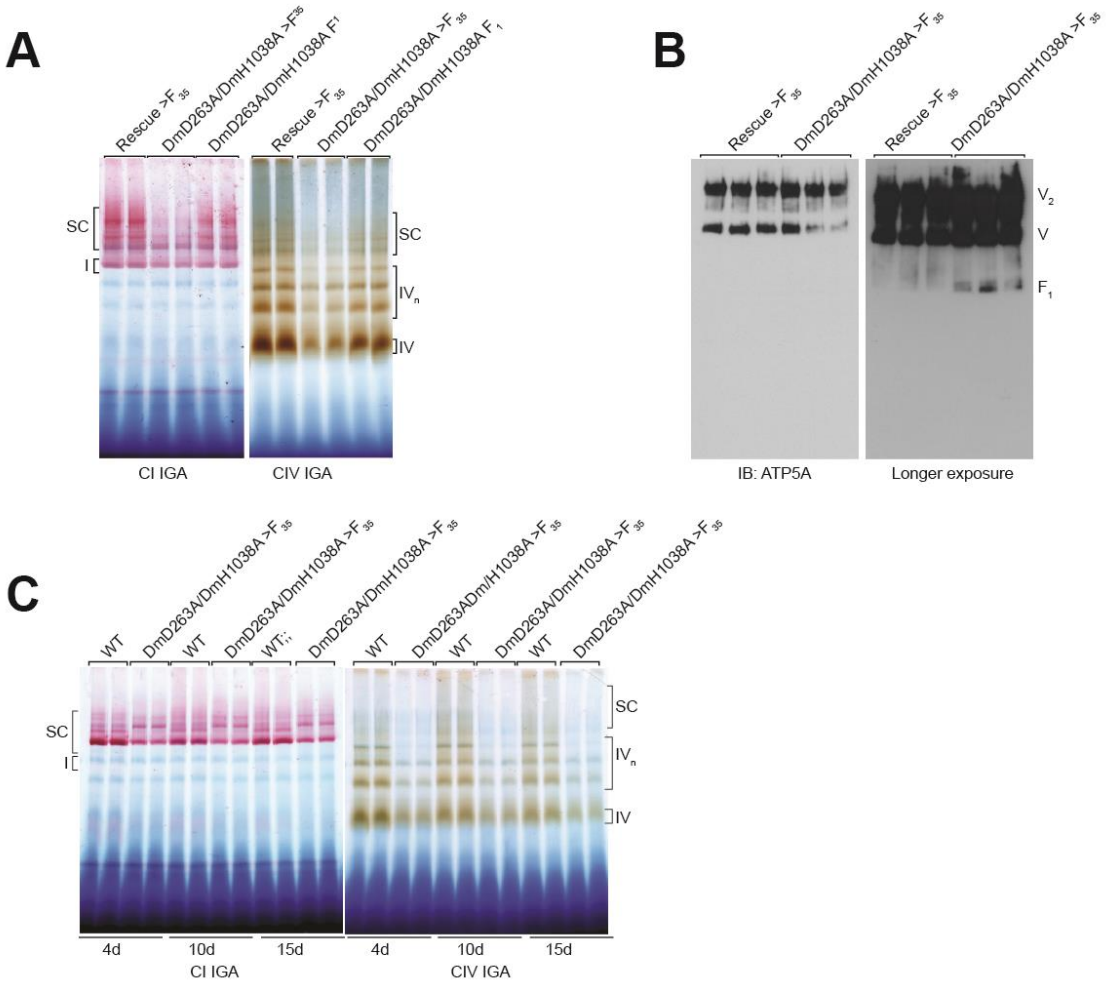

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Figure 3.18: MtDNA mutations lead to loss of OXPHOS complexes
A) Mitochondrial complex I and IV in-gel activity assays in BN-PAGE. SC=Supercomplexes, I = Complex I, IV = Complex IV, IV_n = Complex IV supercomplex. **B**) Immunoblotting against complex V in BN-PAGE gel. V_2 = Complex V dimer, V = Complex V monomer, F_1 = matrix portion of Complex V. **C**) Mitochondrial complex I and IV in-gel activity assays in BN-PAGE of flies of different ages.

3.2.11 Clonally expanded mtDNA mutations cause OXPHOS dysfunction

To verify that the abundant mtDNA mutations in the intercrossed compound heterozygous flies can cause a biochemical defect, the assembly and abundancy of OXPHOS complexes was assessed by in-gel activity assays and western blot (Fig. 3.18). Compound heterozygous flies with only low levels of mtDNA mutations did not show any changes in in-gel activity assay relative to control (Fig. 3.18A) in line with the respiration measurements in compound heterozygous larvae (Fig. 3.7D). Only extensively intercrossed compound heterozygote flies presented with decreased levels of complex I and IV and partially disassembly of complex V (Fig. 3.18B) as well as reduced levels of supercomplexes (Fig. 3.18A) suggesting these defects originate from mtDNA mutations. This decreased OXPHOS activity did not show any age-associated changes (Fig. 3.18C). To further verify these results proteome analysis was performed using Percoll gradient purified mitochondria. Similar to the in-gel activity assays the proteomic data showed a drastic decrease in several complex I and IV subunits in intercrossed compound heterozygote flies (Fig. 3.19A). Some proteins involved in mtDNA gene expression, OXPHOS and metabolism were also significantly upregulated in intercrossed

compound heterozygote flies (Fig. 3.19B). Interestingly, among the OXPHOS components several complex V subunits were upregulated, including bellwether (*blw*) (Fig. 3.19B), the fly ortholog of mouse ATP5A1, which was shown to be upregulated also in the mtDNA mutator mouse (Edgar et al., 2009) suggesting a conserved biochemical response in flies and mice. These data show that clonally expanded mtDNA mutations in flies can lead to OXPHOS dysfunction, which is likely resulting in the observed shortening of fly lifespan.

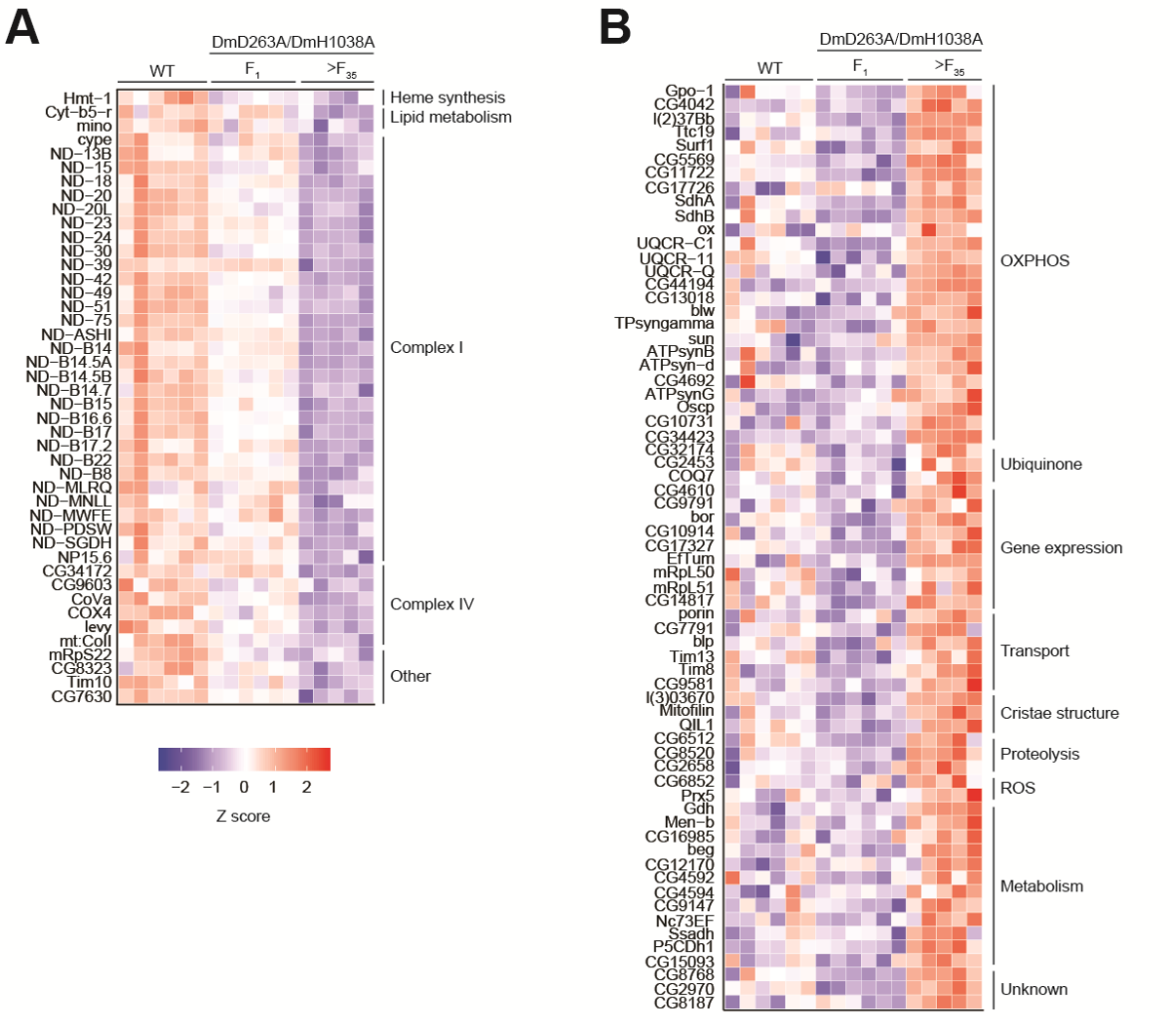

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Figure 3.19: MtDNA mutations cause changes in mitochondrial proteome
A) Mitochondrial proteins downregulated in flies with high levels of mtDNA mutations. **B**) Mitochondrial proteins downregulated in flies with high levels of mtDNA mutations. One-way ANOVA with FDR 0.05.

3.2.12 Decreased fly healthspan due to mtDNA mutations

In humans, mtDNA mutations affect usually first the most energy demanding tissues such as neurons and muscles (Chinnery, 2015). Also in model systems, mutations in nuclear encoded mitochondrial proteins and mutations in mtDNA are known to have negative in both of these tissues (Sen and Cox, 2017). To compare the effects of low and high levels of mtDNA mutations on fly neuromuscular function, the locomotor activity of flies was quantified. All genotypes showed typical activity changes during the day including the morning and evening bouts (Fig. 3.20A). None of the DmPOLγA alleles

of the compound heterozygosity itself had any effects in locomotor activity in comparison with WT control (Fig. 3.20B). However, compound heterozygote flies intercrossed more than 35 generations showed consistently decreased locomotor activity especially during the morning bout (Fig. 3.20B) suggesting these flies have either muscular and/or neuronal defects.

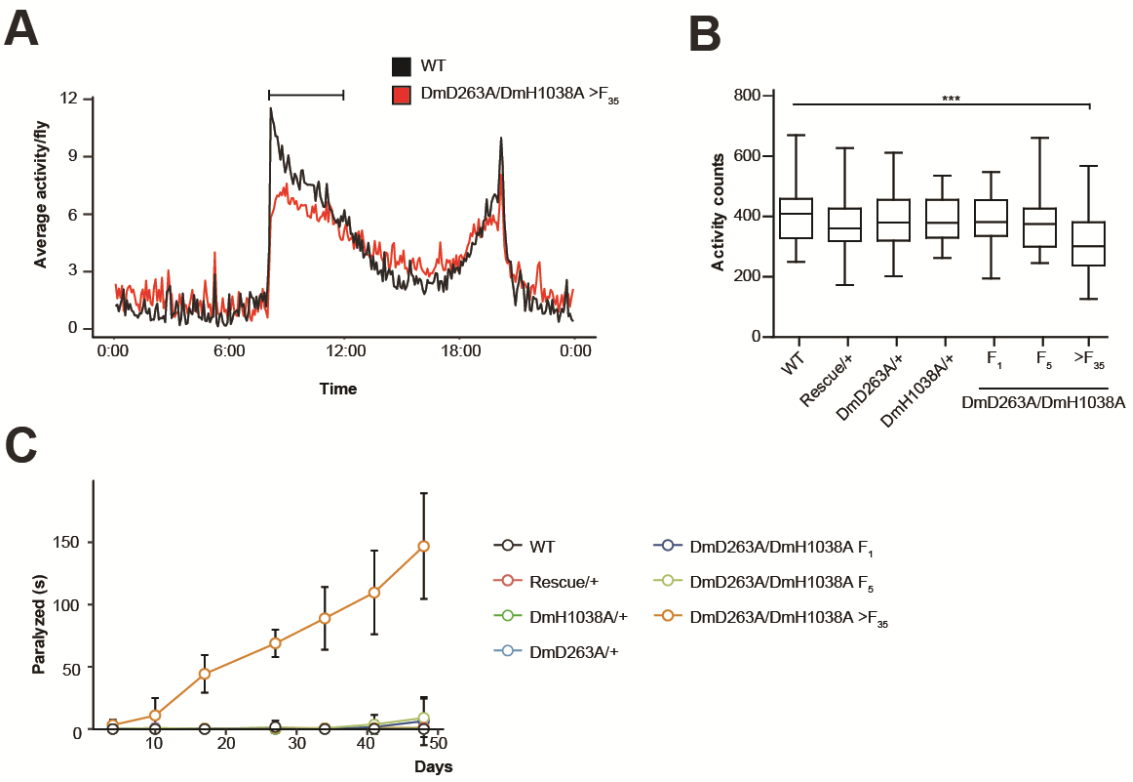

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Figure 3.20: MtDNA mutations decrease locomotor activity and make flies sensitive to mechanical stress
A) A representative example of average locomotor activity of WT and DmD263A/H1038A $>F_{35}$ flies during one day. Locomotor activity during the morning bout used for statistical analysis shown with bar. **B**) Total locomotor activity of flies during the morning bout (8:00-12:00).). One-way ANOVA with Dunnett's post hoc test. ***p<0.001. Data as represented as box plot with whiskers showing maximum and minimum values. **C**) Mechanical stress sensitivity assay for flies during lifespan.

Mutations in several nuclear encoded mitochondrial proteins are known to make flies sensitive to mechanical stress, also known as "bang sensitive". These genes include mRpS12, ANT, citrate synthase and dYme1L (Qi et al., 2016, Fergestad et al., 2006). In most cases these mutant have decreased cellular ATP levels, which likely causes the observed phenotype as most neuronal ATP is consumed in maintaining ion gradients across cell membranes. Indeed, it has been suggested that the bang sensitivity is caused by neuronal hyperexcitability as anticonvulsants can suppress some of these phenotypes (Fergestad et al., 2006). To assess whether mutations in mtDNA can also sensitize flies to mechanical stress the sensitivity of flies carrying different DmPOLγA alleles, compound heterozygote flies and intercrossed compound heterozygote flies were tested. Similar to the locomotor assay only flies with inherited and clonally expanded mutations (DmD263A/DmH1038A $>F_{35}$) showed substantial age-associated increase in sensitivity the mechanical stress (Fig. 3.20C). Together these results show that flies heterozygous for various DmPOLγA alleles or compound heterozygosity itself have no effect on fly physiology, whereas mutations accumulated and clonally expanded over tens of generations can result in marked decrease in fly health span.

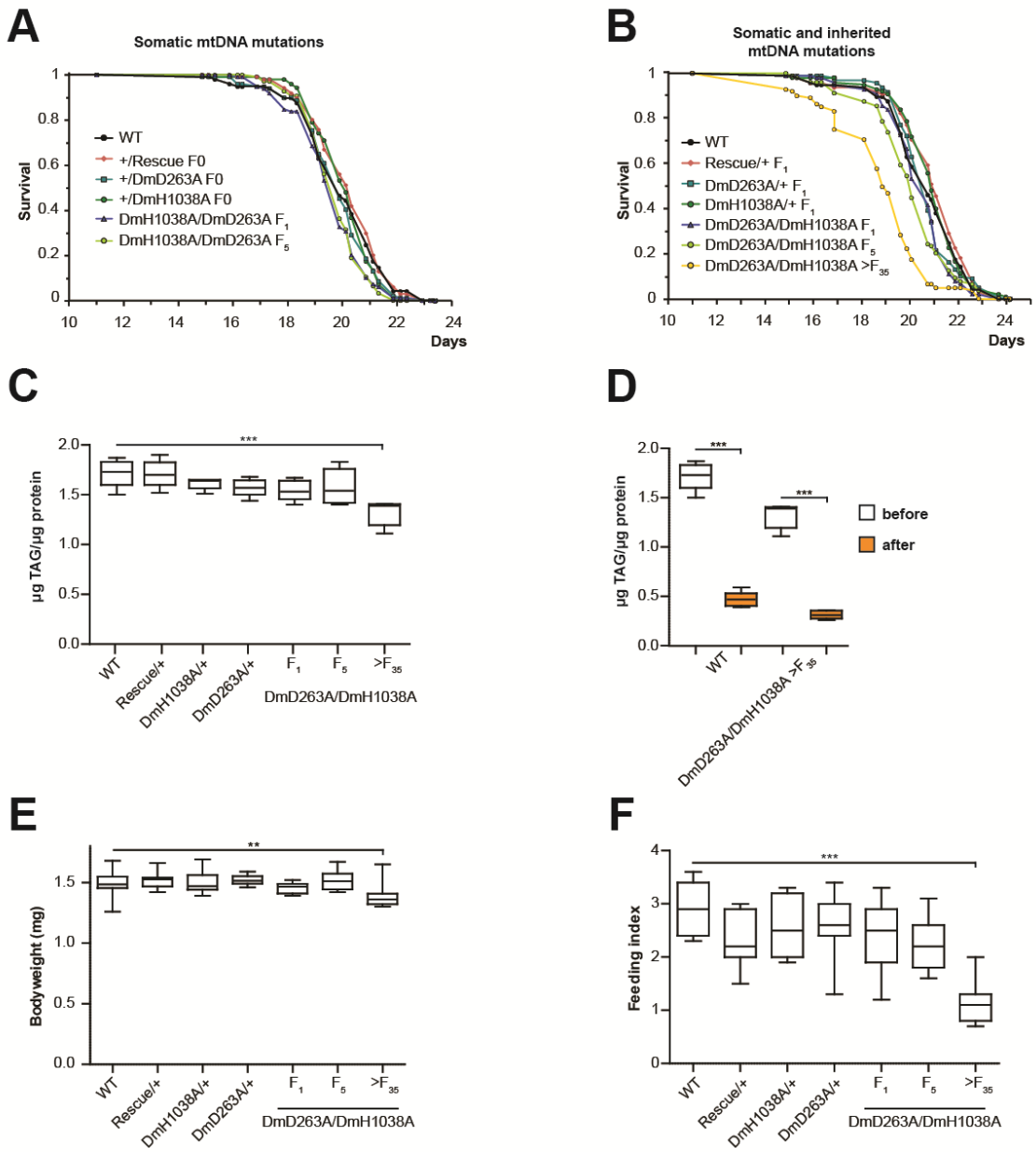

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Figure 3.21: MtDNA mutations make flies starvation sensitive
A/B) Starvation assay of flies with (A) only somatic or (B) both inherited and somatic mtDNA mutations. Log-rank test. **C/D**) Lipid quantification in adult flies (C) before or (D) before and after starvation. Student's two-tailed t-test. **E**) Body weight of young adult flies. One-way ANOVA with Dunnett's post hoc test. **p<0.01 **F**) Feeding activity of young flies. One-way ANOVA with Dunnett's post hoc test. ***p<0.001.

3.2.13 Mitochondrial dysfunction leads to starvation sensitivity

Mitochondria are a hub for several catabolic and anabolic processes including β-oxidation, amino acid degradation and synthesis, TCA cycle, one-carbon metabolism and urea cycle. Impaired mitochondrial function does not only affect the individual cell but can have far reaching consequences when the function of this sensory organelle is impaired in cells controlling metabolism of the whole organism (Sharoyko et al., 2014). Our mass spectrometry analysis of mitochondrial proteome showed that mtDNA mutations result in the upregulation of several enzymes related to mitochondrial metabolism (Fig. 3.19B). To detect whether these changes have any physiological effects, flies with DmPOLγA alleles and variable levels of mtDNA mutations were exposed to starvation. The presence of different

DmPOLγA alleles as heterozygous, compound heterozygosity or small increase in mtDNA mutation load had no effect on starvation sensitivity (Fig. 3.21A, Table S20). In contrast, flies with clonally expanded mtDNA mutation had significantly decreased sensitivity to starvation (Fig. 3.21B, Table S21). This is in line with the studies done in *D. simulans* showing that mtDNA haplotypes can affect fly metabolism and that a homoplasmic ND2 mutation in *D. melanogaster* is known to impair fat storage (Wang et al., 2016, Ballard et al., 2007).

This increase in starvation sensitivity could be caused by decreased amount of stored lipids or failure to mobilize and utilize those lipids upon starvation. Therefore, the fly lipid content was quantified both before and after starvation (Fig. 3.21C and D). The presence of any of the DmPOLγA alleles or compound heterozygosity did not have any effect on fly lipid content (Fig. 3.21C). Intercrossed compound heterozygous flies showed a small but significant decrease in the lipid content before starvation but these flies were able to mobilize lipid storages upon starvation (Fig. 3.21D). These changes in the lipid content correlated also with changes in body weight (Fig. 3.21E). Therefore the presence of mtDNA mutations and OXPHOS dysfunction in intercrossed compound heterozygous flies does not impair β-oxidation. What then could cause a decrease in fly body weight and lipid content? One of the simplest explanations could stem from studies done using the classical nematode *eat-2* mutant. This nematode present with a swallowing defect due to acetylcholine receptor mutation leading to loss of pharyngeal pumping (Lakowski and Hekimi, 1998). Indeed flies with high heteroplasmy mtDNA mutations have neuromuscular defects that could also affect the feeding behavior of the fly. To address this possibility the proboscis-extensions onto food surface was counted for flies with variable levels of mtDNA mutations. Only flies with high heteroplasmy level mutations showed decreased feeding activity (Fig. 3.21F) which could be caused by the neuromuscular defects and would likely contribute to the decreased lipid content and body weight of these flies.

3.2.14 Mitochondrial dysfunction can attenuate DR-mediated lifespan extension

One of the most consistent ways to improve organismal health is dietary restriction, chronic reduced intake of dietary constituents with the exception of essential nutrients (Fontana and Partridge, 2015). This effect is likely achieved via multiple pathways including improved insulin signaling and target of rapamycin (TOR). To study whether flies with mtDNA mutations respond properly to DR, flies with the various DmPOLγA alleles, compound heterozygote flies and flies with clonally expanded mtDNA mutations were subjected to varying yeast concentrations (0.1x, 0.5x, 1.0x and 2.0x). As expected, the lifespan of WT flies was longest under 0.5x and 1.0x yeast conditions whereas high and low yeast concentrations caused mild and substantial decrease in lifespan, respectively (Fig. 3.22A and B). Flies with low levels of mtDNA mutations were able to respond properly to DR whereas flies with clonally expanded mtDNA mutations had similar lifespan under 0.5x, 1.0x and 2.0x conditions suggesting a failure to respond to DR (Fig. 3.22A and B, Table S22). Interestingly, some studies have shown that knockdown of some nuclear encoded OXPHOS proteins attenuates the effects of DR (Bahadorani et

al., 2010, Zid et al., 2009). These results suggest that at least in flies mitochondrial function is necessary for DR mediated lifespan extension as mitochondrial dysfunction originating from either nuclear encoded mitochondrial genes or mtDNA can suppress the effects of DR. How mitochondria could participate in DR-mediated lifespan extension is still an open question. This could potentially take place through altered ROS signaling as ROS production is decreased in rodent heart, liver and skeletal muscles upon dietary restriction (DeBalsi et al., 2017). This altered signaling would affect especially stem cells, which maintenance is highly sensitive to changes in ROS levels (Ahlqvist et al., 2015b).

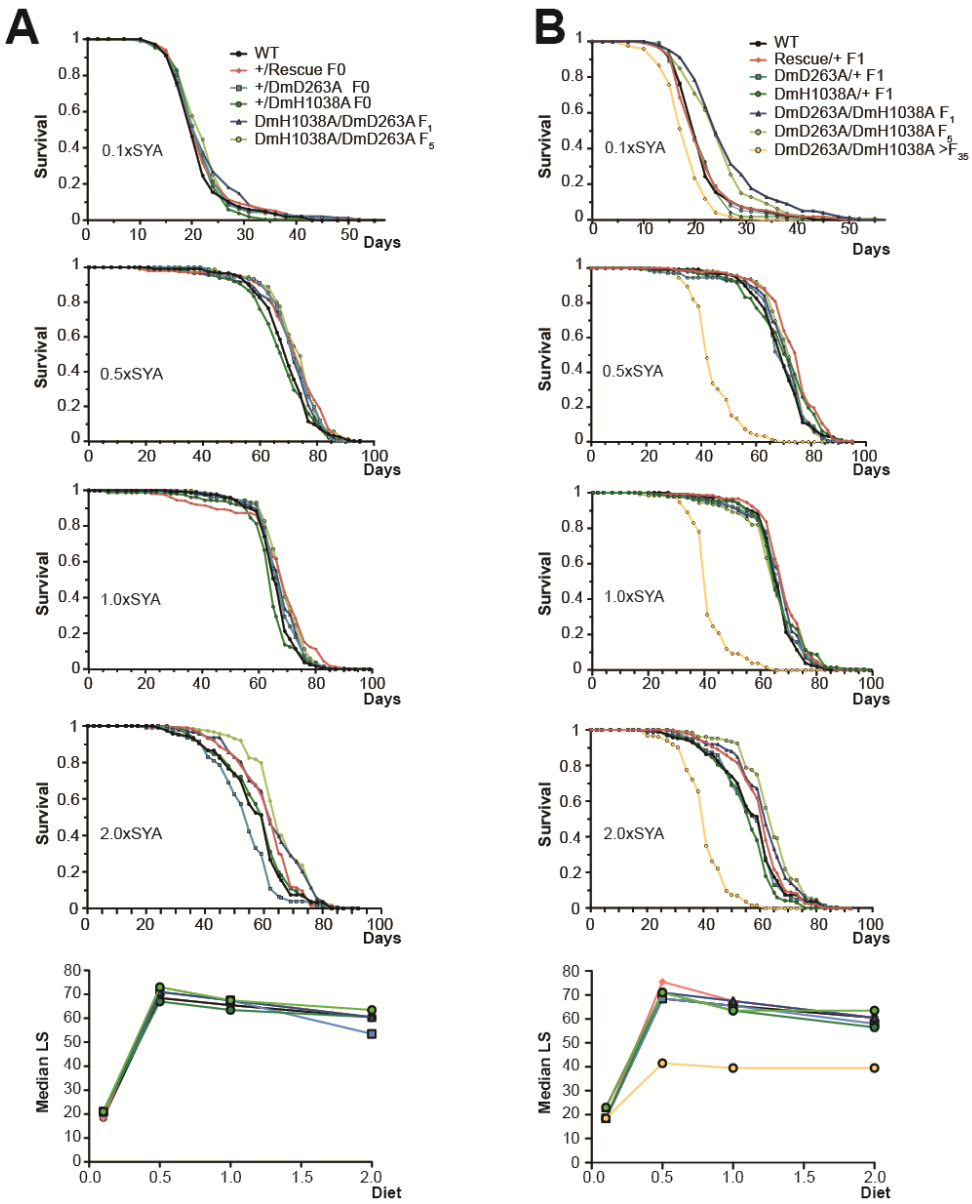

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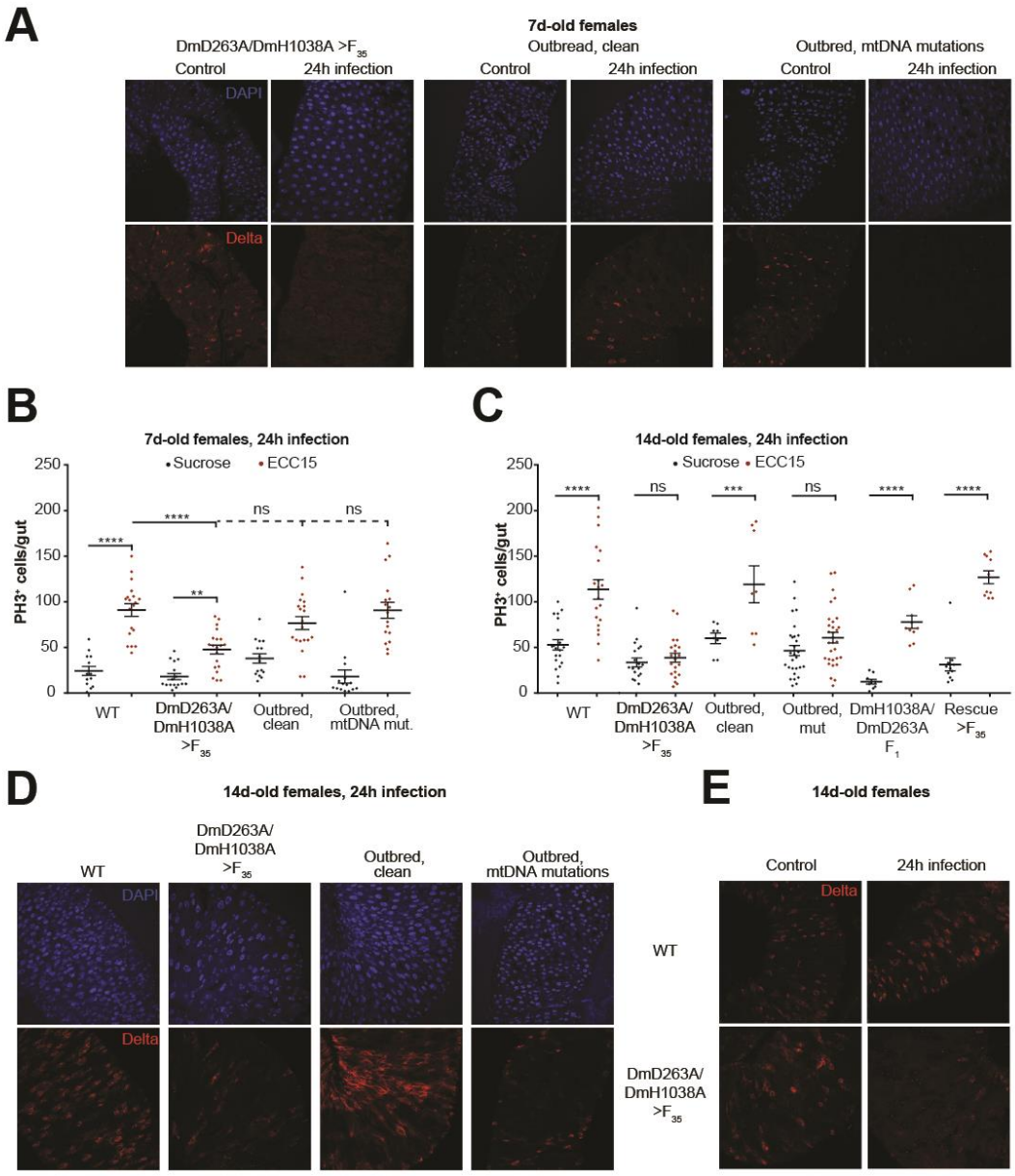
Figure 3.22: Dietary restriction assay for flies with mtDNA mutations
A/B) Lifespan analysis under varying yeast concentrations (0.1x, 0.5x, 1x and 2x SYA). Flies had either (A) only somatic mtDNA mutations or (B) both somatic and inherited mtDNA mutations. Log-rank test. Parametric survival analysis with logistic distribution (lowest Akaike information criterion) was used to analyze responses to DR.

3.2.15 Impaired proliferation of ISCs in the presence of mtDNA mutations

Fruit flies are mostly post-mitotic organism with the exception of few stem cell niches, such as the gut (Gonen and Toledano, 2014). The fruit fly gut is a single cell layer epithelium consisting of pluripotent ISCs, which can produce transient enteroblasts (EB) that further differentiate into enterocytes (EC) and enteroendocrine cells (EE). The ability of these ISCs to proliferate has been shown to be important for protecting the integrity of the gut in the presence of different stressors, varying dietary conditions and ageing (Jasper, 2015, Biteau et al., 2010). Additionally, increasing mitochondrial biogenesis in fly ISCs might have beneficial effects (Rera et al., 2012), whereas impaired mitochondrial function can cause the ISCs to enter senescence (Koehler et al., 2017). The observed attenuated DR response and decreased fly lipid content in flies with mtDNA mutations are often linked to impaired intestinal barrier function (Regan et al., 2016, Rera et al., 2012). In addition, mtDNA mutations are known to accumulate in the colonic crypts of ageing humans (Baines et al., 2014) leading to decreased proliferation and increased apoptosis (Nooteboom et al., 2010). Similar changes seen in ageing humans have been also observed in the mtDNA mutator mouse (Fox et al., 2012). Therefore, several physiological changes observed in the flies with abundant mtDNA mutations could stem from changes in intestinal stem cell (ISC) function.

To study whether mtDNA mutations can impair ISC homeostasis, 7 d and 14 d flies with different levels of mtDNA mutations were exposed to mitogenic condition by feeding flies *Erwinia carotovora carotovora 15* (Ecc15). This bacterium evokes a strong immune response in fly gut which is accompanied by increased proliferation of the ISCs (Buchon et al., 2009). In young flies only ISCs of flies with high levels of mtDNA mutations (DmD263A/DmH1038A $>F_{35}$) were not able to proliferate in response to Ecc15 as verified by the lack of Delta marker and $PH3^{+}$ cells (Fig. 3.23A and B). Interestingly, older flies with WT nuclear background but inherited mtDNA mutations showed also a slight decrease in ISC proliferation in response to the pathogenic bacteria (Fig. 3.23C and D) although the lifespan of these flies is not affected by the mtDNA mutations (Fig. 3.17D). These results suggest that somatic mtDNA mutations would not affect the maintenance of ISCs in WT flies and only multiple generations inherited mtDNA mutations can decrease ISCs proliferation in response to environmental stress. Further work would be needed to show whether DR can have positive effects on ISC maintenance in the presence of mtDNA mutations.



Adapted from Kauppila et al., manuscript

Figure 3.23: Intestinal stem cell (ISC) proliferation
A) DAPI (blue) staining for nDNA and Delta immunohistochemistry to detect intestinal stem cells in young flies before and after EEC15 infection. **B**) Quantification of the experiment shown in (A) using immunohistochemistry against $PH3^+$. One-way ANOVA, with Sidak's post hoc test. **C**) Quantification of $PH3^+$ cells as in (B) but in older females One-way ANOVA, with Sidak's post hoc test **D**) Immunohistochemistry as in (A) but only after EEC15 infection using older females. **E**) Immunohistochemistry as in (A) showing Delta staining before and after infection for WT and intercrossed complementing flies.

3.3 Discussion

Accurate mtDNA replication is essential for mtDNA maintenance especially due to the absence of recombination (Cooper et al., 2015, Hagström et al., 2014). Nevertheless, mtDNA is known to accumulate mutations an order of magnitude faster than the nDNA (Haag-Liautard et al., 2008). There is accumulating evidence showing that these mtDNA mutations originate from replication errors and not, as was previously thought, from oxidative damage (Kauppila et al., 2017). This difference in the mutation accumulation between nDNA and mtDNA is not due to error proneness of POLγ which, in

fact, is one of the most accurate mammalian DNA polymerases (Lynch, 2011). Instead, the high mutation rate is likely contributed by the constant mtDNA turnover in mitotic and post-mitotic tissues and the polyploidic nature of mtDNA allows even harmful mtDNA mutations to drift in the population.

Increasing the accuracy of POLγ is a tempting approach to decrease mtDNA mutagenesis. To date, all efforts to improve the accuracy of DNA polymerases have been performed in single cell organisms, such as *E. coli* and *S. cerevisiae* (Foury and Szczepanowska, 2011, Minnick et al., 1999). Therefore we wanted to address whether it would be possible to decrease mtDNA mutagenesis in a metazoan model by increasing the proofreading/polymerase activity ratio of POLγ. A detailed biochemical characterization showed that introducing single amino acid changes to the polymerase domain of POLγA (pol$^-$ variants) can increase the proofreading/polymerase activity ratio of the polymerase. This, however, came with a cost as the *in vitro* polymerase activity of these variants was substantially decreased and *in vivo* led to severe mtDNA depletion. Further studies are required to assess whether mutations affecting, for instance, the primer partitioning between the polymerase and exonuclease domains (HsPOLγA amino acids 842-856) could be mutated to favor the latter (Szymanski et al., 2015, Euro et al., 2011). Indeed, in WT POLγA the cost of proofreading is exceptionally low (Johnson and Johnson, 2001) so it might be possible to increase the exonuclease activity with the cost of increasing the removal of correctly paired bases.

The proofreading activity of POLγA can be decreased by introducing single amino acid changes in the exonuclease domain from yeast to mice (Kujoth et al., 2005, Trifunovic et al., 2004, Vanderstraeten et al., 1998). Both in mtDNA mutator mice and flies this leads to increased mtDNA mutation load, most of which are transition mutations similar to observed in WT animals again suggesting that mtDNA mutations originate from replication errors. In exo$^-$ flies, however, a quarter of all mutations detected were indels. Whether this high proportion of indels is caused by something intrinsic to the DmPOLγA or fly mtDNA (e.g. high number of poly-dT and poly-dA repeats) is not known. In addition, our results argue that flies are more sensitive to mtDNA maintenance defects than mice as flies homozygous for the exo$^-$ allele die at late larval stage whereas the mtDNA mutator mouse is viable despite the larvae carrying threefold less mtDNA mutations than the mtDNA mutator mouse. These differences could stem from different levels of mtDNA with linear deletions in flies and mice or because DmPOLγA seems to have dominant negative phenotype as hemizygous larvae develop further than homozygous larvae. At the physiological levels it was also clear that mtDNA mutations have little effect on adult flies whereas they cause a strong developmental delay in larvae. This could be taken to suggest that the energy needed for fly development is so high that even mild disturbances in mitochondria lead to lethality.

Interestingly, homozygous exo$^-$ larvae presented with linear mtDNA deletions. It has been previously characterized how the mtDNA mutator mouse carries linear mtDNA with deletions spanning the major

arc (Bailey et al., 2009, Trifunovic et al., 2004). An explanation for these deletions comes from a recent elegant biochemical work showing that upon reaching 5'-end of the newly synthesized DNA, POLγ displaces it partially before backtracking to form ligatable DNA ends (Macao et al., 2015). However, exonuclease deficient POLγ cannot backtrack after displacing the 5'-end resulting in flap structure that cannot be ligated. Using this nicked mtDNA as a template for mtDNA replication can lead to the formation of mtDNA with linear deletions between the replication origins. Although the origins of replication of fly mtDNA have not been fully defined, free 5'-end mapping suggests both replication origins are located in the A+T-rich region (Saito et al., 2005). The detected linear mtDNA fragments in the exo$^-$ larvae coincide with these sites supporting the view that fly mtDNA has dedicated origins of replication in the A+T-rich region.

POLγA HsA467T is one of the most common human POLγA mutations and it is often found alone or *in trans* with other mutations (Rajakulendran et al., 2016, Tzoulis et al., 2006). Heterozygote carriers are healthy although this mutant has only 4% of the WT polymerase activity (Chan et al., 2005) suggesting that WT allele can complement this defect. Surprisingly, our complementation studies suggest that polymerases carrying defects in different domains can complement each other both *in vivo* and *in vitro*. Based on the mutation load analysis it doesn't seem that pol$^-$ variants are able to remove any mismatches introduced by the exo$^-$ allele as the mtDNA mutation loads were similar between heterozygous exo$^-$ flies and complementing flies with only somatic mtDNA mutations. Instead, it is likely that the presence of the pol$^-$ allele enables the removal of flap structures formed by the exo$^-$ allele thereby creating ligatable mtDNA ends and avoiding the formation of mtDNA with linear deletions. In addition, it has been argued whether the switch between mtDNA synthesis and proofreading takes place intra- or intermolecularly (Johnson and Johnson, 2001, Olson and Kaguni, 1992). Although our results do not provide a definitive answer, the results suggest the intermolecular change is sufficient to maintain WT mtDNA levels.

MtDNA mutation load is known to increase with age in several species (Itsara et al., 2014, Williams et al., 2013, Yui et al., 2003, Khaidakov et al., 2003, Schwarze et al., 1995). It has been unclear, however, how mtDNA mutations accumulate in short-lived species and whether they contribute to ageing phenotypes. We showed here that fruit flies acquire most mtDNA mutations in the early development, which is similar to what has been observed in vertebrates. In long-lived species these mutations can drift randomly (Baines et al., 2014, Greaves et al., 2012) and when present above a critical threshold level lead to focal OXPHOS dysfunction (Larsson, 2010). Mathematical models have suggested that in humans it can take even decades for a *de novo* mutation to drift to reach this threshold level (Elson et al., 2001). This is supported also by mouse studies showing that rodents have limited mtDNA mutation accumulation with age probably owing to the relative short lifespan of the organisms (Kowald and Kirkwood, 2013, Greaves et al., 2011). Our results suggest that in fruit flies

accumulation of mtDNA mutations with age is limited. Accumulating mtDNA mutations for multiple generations had no effect on fly lifespan due to the slow clonal expansion of mtDNA mutations.

Only compound heterozygote flies with increased mtDNA mutagenesis and decreased size of the genetic bottleneck showed high heteroplasmy level mtDNA mutations after extensive intercrossing. This slow clonal expansion of mtDNA mutations between generations is in stark contrast to humans where rapid shifts in heteroplasmy levels across generations can lead to mitochondrial diseases (Wilson et al., 2016, Elliott et al., 2008). The number of female germ cell divisions per generation is similar in flies (36.5) and mice (31) (Drost and Lee, 1995) and cannot therefore solely explain the observed differences. Instead contribution of the fly nurse cells to the total mitochondrial pool of the egg (Cox and Spradling, 2003) and differences in the formation of the primordial germ cells likely explain these differences (Hurd et al., 2016, Strome and Updike, 2015).

In the mtDNA mutator mouse abundant mtDNA mutations lead to OXPHOS instability and mitochondrial dysfunction (Edgar et al., 2009). Similarly in intercrossed complementing flies mtDNA mutations lead to the loss of OXPHOS complexes causing several physiological alterations such as starvation sensitivity, decreased feeding and locomotor activity and increase seizure susceptibility, last of which has also been reported in many patients (Gorman et al., 2016). In addition, these flies mimic the well documented stem cell dysfunctions reported in the mtDNA mutator mice. It will be of high interest in the future to take advantage of the genetic tools of the fly community to better understand how mitochondrial dysfunction leads to loss of stem cell homeostasis.

TESTING THE "MAINTENANCE OF WILD TYPE" HYPOTHESIS IN mtDNA MUTATOR MOUSE

4.1 Introduction

According to the classical model, pathogenic mtDNA mutations cause a disease only when they reach a mutation and tissue specific threshold level. However, it has been also suggested that it is not the mutation levels *per se* that determines the disease penetrance but rather the absolute number of WT molecules. This theory has been called "maintenance of wild type hypothesis" (Stewart and Chinnery, 2015). To date, there has been limited evidence to support the latter model (Durham et al., 2006, Chinnery and Samuels, 1999) mostly due to the experimental difficulties in addressing it.

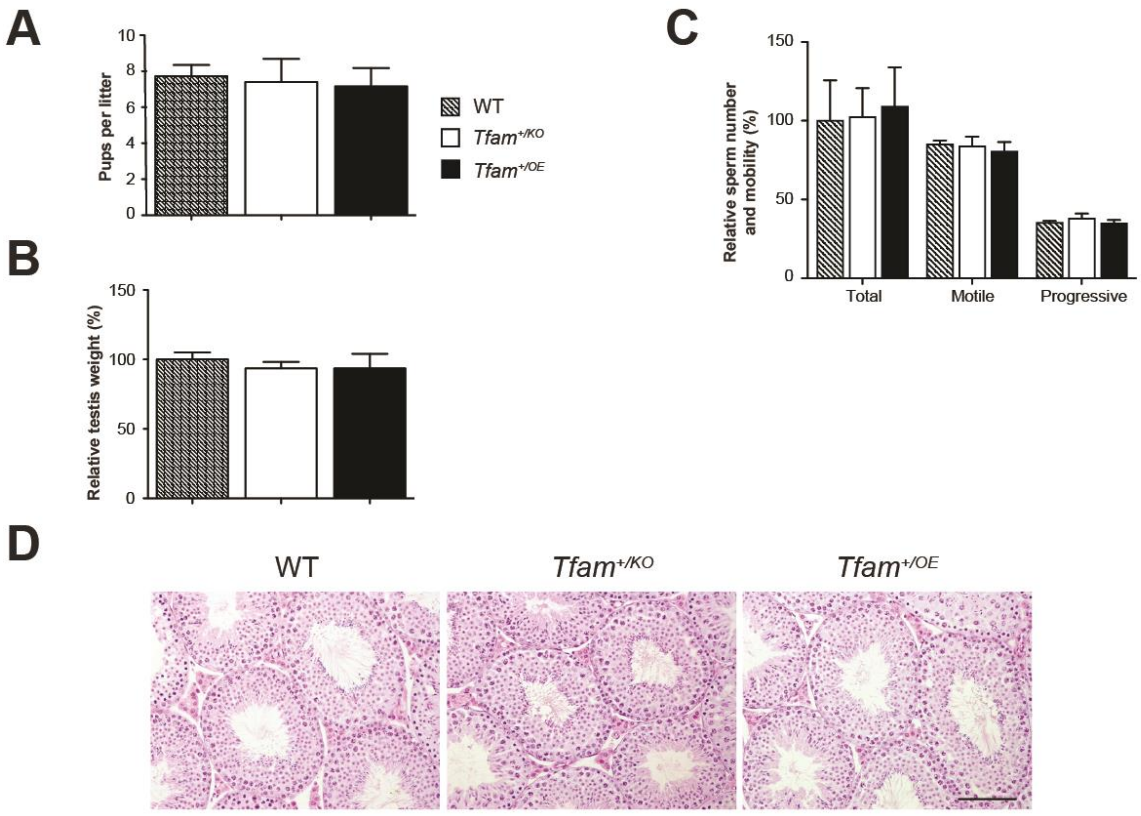
Mitochondrial dysfunction is a common cause for inherited diseases (Gorman et al., 2015). These diseases are well known to affect the most energy demanding tissues such as the neuromuscular system but less in known regarding the effects of mitochondrial dysfunction on fertility, a common human health problem (Boivin et al., 2007). Studies done in females suggests that mtDNA mutations do not limit the fertility of female carriers (Gorman et al., 2015, Moilanen and Majamaa, 2001), whereas in males the same mutation can decrease sperm motility (Spiropoulos et al., 2002) suggesting that sperm are more sensitive to mitochondrial dysfunction. Indeed, several lines of evidence from clinical studies and animal models suggest that impaired mitochondrial function can cause male infertility (Ji et al., 2017, Gao et al., 2016, Baklouti-Gargouri et al., 2014, Nakada et al., 2006, Spiropoulos et al., 2002, Ruiz-Pesini et al., 2000, Kao et al., 1998). A recent larger study discovered that males with mitochondrial disease have decreased fertility which correlates with the severity of the disease (Martikainen et al., 2017).

The mtDNA mutator mouse carries a proofreading-deficient $PolgA^{mut/mut}$ leading to accumulation of mtDNA mutations (Kujoth et al., 2005, Trifunovic et al., 2004). One of the mtDNA mutator models is also completely male sterile but shows partial rescue as hemizygous when the amount of maternally limited mutations is limited (Ross et al., 2013) suggesting that inherited and clonally expanded mtDNA mutations are an important factor contributing to male infertility. The mtDNA mutator mouse offers an interesting model to study the "maintenance of wild type hypothesis" as, at least in theory, increasing the mtDNA copy number in this mouse models should have beneficial effects. It is not fully understood how mtDNA copy number is regulated, but it has been shown to correlate well with TFAM levels (Ekstrand et al., 2004, Larsson et al., 1998). In this study, we took advantage of TFAM overexpressing mice and TFAM hemizygous mice in combination with the mtDNA mutator mice to study how the manipulation of TFAM levels, and thereby mtDNA copy number, affects the male infertility phenotype. Interestingly, increasing mtDNA copy number partially rescued male infertility of the mtDNA mutator mice without affecting total mtDNA mutation load suggesting that the number of WT mtDNA molecules determines the disease penetrance. Additionally, the rescue of this stem-cell-driven phenotype suggests that manipulating mtDNA copy number could be a powerful therapeutic strategy to alleviate the effects of heteroplasmic and pathogenic mtDNA mutations.

4.2 Results

4.2.1 Sperm defects correlate negatively with TFAM expression levels in mtDNA mutator mouse

To study how decreased and increased TFAM levels affect male fertility in WT mice, litter sizes and various sperm parameters of TFAM hemizygous (*TFAM*$^{+/KO}$) and TFAM overexpressing mice (*TFAM*$^{+/OE}$) were studied. When mated with WT females, both *TFAM*$^{+/KO}$ and *TFAM*$^{+/OE}$ mice produced similar litter sizes to WT control (Fig. 4.1A). Also the testes weight, sperm count, sperm motility and testes histology were unchanged (Fig. 4.1B, C and D). These results suggest that slight decrease or increase in mtDNA copy number has no effect on male fertility and that these mouse models can be used to study how differences in mtDNA copy number affect the fertility of mtDNA mutator mice.



Adapted from Jiang et al. 2017

Figure 4.1: Assessing TFAM^{OE} and TFAM^{KO} fertility
A) The shown phenotypes were crossed with WT females and the number of pups was scored. **B**) Testes relative weight. **C**) Relative sperm number and motility of the shown genotypes. **D**) Hematoxylin and eosin (HE) staining of testes sections. Whiskers represent SEM. Scale bar = 100 µm.

Designing experiments using the mtDNA mutator mouse is challenging because maternally transmitted mtDNA mutations can have confounding effects (Kauppila et al., 2017, Ross et al., 2013). Therefore, a specific mating strategy was designed to minimize the effect of maternal contribution of mtDNA mutation load (Fig. 4.2A). WT, *TFAM*$^{+/KO}$ and *TFAM*$^{+/OE}$ females were crossed with heterozygous mtDNA mutator males *PolgA*$^{+/mut}$ to obtain mtDNA mutator mice with decreased, normal or increased mtDNA copy number. One additional cross was carried out to obtain homozygous

mtDNA mutator mice with decreased (*PolgA*$^{mut/mut}$, *Tfam*$^{+/KO}$ (henceforth *PolgA*^{mut}*Tfam*^{KO})), normal (*PolgA*$^{mut/mut}$, *Tfam*$^{+/+}$ (henceforth *PolgA*^{mut}*Tfam*^{WT})) and increased (*PolgA*$^{mut/mut}$, *Tfam*$^{+/OE}$ (henceforth *PolgA*^{mut}*Tfam*^{OE})) mtDNA copy number.

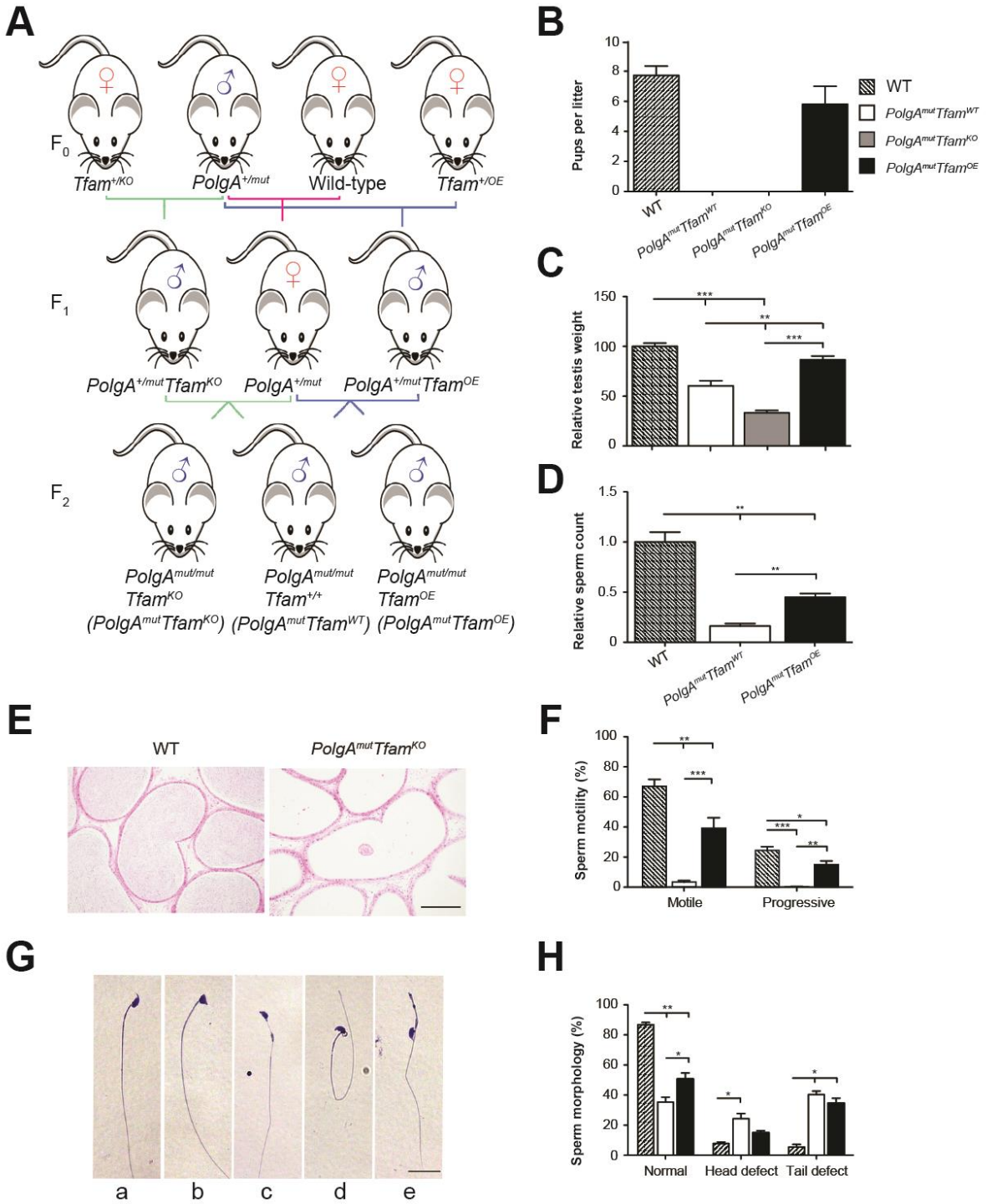


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Figure 4.2: TFAM overexpression partially rescues mtDNA mutator mouse infertility
A) Mating strategy to obtain homozygous mtDNA mutator mice with minimal amount of inherited mtDNA mutations and variable TFAM levels. **B**) Male mice of the shown genotypes were crossed with WT females and the number of pups per litter was scored. **C**) Relative testes weight. **D**) Relative sperm count **E**) Hematoxylin and eosin (HE) staining of cauda epididymis. Scale bar = 100 µm. **F**) Sperm motility. **G**) Examples of variable sperm morphology including a) normal sperm, b/c) sperm head defects, c/d/e) sperm tail defects. Scale bar = 20 µm. **H**) Quantification of sperm morphology. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

PolgA^{mut}*Tfam*^{KO}, *PolgA*^{mut}*Tfam*^{WT} and *PolgA*^{mut}*Tfam*^{OE} mice did not show any changes in mating behavior as was evident by the presence of copulatory plugs in wild type females after overnight mating. Despite normal mating behavior *PolgA*^{mut}*Tfam*^{WT} mice did not sire any offspring during a 3-month fertility test which is in accordance with the published results (Ross et al., 2013, Trifunovic et al., 2004). *PolgA*^{mut}*Tfam*^{KO} mice were similarly sterile but interestingly *PolgA*^{mut}*Tfam*^{OE} mice were able to produce offspring near WT levels (Fig. 4.2B). In agreement with the infertility phenotype, the testes of *PolgA*^{mut}*Tfam*^{WT} and *PolgA*^{mut}*Tfam*^{KO} were smaller in comparison with WT control and *PolgA*^{mut}*Tfam*^{WT} had a substantially decreased sperm count (Fig. 4.2C and D). In fact, the cauda epididymis of the PolgA^{mut}Tfam^{KO} mice did not contain almost any sperm (Fig. 4.2E) showing even stronger phenotype than the mtDNA mutator mouse alone. Sperm left in *PolgA*^{mut}*Tfam*^{WT} mice showed decreased motility (Fig. 4.2F) and significant increase in morphological defects, including typical hook and banana-like structure and irregular forms (Fig. 4.2G) in line with the infertility phenotype of this mouse model. *PolgA*^{mut}*Tfam*^{OE} mice, in contrast, showed WT-like testes weight (Fig. 4.2C) and partial rescue in sperm count (Fig. 4.2D). The sperm motility of *PolgA*^{mut}*Tfam*^{OE} mice was only slightly decreased (Fig. 4.2F) and higher proportion of sperm showed normal morphology (Fig. 4.2H). These results show that the infertility phenotype of the mtDNA mutator mice can be aggravated by decreasing mtDNA copy number and increasing mtDNA copy number can at least partially rescue many of the infertility phenotypes.

4.2.2 Spermatogenesis and OXPHOS dysfunction partially rescued by TFAM overexpression

Spermatogenesis can be divided into three stages: proliferation of spermatogonial stem cells, meiosis of spermatocytes and morphological maturation of spermatids (spermiogenesis). Spermatogenesis takes place within the seminiferous tubule of testes, where it is supported by Sertoli cells producing various growth factors and nutrients for the developing germ cells. Further histological analysis was carried out to better understand the effects of decreased and increased TFAM levels on mtDNA mutator mouse spermatogenesis. Before the onset of sexual maturity, testes of young WT, *PolgA*^{mut}*Tfam*^{KO}, *PolgA*^{mut}*Tfam*^{WT} and *PolgA*^{mut}*Tfam*^{OE} mice showed similar histology (Fig. 4.3A). After first round of spermatogenesis (6 weeks) mild and more severe loss of late stage spermatocytes and round spermatids was observed in *PolgA*^{mut}*Tfam*^{WT} and *PolgA*^{mut}*Tfam*^{KO} mice, respectively (Fig. 4.3A and 4.4C). This phenotype was worsened by time as at 4 months of age both *PolgA*^{mut}*Tfam*^{WT} and *PolgA*^{mut}*Tfam*^{KO} mice presented with Sertoli-cell-only syndrome (Fig. 4.3A). In contrast, *PolgA*^{mut}*Tfam*^{OE} testes were indistinguishable from WT control at 6 weeks of age and even at 4 months of age only mild defects were apparent reflecting a remarkable rescue of the phenotype (Fig. 4.3A and 4.4C).

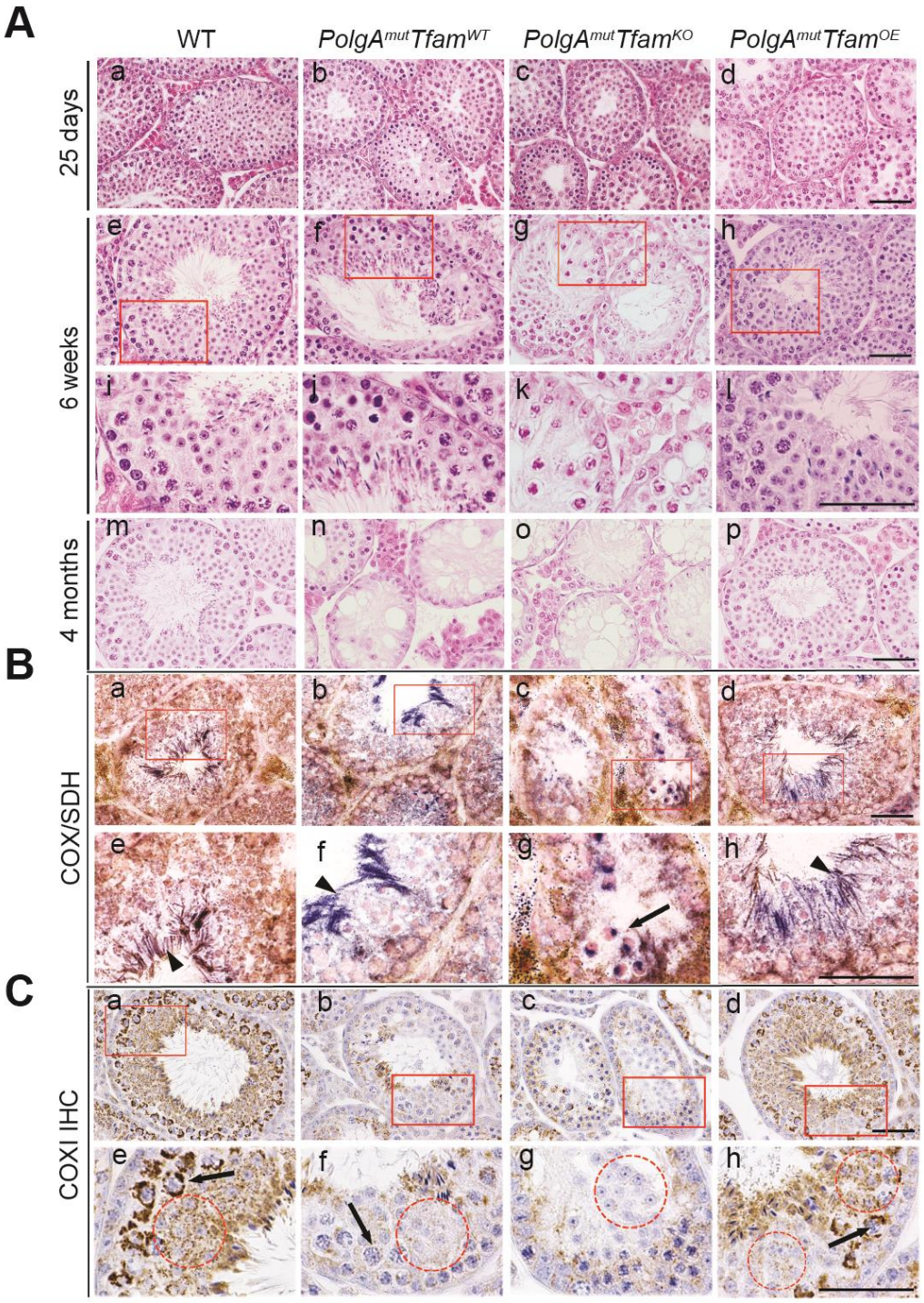

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Figure 4.3: TFAM overexpression partially rescues structural and OXPHOS defects in seminiferous epithelium
A) HE staining of testis sections of 25 d (a-d), 6 weeks (e-l) and 4 month (m-p) old mice. i-l are magnifications of red frames in e-h. **B**) COX/SDH staining of testis sections of 8-10 weeks old mice. e-h are magnifications of red frames in a-d. Arrowheads and arrows indicate sperm tails and round spermatids, respectively. **C**) Immunohistochemistry of mtDNA encoded COXI from testis sections. e-h are magnifications of red frames in a-d. Red circles indicate round spermatids and arrows pachytene spermatocytes. Scale bar = 50 µm.

To assess mitochondrial function in the presence of mtDNA mutations and variable TFAM levels, an enzyme histochemical in situ approach was chosen. Standard COX/SDH staining was performed on frozen tissue sections from 8-10 weeks old mice. The WT tissue sections showed infrequent COX negative cells (blue) whereas *PolgA*^{mut}*Tfam*^{WT} presented with COX negative round and later stage spermatids (Fig. 4.3B). This phenotype was exacerbated in the *PolgA*^{mut}*Tfam*^{KO} mice which showed

both COX negative spermatocytes and spermatids (Fig. 4.3B). TFAM overexpression in the *PolgA*^{mut}*Tfam*^{OE} mice was again able to improve the observed phenotypes as reflected by the higher COX activity relative to mtDNA mutator mouse although small COX negative sections were still visible (Fig. 4.3B).

Further immunohistochemistry and semi-quantification against mtDNA encoded COXI (COXI -IHC) was performed to verify that the reduction in COX activity was cause by reduction in mtDNA encoded proteins. COXI was highly expressed in WT control, especially in the pachytene spermatocytes (Fig. 4.3C and 4.4A). Both *PolgA*^{mut}*Tfam*^{WT} and *PolgA*^{mut}*Tfam*^{KO} mice showed significant decrease in COXI expression both in pachytene spermatocytes and round spermatids consistent with the COX/SDH staining (Fig. 4.3C, 4.4A and B). *PolgA*^{mut}*Tfam*^{OE} mice showed near WT level COXI expression although, again, some seminiferous epithelium sections showed decreased COX expression (Fig. 4.3C).

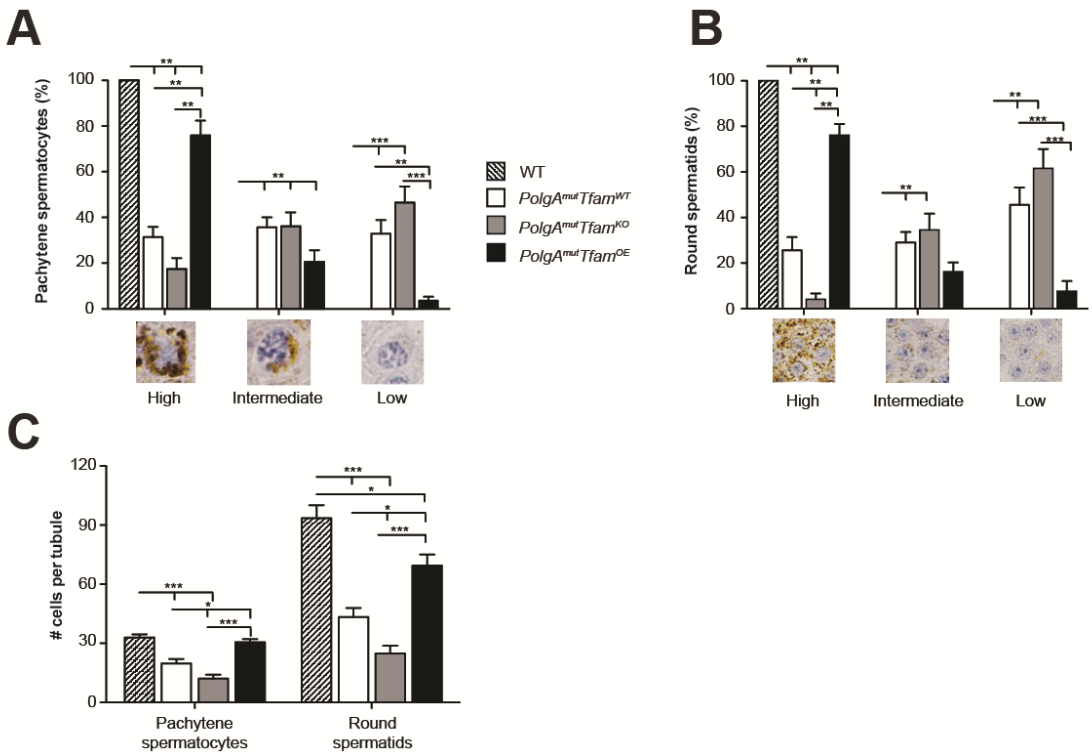

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Figure 4.4: Semi-quantification of COXI-IHC and cell count
A) Pachytene spermatocytes and **B**) round spermatids were categorized into high, intermediate and low COX expression groups based on IHC staining. **C**) Total number of each cell type in seminiferous tubules. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

4.2.2 TFAM overexpression partially rescues cristae remodeling during spermatogenesis

Mitochondria are known to take specific mitochondrial ultrastructures in many mitochondrial diseases (Jiang et al., 2017b, Vincent et al., 2016) but ultrastructure also changes depending on the energetic state of mitochondria (Benard and Rossignol, 2008, Hackenbrock, 1966). Depending on the energetic state mitochondria take usually three major forms: orthodox, intermediate and condensed. The orthodox form has larger matrix volume and correlates with low respiratory activity whereas the

condensed form has decreased matrix volume, increased cristae and is associated with high respiratory activity.

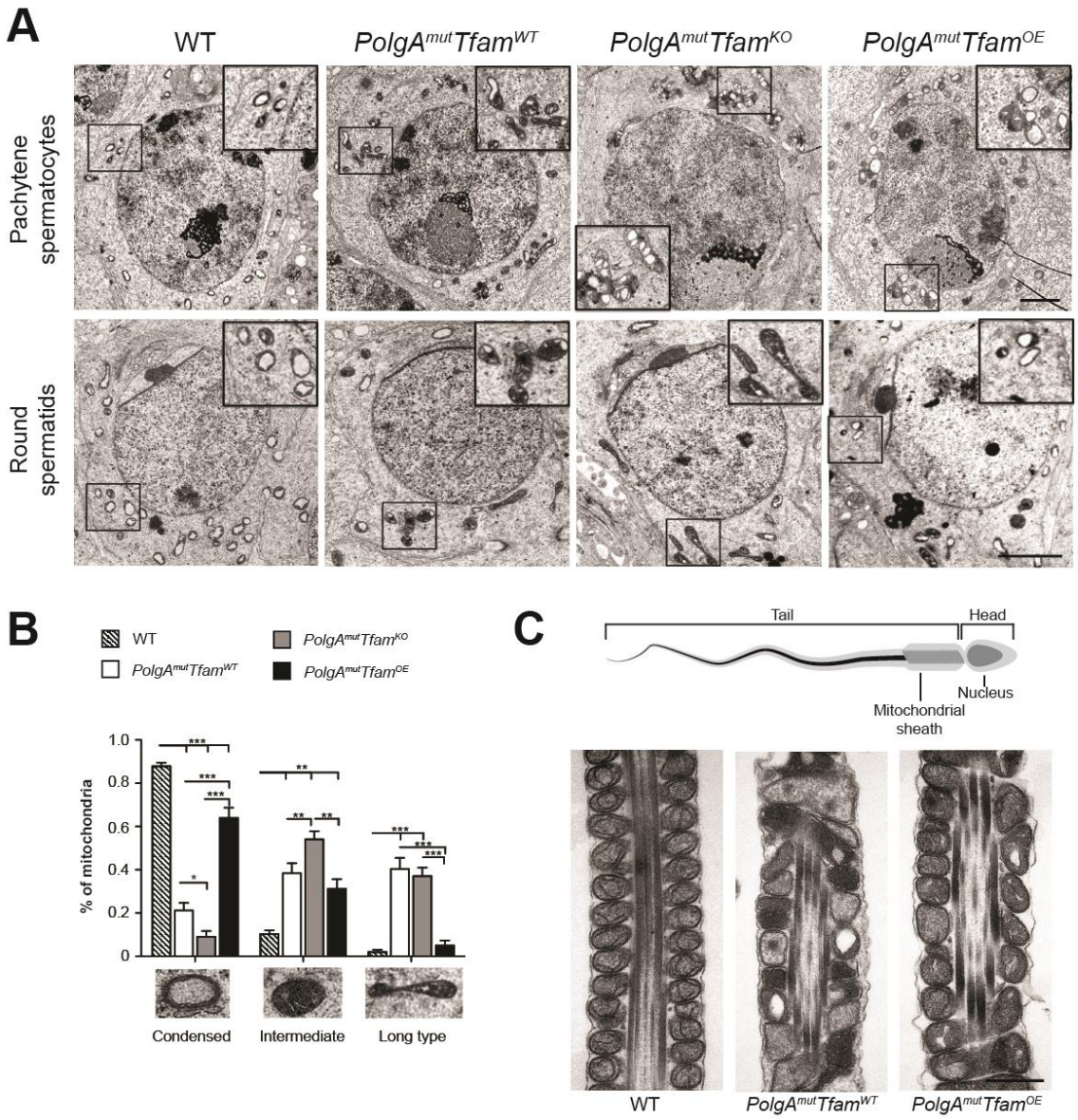

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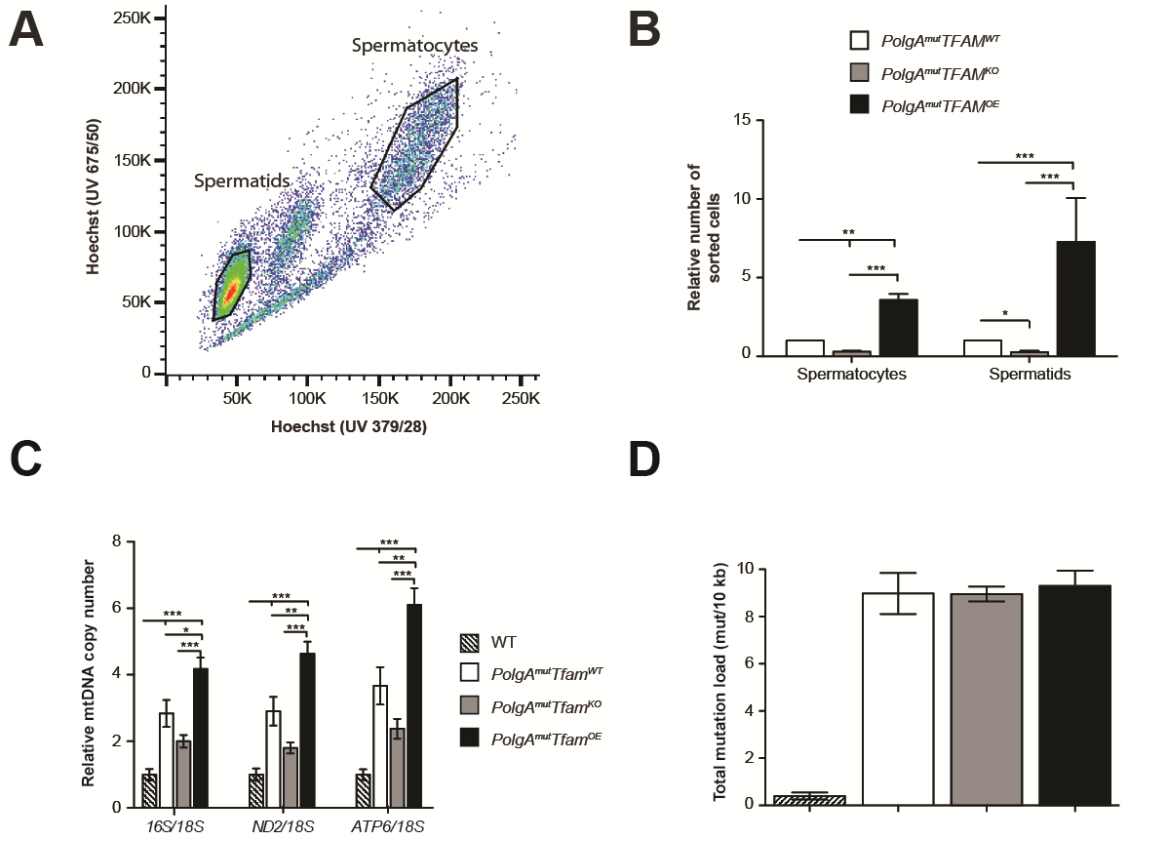
Figure 4.5: TFAM overexpression partially rescues changes in mitochondrial ultrastructure
A) Electron microscopy pictures of late pachytene spermatocytes and round spermatids. Black frames represent magnified regions. Scale bar = 2.5 µm. **B**) Quantification of mitochondrial ultrastructure in round spermatids, where default form in WT cells in condensed and intermediate and long types represent abnormal forms. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 **C**) Scheme of sperm structure showing the mitochondrial sheath which was used for electron microscopy. Scale bar = 500 nm.

In testes mitochondria harbor all of these forms depending on the cell type. Sertoli cells, spermatogonia, and preleptotene and leptotene spermatocytes take the low respiratory orthodox forms, zygotene spermatocytes, late spermatids and spermatozoa take the intermediate form and pachytene and secondary spermatocytes and early spermatids take the high respiratory condensed form (Ramalho-Santos et al., 2009). Mouse knockout for mitochondrial elongation factor 4 has shown that mitochondrial dysfunction can disturb proper transitions between different mitochondrial forms during spermatogenesis (Gao et al., 2016). To study how mtDNA mutations affect mitochondrial ultrastructure, electron microscopy pictures were taken from mtDNA mutator mice with different

levels of TFAM. The mitochondrial ultrastructure was unchanged in Sertoli cells, spermatogonia and early-stage spermatocytes in all mtDNA mutator mouse variants relative to WT control. However, the cristae of late spermatids of *PolgA*^{mut}*Tfam*^{WT} and *PolgA*^{mut}*Tfam*^{KO} mice were either long dumbbell shaped or of intermediate form instead of the normal condensed form as observed in WT control and *PolgA*^{mut}*Tfam*^{OE} mice (Fig. 4.5A and B). Additionally, mitochondrial sheath in the spermatozoa of *PolgA*^{mut}*Tfam*^{WT} presented with abnormal cristae with vacuoles and other abnormalities (Fig. 4.5C). The presence of these abnormalities was partially rescued by TFAM overexpression (Fig. 4.5C).



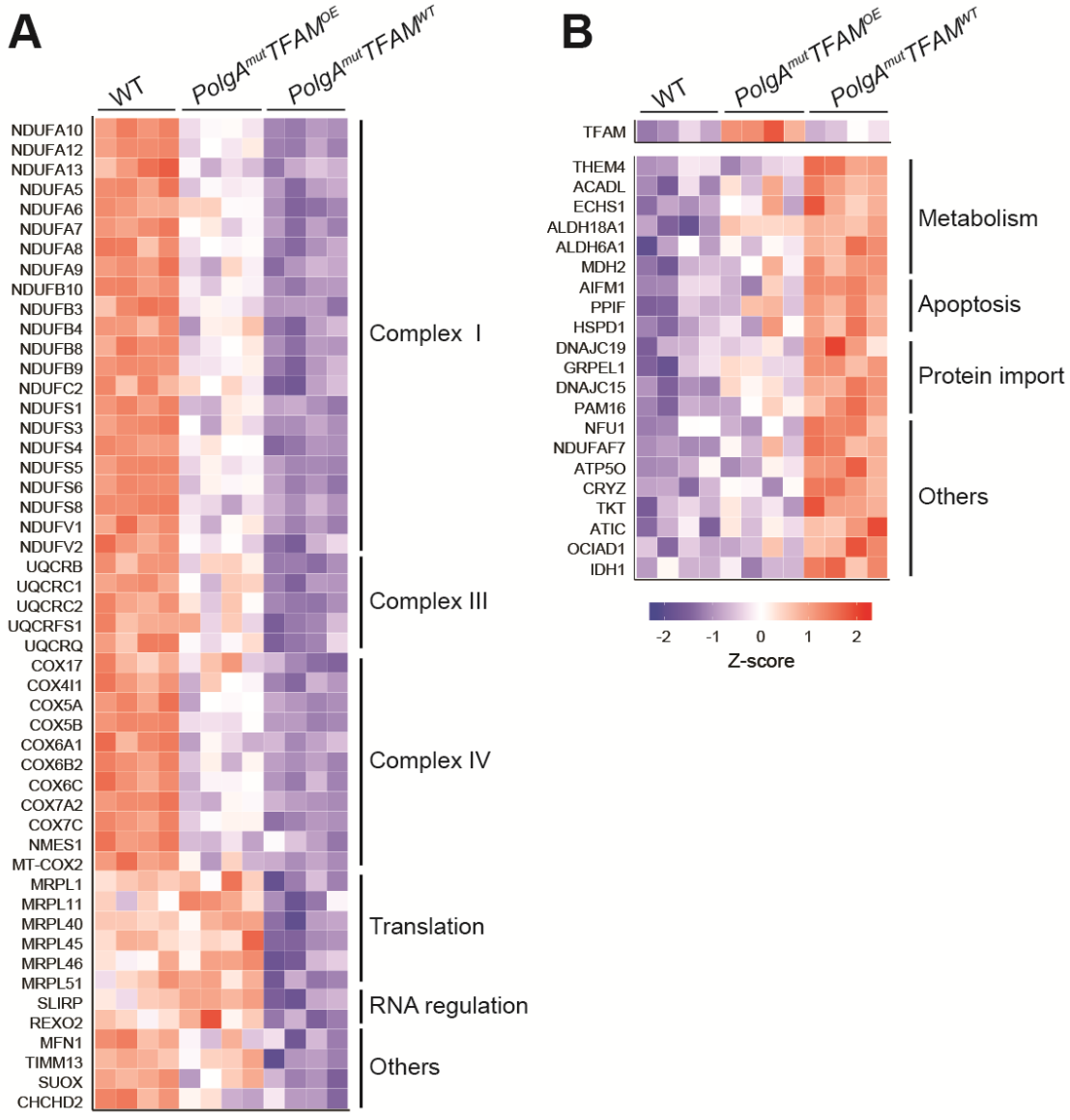
Adapted from Jiang et al. 2017

Figure 4.6: FACS sorted spermatocytes and spermatids
A) Spermatocytes (tetraploid) and spermatids (haploid) were FACS sorted. **B**) Quantification of the isolated spermatocytes and spermatids from each genotype. **C**) Relative mtDNA copy number quantified by qPCR from FACS sorted spermatocytes. Three different mtDNA genes (16S rRNA, ND2 and ATP6) were compared against nuclear 18S rRNA. **D**) Total mtDNA mutation load quantified by post-PCR cloning and sequencing from FACS sorted spermatocytes. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001

4.2.3 Modifying mtDNA levels does not affect mtDNA mutation load

Spermatocytes and spermatids of mtDNA mutator mice show marked changes in mitochondrial structure (Fig. 4.5A and B) and respiration (Fig. 4.4A and B). The better understand changes taking place at the molecular level, spermatocytes (tetraploid) and spermatids (haploid) were separated using fluorescence activated cell sorting (FACS)(Fig. 4.6A). As shown above the *PolgA*^{mut}*Tfam*^{KO} had barely any spermatocytes or spermatids whereas TFAM overexpression increased the number of both relative to the mtDNA mutator mouse (Fig. 4.6B). Quantification of relative mtDNA copy number in spermatocytes showed that the mtDNA mutations alone cause an increase in mtDNA levels (Fig. 4.6C), probably as a compensatory biogenesis response (Kauppila et al., 2017). As expected, mtDNA

mutator mice hemizygous for TFAM had slightly decreased mtDNA copy number whereas TFAM overexpression led to increased mtDNA levels (Fig. 4.6C). MtDNA mutation load was substantially increased in all mtDNA mutator mouse lines relative to WT control but surprisingly decreased or increased levels of TFAM did not affect mtDNA mutation load (Fig. 4.6D).



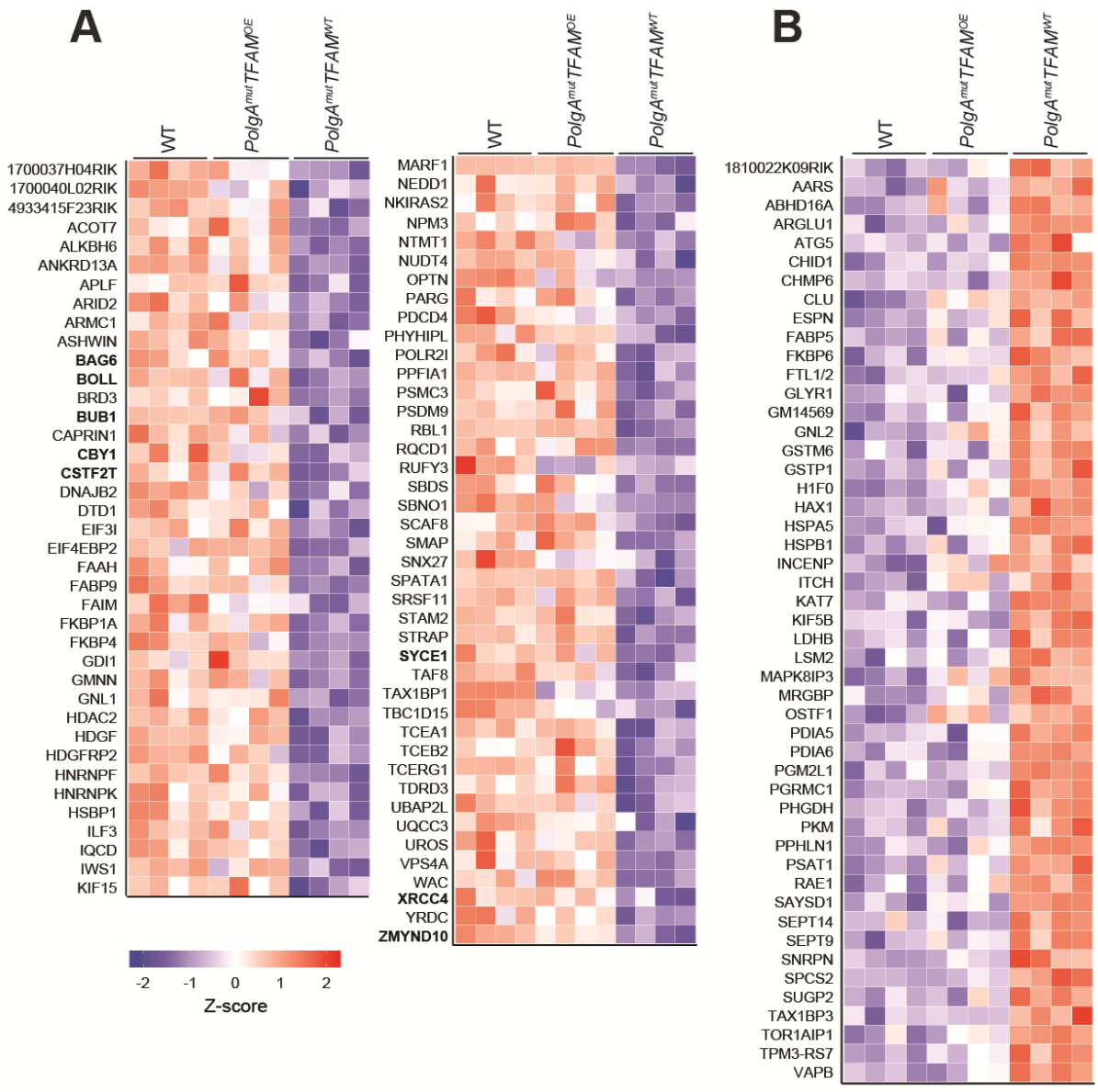
Adapted from Jiang et al. 2017

Figure 4.7: Mitochondrial proteins from mass spectrometry analysis of spermatocyte proteome
A) Mitochondrial proteins downregulated in mtDNA mutator mice spermatocytes. **B**) TFAM levels and mitochondrial proteins upregulated in mtDNA mutator mice spermatocytes. FRD = 0.05.

4.2.4 TFAM overexpression reverses changes in cellular proteome

FACS sorted spermatocytes from WT, *PolgA*^{mut}*Tfam*^{OE} and *PolgA*^{mut}*Tfam*^{WT} mice were further used for label-free quantitative proteomics. *PolgA*^{mut}*Tfam*^{KO} mice were not included in this experiment as it was not possible to obtain sufficient amount of material for analysis due to the severe phenotype (Fig. 4.2E). All significantly changed proteins were categorized into mitochondrial (Fig. 4.7) and non-mitochondrial (Fig. 4.8) based on their presence or absence in MitoCarta 2.0 database, respectively

(Calvo et al., 2016). As expected, TFAM levels were increased in *PolgA*^{mut}*Tfam*^{OE} mice (Fig. 4.7B). MtDNA mutator mouse showed a decrease in OXPHOS subunits, mainly complex I, and mitochondrial ribosomal proteins (Fig. 4.7A). In contrast, some proteins involved in apoptosis, metabolism and mitochondrial protein import were increased (Fig. 4.7B). Interestingly, overexpressing TFAM in the mtDNA mutator mouse background was sufficient to reverse many of these changes (Fig. 4.7A and B).



Adapted from Jiang et al. 2017

Figure 4.8: Non-mitochondrial proteins from mass spectrometry analysis of spermatocyte proteome
A) Non-mitochondrial proteins downregulated in mtDNA mutator mice. Bolded genes have been linked to male infertility in mouse and fly models. **B**) Non-mitochondrial proteins upregulated in mtDNA mutator mice. FRD = 0.05.

Several non-mitochondrial proteins were up- or down-regulated in mtDNA mutator mouse relative to WT control (Fig. 4.8). Remarkably, TFAM overexpression was able to reverse these changes in global cellular proteome (Fig. 4.8) suggesting that secondary changes caused by mitochondrial dysfunction can be reversed be partially rescuing mitochondrial function. Although some of these secondary changes might be adaptive, some of them might even aggravate the cellular problems. For instance,

several proteins connected to male infertility were downregulated in mtDNA mutator mouse, including Syce1 (Bolcun-Filas et al., 2009), Bag6 (Sasaki et al., 2008), Cstf2t (Dass et al., 2007), Boll (Xu et al., 2003), Bub1 (Perera et al., 2007), Cby1 (Enjolras et al., 2012), Zmynd10 (Moore et al., 2013), and Xrcc4 (Li et al., 2016) (Fig. 4.8A) and are likely to contribute to the infertility phenotype.

4.3 Discussion

The mtDNA mutator mouse accumulates high number of mtDNA mutations due to the abolished proofreading ability of POLγA (Ross et al., 2013, Trifunovic et al., 2004) leading to OXPHOS instability and mitochondrial dysfunction (Edgar et al., 2009). Several studies have shown that various stem cell pools are especially vulnerable to mtDNA dysfunction (Ahlqvist et al., 2015a). This seems to be the case also for the stem cells in testis as mtDNA mutations lead to male infertility in the mtDNA mutator mouse. However, the question remains whether it is the high mtDNA mutation levels or low absolute levels of WT mtDNA that leads to impaired mitochondrial function. We decided to address this question by modifying TFAM levels, a known regulator of mtDNA copy number, in the mtDNA mutator mouse.

Decreased mtDNA levels in the TFAM hemizygous mtDNA mutator mice aggravated all of the tested phenotypes, including testis weight, histology and mitochondrial ultrastructure, to the extent that no spermatozoa could be detected. In contrast, overexpression of TFAM partially rescued the infertility phenotype of the mtDNA mutator mouse as illustrated by the increased litter sizes, sperm function and morphology, histology, mitochondrial morphology and proteome. Most importantly, this rescue took place without changes in the mtDNA mutation load showing that the absolute amount of functional mtDNA copies and not the levels of mutated mtDNA determines the disease penetrance. This increase in functional mtDNA copies can ensure the production of sufficient amount of functional gene products and thereby counter the negative effects of increased mtDNA mutagenesis.

We have previously shown that heart specific TFAM conditional knockout mice have progressive OXPHOS deficiency and additionally show a metabolic switch at an early stage of the disease before the onset of mitochondrial changes (Hansson et al., 2004). This shift from oxidative to glycolytic metabolism is likely to aggravate the disease. Quantification of the spermatocyte proteome of mtDNA mutator mouse showed how the levels of several mitochondrial and non-mitochondrial proteins change upon mitochondrial dysfunction. Of special interest were several factors previously connected to male infertility that were downregulated in mtDNA mutator mice (*Syce1*, *Bag6*, *Cstf2t*, *Boll*, *Bub1*, *Cby1*, *Zmynd10*, and *Xrcc4*), which likely contribute to the male infertility of this mouse model. Interestingly, overexpressing TFAM not only restored changes in mitochondrial proteome but also changes in these secondary factors showing how rescuing the original mitochondrial defect originating from mtDNA mutations can have global effects on cellular function.

There is a high interest in developing treatments against mitochondrial diseases to which there is currently none available (Pfeffer et al., 2012). Gene therapy base methods to specifically destroy mutated mtDNA molecules using transcription activator-like effector nucleases (TALENs) and zinc fingers are being developed (Viscomi, 2016). Although these might be functional in removing the mtDNA molecules with specific mutations, it has not been addressed yet whether molecules used to

repopulate the mitochondrial pool might carry other pathogenic mutations which is likely because all humans carry low level heteroplasmy (Payne et al., 2013). Several studies have been also done using mice overexpressing PGC-1α, which leads to muscle fiber-type switch towards more oxidative fibers with more mitochondria (Kauppila et al., 2017). Although this approach is limited to muscle, it has been shown improve the exercise capacity of mice with COX deficiency (Viscomi et al., 2011).

Other approaches have relied on small molecule supplementation, such as nicotine amide riboside (NR) and n-acetyl-L-cysteine (NAC). NR is a precursor for nicotinamide adenine dinucleotide (NAD^+), which has been shown to induce mitochondrial biogenesis (Cerutti et al., 2014, Khan et al., 2014) whereas NAC increases glutathione levels which have been shown to be beneficial in multiple stem cell models of the mtDNA mutator mouse (Ahlqvist et al., 2015a, Ahlqvist et al., 2012). Although these concepts are promising, they are rather unspecific in targeting dysfunction originating from mtDNA mutations and might have some undesired side effects.

Based on this study we propose an alternative way to rescue OXPHOS dysfunction caused by mtDNA mutations by increasing total mtDNA copy number. Screens to identify regulators of mtDNA copy number (Fukuoh et al., 2014) could help to find targets for pharmacological agents that directly increase mtDNA copy number. Indeed it has been shown previously that it is feasible to specifically increase mtDNA copy number without affecting mitochondrial biogenesis (Ekstrand et al., 2004). Our study provides a proof-of-concept that manipulating mtDNA copy number might be an efficient strategy to treat patients with pathogenic mtDNA mutations.

DISCUSSION AND FUTURE PESPECTIVES

In this work, I characterized *in vitro* and *in vivo* several POLγA variants focusing on the proofreading-deficient POLγA and how the negative effects of mtDNA mutations can be rescued. Most importantly, *in vitro* work using recombinant human POLγA and *in vivo* work in fruit flies and mice showed that combining these three approaches can have synergistic effects when trying to understand basic biology and even when developing potential treatments.

First, *in vitro* characterization of POLγA variants showed that single amino acid changes are sufficient to increase and decrease the proofreading/polymerase activity ratio of POLγA. Next I proceeded to establish genomically engineered POLγA flies, which enable the introduction of any POLγA variant to the endogenous fly locus. By introducing *in vitro* characterized mutations into fruit flies I was able to show that increasing the proofreading/polymerase activity ratio of POLγA *in vivo* leads to mtDNA depletion whereas decreasing this ratio leads to increased mtDNA mutagenesis. Interestingly, the mtDNA mutations and deletions observed in mtDNA mutator fly and mtDNA mutator mouse share high similarity. Given our understanding regarding the formation of linear mtDNA deletions, these results suggest that fruit flies, like mice, use the classical strand-displacement mode of mtDNA replication. In addition, these *in vitro* and *in vivo* findings show that polymerase-deficient and proofreading-deficient POLγA variants can complement each other during DNA replication. This suggests that the polymerase activity and proofreading activity of POLγA do not have to be present in the same molecule for successful mtDNA replication to take place.

Using these fly models I was able to show that the accumulation of mtDNA mutation is slow both within individuals and across generations. Indeed, I show here that mtDNA is turned over in adult flies resulting in random drift of already existing mutations. Flies compound heterozygous for polymerase-deficient and proofreading-deficient alleles have increased mtDNA mutagenesis and clonal expansion of mtDNA mutations across generations. Using the compound heterozygote flies it is therefore possible to obtain fly lines with high levels of mtDNA mutations. These compound heterozygote flies showed several changes in fly physiology, including developmental delay, decreased lifespan and locomotor activity, sensitivity to mechanical stress and starvation sensitivity. Compound heterozygote flies presented also with failed proliferation of intestinal stem cells similar to what has been observed in mtDNA mutator mouse. Owing to the plethora of genetic tools and well defined cell markers in flies, this model can be used in to future to better assess how mtDNA mutations impair stem cell proliferation. Especially in natural fly populations, however, the high accuracy of WT POLγA and short lifespan of this model organism limits the extent of clonal expansion of mtDNA mutations. Therefore, it is unlikely that mtDNA mutations can reach high enough heteroplasmy levels to cause a focal OXPHOS dysfunction in flies with age. In other words, mtDNA mutations are not a major factor in determining fruit fly lifespan.

It is often a challenge in the clinics to identify potential variants as pathogenic due to the low number of patients carrying the same variant. One option to improve diagnosis would be to study potentially pathogenic mutation in model organisms. Using the genomically engineered POLγA founder line established in this work it is now possible to introduce POLγA variants of interest to the endogenous genetic locus and study their effects *in vivo*. Indeed, we have recently characterized one well studied and one *de novo* POLγA patient mutation using this fly model (Siibak et al., 2017). In addition to studying the effects of mtDNA mutations and characterizing potential patient mutation, the genomically engineered flies could be further applied to answer some basic biological questions. For instance, studies done in HIV patients suggest that inhibiting POLγA by anti-retroviral drugs can increase the clonal expansion of low level mtDNA mutations. Fly models established in this study could be used to analyze whether mtDNA damage normally stalling POLγA would have this same effect. From the developmental point-of-view, to date it has been infeasible to perform a quantitative or qualitative assessment of POLγA expression during development and in response to environmental changes due to the lack of antibodies and low expression level of POLγA. Adding an epitope tag to POLγA would overcome this problem. In addition, an epitope tagged POLγA could be used to perform co-immunoprecipitation assays to find new POLγA interaction partners.

It is a common knowledge that pathogenic mtDNA mutations have to reach a certain mutation and tissue specific threshold level before causing an OXPHOS dysfunction. However, it has been also hypothesized that the actual threshold level is in some sense irrelevant and it is the absolute number of WT mtDNA copies that determines disease penetrance. To study the latter hypothesis I took advantage of the mtDNA mutator mouse model which shows complete male sterility. Decreasing mtDNA copy number of this mouse model aggravated all the observed phenotypes, including testis weight, sperm count, sperm morphology and histology. In contrast, increasing mtDNA copy number of the mtDNA mutator mice was able to partially rescue all of these phenotypes. Importantly, this rescue was not only observable at the mitochondrial function and proteome, but changes in global proteome were similarly normalized. These results suggest that increasing mtDNA copy number could be beneficial in alleviating mitochondrial dysfunction originating from mtDNA mutations. The question still remains how increasing mtDNA copy number in the presence of increased mtDNA mutagenesis can have beneficial effects? Comparing total mtDNA mutation load between mtDNA mutator mice and mtDNA mutator mice with increased mtDNA copy number did not show any differences. These results suggest that an increase in the amount of functional mtDNA was resulting in this phenotypic rescue of the mtDNA mutator mice and support the model that the absolute number of WT mtDNA molecules determines disease penetrance of mtDNA mutation caused diseases.

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SUPPLEMENT

Table S1: Primers used to clone *DmPOLγA* donor constructs for ends out homologous recombination	
Primer	**5‘-3‘**
ET-cloning_tamas_right_For	atacgaaggccgagcatacaacac|CCGCGCGG|AATTGTAAACTAAAA TGCTTATTTTTTGTAAAATAAGTCAAACGATATTT
ET-cloning_tamas_right_Rev	gtttatccccaaggcgcgtgta|TCCGGA|ATCGAGACGAAGACCTACC GACACTAGTACCGGCCCTGAGCGTTAGGGAC
ET-cloning_tamas_left_For	atacgaaggccgagcatacaacac|CGTACG|CCGGGCACTATTGCACA AAGGATTGGGTTGGTAGGATAGGCAAACGTCTA
ET-cloning_tamas_left_Rev	gtttatccccaaggcgcgtgta|GGCGCC|AGTGATACAAAAATTTATTG GAACGTAAATTTCGTAGGACAACGGCTATG

Table S2: Primers used for genotyping genomically engineered *DmPOLγA* flies	
Primer	**5‘-3‘**
PCR1 For	TCATTTGGAATGTGGAGCAG
PCR1 Rev	AAGGAGGGCATGATCAAGAA
PCR2 For	CACCCGAAATTAGAGCTGGA
PCR2 Rev	GAACGCAGTGGTCCAGCTAT
PCR3 For	ACCTGCGGTAAGTGGTCATC
PCR3 Rev	CACTACGCCCCCAACTGAGAGAAC
PCR4 For	AGAAGTGACCGTGGAGCAAC
PCR4 Rev	CTCGACACCGGTATAACTTCGTATAATG
PCR5 For	TGTCAGTCAGTGGGATTGGA
PCR5 Rev	AAGGAGGGCATGATCAAGAA
PCR6 For	TCATTTGGAATGTGGAGCAG
PCR6 Rev	GGGAATAAGGGCGACACGGA
PCR7 For	TCATTTGGAATGTGGAGCAG
PCR7 Rev	GTGGAACTGCATCCTCGTTT
PCR8 For	TTTCTCGAGTTAAGTTTGCAAACCCTTAAC
PCR8 Rev	TTTGGCGCGCCGTGTTTGTTTTTTAATAATTAATCG

Table S3: Primers for fruit fly site-specific mutagenesis	
Primer	**5‘-3‘**
DmD263A For	CACAATGTCTCCTACGCCAGGGCGCGACTGAAG
DmD263A Rev	CTTCAGTCGCGCCCTGGCGTAGGAGACATTGTG
DmQ1009A For	CAATTGGGTGGTAGCGAGCGGTGCAGTG
DmQ1009A Rev	CACTGCACCGCTCGCTACCACCCAATTG
DmH1038A For	CTGCTTGAGCTTCGCTGATGAATTGCGC
DmH1038A Rev	GCGCAATTCATCAGCGAAGCTCAAGCAG

Table S4: Primers for fruit fly mtDNA Southern blot analysis	
Primer	**5‘-3‘**
ND2 For	CTTGGTTAGGAGCTTGAATAGGT
ND2 Rev	AATGGAGGTAATCCTCCTAATGA
12S rRNA For	TCATTCTAGATACACTTTCCAGTACATC
12S rRNA Rev	ACTAAATTGGTGCCAGCAGTCGCGGT
COXI For	AATGGAGCTGGAACAGGATG
COXI Rev	TCGAGGTATTCCAGCCAATC

Table S5: Primers for fruit fly relative mtDNA copy number determination	
Primer	**5‘-3‘**
CytB For	TTAATCATATTTGTCGAGACG
CytB Rev	AATGATGCACCGTTAGCAT
RpL32 For	GACGCTTAAGGGACAGTATCTG
RpL32 Rev	AAACGCGGTTCTGCATGAG

Table S6: Primers for fruit fly post-PCR cloning and sequencing	
Primer	**5‘-3‘**
2194 For	TTGATTTTTTGGTCACCCTGAAGT
3382 Rev	TAACTTCAATATCATTGATGGCCG
M13 For	TGTAAAACGACGGCCAGT
M13 Rev	CAGGAAACAGCTATGACC

Table S7: Primers for fruit fly mtDNA sequencing	
Primer	**5‘-3‘**
36 For	AAAAAGGATTACCTTGATAGG
498 Rev	GGATGTAAAAGATTCATTAATTTC
538 For	TTACATCCATAATTATTATATCAGC
993 Rev	GGTAAAAATCCTAAAAATGG
1124 For	TTTATTTACGAATTTGTTATTCC
1555 Rev	AATTAAAATTCTTAAAGATGTTCC
1775 Rev	AAAGAGCAGGAGGTAGTAGTC
1653 For	TAATTGTAACTGCACATGCT
1831 For	AAGTAGAATAGTTGAAAATGGAG
2191 Rev	ATATAAACTTCAGGGTGACC
2333 For	GATTATTAGGATTTATTGTATGAGC
2440 Rev	TTGAGTTCCATGTAAAGTAGC
2908 For	AGAAAGTTTAGTATCACAACGAC
3087 Rev	GTAAACCTAAATTAGCTCATGTAG
3437 Rev	ATTTGTTGGAATTATATATGAATC
3162 For	ATTTTTTTTCATGATCATGC
3726 For	GATTGTAATTGAAAGTGTTCC
3823 Rev	TTTTTTATCATTAGAAGTAAGTGC
4254 Rev	TTAAAAGTAGATCCATTATGACC
4183 For	ATTTATTGATTAATACCTTCTCG
4701 For	TCAATCTTATGTGTTTGCTG
5045 Rev	CGGGTGATAAACTTCTGTG

5195 For	AGCCCACCATAGACTTATAG
5448 Rev	GTCAATATCATGCAGCTG
5922 Rev	CCTTGATTTCATTCATGG
5701 For	TATCAAAAAAAGCTTTAATCG
6016 For	ATATAACATTTGATTTGCATTC
6207 Rev	CATTAAAAGAAAAAAGTTAGCAG
6376 For	TCCATAACATCTTCAATGTC
6864 Rev	TGATGAAAGTTGAATTATACTCC
6735 For	TCCAAATAAACCCCCTAC
7106 Rev	ATTCATATACCTTTAACTTCAGC
7317 For	CCTAATTGACTTAAAGTAGATAAAGC31
7722 Rev	CAAAATATTAAATCTTATAATGCTG
7870 Rev	TGAAAATCATATTAATCGATTC
7779 For	CTAAACAATAAGAAACAAAGTCC
8421 For	AAAATTATAGAAATTCAAGATCAAG
8449 Rev	TCCAACATTAAATTTATTAGGAG
8863 For	AATAACTCGTAATATTCCATAACC
8976 Rev	TTATATTTTTGTTTATTGTGTGC
9472 Rev	GTTTTATTAATAATATATATTGAATGGTAC
9196 For	AATAATGTAGGAATTAATCTTCTTTC23
9641 For	CCCCTTCACATACTCTAAATG
9806 Rev	TGATTATAATTTTATATTGAAGTTTACC
10195 For	GTAACATCTTTAGCCTCTAATG
10471 Rev	TTAAGATATTATTCGAATAGGTCC
10667 Rev	GAAAGCTAGATTAATATCAGCTG
10568 For	ATGCTTTAGTAGATTTACCAGC
11030 For	GATAATGCCACTTTAACTCG
11237 Rev	TCTCCCAATAAATTTGGTC
11650 For	ATTAAATTAAATAGTTAATGAGCTTG
11835 Rev	GAGTTCGAGGAACTTTACC
12378 Rev	TTTGTTTGAATATGTATGCC
12021 For	TCTTCTATATTCTACATTAAATCCTG
12612 For	TTAAAAAAGCTACACTTACTAATACAC
12936 Rev	TATCGATAAAAAAGATTGCG
12987 For	AAAAATTACGCTGTTATCCC
13576 Rev	ATTTAATAAATATATGCTTAGAATTAGC
13391 For	ACATGTTTTTGTTAAACAGG
13877 For	TTAATAAACACTGATACACAAGG
14076 Rev	CATTGAAAAGATTTTTGTGC
14447 For	AAACTGATTACAAATTTAAGTAAGG
14716 Rev	CGCGGTTATACCATTAATAC
14899 Rev	AAATTTAAAGTTTTATTTTGGC

Table S8: Primers for mouse post-PCR cloning and sequencing	
Primer	**5‘-3‘**
WANCY-N2-F4921 For	CCTACCCCTAGCCCCCC
WANCY-C1-R5953 Rev	TCATATCATTACGGACGCCG
M13 For	TGTAAAACGACGGCCAGT
M13 Rev	CAGGAAACAGCTATGACC

Table S9: Chi-squared p-values for log-rank in Fig. 3.15C					
	WT	**+/Rescue F_0**	**+/DmQ1009A F_0**	**+/DmH1038A F_0**	**+/DmD263A F_0**
median (d)	74	74	71	74	74
mean (d)	72	72	72	72	76
WT		0.0246	0.0348	0.118	0.514
+/Rescue F_0			0.877	0.617	0.116
+/DmQ1009A F_0				0.710	0.167
+/DmH1038A F_0					0.224

Table S10: Chi-squared p-values for log-rank in Fig. 3.15D			
	WT	**+/+ (mother: Rescue/+ F_1)**	**+/+ (mother: DmD263A/+ F_1)**
median (d)	73	75	78
mean (d)	71	72	72
WT		0.257	0.815
+/+ (mother: Rescue/+ F_1)			0.111

Table S11: Chi-squared p-values for log-rank in Fig. 3.15E			
	WT	**+/+ (mother: Rescue/+ F_1)**	**+/+ (mother: DmD263A/+ F_1)**
median (d)	74	74	74
mean (d)	72	72	72
WT		0.617	0.025
+/+ (mother: Rescue/+ F_1)			0.118

Table S12: Chi-squared p-values for log-rank in Fig. 3.17A					
	WT	**Rescue/+ F_1**	**DmQ1009A/+ F_1**	**DmH1038A/+ F_1**	**DmD263A/+ F_1**
median (d)	74	74	74	74	74
mean (d)	73	72	73	74	71
WT		0.550	0.370	0.809	0.010
Rescue/+ F_1			0.983	0.347	0.111
DmQ1009A/+ F_1				0.227	0.157
DmH1038A/+ F_1					0.009

Table S13: Chi-squared p-values for log-rank in Fig. 3.17B					
	WT	**Rescue/+ F_6**	**DmQ1009A/+ F_6**	**DmH1038A/+ F_6**	**DmD263A/+ F_6**
median (d)	78	78	74	72	76
mean (d)	77	76	74	70	76
WT		0.0427	**1.153E-06**	**8.546E-08**	0.070
Rescue/+ F_6			0.001	1.162E-4	0.780
DmQ1009A/+ F_6				0.339	4.771E-4
DmH1038A/+ F_6					**4.370E-05**

Table S14: Chi-squared p-values for log-rank in Fig. 3.17C					
	WT	**Rescue/+ F_{15}**	**DmQ1009A/+ F_{15}**	**DmH1038A/+ F_{15}**	**DmD263A/+ F_{15}**
median (d)	68	74	76	74	76
mean (d)	68	74	75	73	75
WT		**1.8733E-18**	**2.1002E-27**	**2.9613E-15**	**7.5621E-24**
Rescue/+ F_{15}			0.184	0.699	0.694
DmQ1009A/+ F_{15}				0.054	0.270
DmH1038A/+ F_{15}					0.400

Table S15: Chi-squared p-values for log-rank in Fig. 3.17D			
	WT	**+/+ (clean mtDNA)**	**+/+ (mut mtDNA)**
median (d)	69	74	67
mean (d)	70	72	69
WT		0.041	0.107
+/+ (clean mtDNA)			1.525E-4

Table S16: Chi-squared p-values for log-rank in Fig. 3.17F						
	WT	**Rescue**	**Rescue/ DmD263A**	**DmD263A/ Rescue**	**DmH1038A/ DmD263A**	**DmD263A/ DmH1038A**
median (d)	69	71	73	76	73	77
mean (d)	66	70	69	73	74	73
WT		0.008	1.660E-4	**5.64E-06**	**5.18E-07**	**1.04E-07**
Rescue			0.247	0.064	0.011	0.007
Rescue/ DmD263A				0.859	0.137	0.214
DmD263A/ Rescue					0.261	0.324
DmH1038A/ DmD263A						0.840

Table S17: Chi-squared p-values for log-rank in Fig. 3.17G						
	WT	**Rescue**	**Rescue/ DmD263A**	**DmD263A/ Rescue**	**DmD263A/ DmH1038A F_4**	**DmH1038A/ DmD263A F_4**
median (d)	76	80	83	78	76	80
mean (d)	73	79	82	75	73	75
WT		**8.107E-06**	**3.628E-13**	0.031	0.816	1.056E-4
Rescue			4.938E-4	0.0512	**2.572E-05**	0.779
Rescue/ DmD263A				**6.941E-07**	**1.158E-12**	0.002
DmD263A/ Rescue					0.052	0.043
DmD263A/ DmH1038A F_4						2.661E-4

Table S18: Chi-squared p-values for log-rank in Fig. 3.17I		
	WT	**DmD263A/DmH1038A F_5**
median (d)	77	75
mean (d)	75	74
WT		**0.002**

Table S19: Chi-squared p-values for log-rank in Fig. 3.17J			
	WT	**DmD263A/DmH1038A >F_{35}**	**Rescue >F_{35}**
median (d)	52	36	58
mean (d)	53	38	58
WT		**1.531E-30**	0.001
DmD263A/ DmH1038A >F_{35}			**1.987E-44**

Table S20: Chi-squared p-values for log-rank in Fig. 3.21A						
	WT	**+/Rescue F_0**	**+/DmD263A F_0**	**+/DmH1038A F_0**	**DmH1038A/ DmD263A F_1**	**DmH1038A/ DmD263A F_5**
WT		0.246	0.285	0.540	0.017	0.010
+/Rescue F_0			0.022	0.069	1.922E-4	**8.147E-05**
+/DmD263A F_0				0.534	0.132	0.086
+/DmH1038A F_0					0.017	0.007
DmH1038A/ DmD263A F_1						0.983

Table S21: Chi-squared p-values for log-rank in Fig. 3.21B							
	WT	**Rescue/+ F_1**	**DmD263A/+ F_1**	**DmH1038A/+ F_1**	**DmD263A/ DmH1038A F_1**	**DmD263A/ DmH1038A F_5**	**DmD263A /DmH1038A $>F_{35}$**
WT		0.248	0.639	0.383	0.060	0.0233	**7.98E-11**
Rescue/+ F_1			0.091	0.736	0.001	6.495E-4	**3.11E-14**
DmD263A/+ F_1				0.077	0.239	0.037	**4.33E-12**
DmH1038A/+ F_1					0.004	4.836E-4	**6.15E-15**
DmD263A/ DmH1038A F_1						0.412	**9.23E-09**
DmD263A/ DmH1038A F_5							**2.56E-06**

Table S22: p-values for parametric survival analysis in Fig. 3.22B							
	WT	**+/Rescue F_0**	**Rescue/+ F_1**	**+/DmH1038A F_0**	**DmH1038A/+ F_1**	**+/DmD263A F_0**	
WT		1.098E-02	9.418E-04	1.317E-01	8.446E-02	1.161E-06	
+/Rescue F_0			8.103E-01	3.595E-03	3.980E-03	1.263E-08	
Rescue/+ F_1				4.405E-05	3.445E-03	1.267E-08	
+/DmH1038A F_0					8.170E-04	1.701E-10	
+/DmH1038A F_1						4.422E-02	
	DmD263A/+ F_1	**DmH1038A/ DmD263A F_0**	**DmD263A/ DmH1038A F_1**	**DmH1038A/ DmD263A F_5**	**DmD263A/ DmH1038A F_5**	**DmD263A/ DmH1038A $>F_{35}$**	
WT	8.413E-01	3.435E-03	1.999E-02	3.261E-07	3.349E-07	**2.203E-92**	
+/Rescue F_0	4.116E-02	2.599E-01	6.777E-04	3.619E-02	1.168E-05	**1.918E-95**	
Rescue/+ F_1	1.072E-02	2.180E-02	5.566E-06	1.406E-03	8.002E-09	**8.884E-118**	
+/DmH1038A F_0	6.456E-02	3.398E-02	7.518E-01	1.744E-05	6.890E-03	**4.754E-77**	
+/DmH1038A F_1	3.981E-01	2.127E-05	9.672E-05	5.539E-10	2.484E-10	**1.964E-92**	
	DmD263A/+ F_1	**DmH1038A/ DmD263A F_0**	**DmD263A/ DmH1038A F_1**	**DmH1038A/ DmD263A F_5**	**DmD263A/ DmH1038A F_5**	**DmD263A/ DmH1038A $>F_{35}$**	
+/DmD263A F_0	9.545E-05	3.033E-13	4.018E-11	6.338E-21	7.598E-21	**5.715E-112**	
DmD263A/+ F_1		4.130E-03	8.305E-03	8.465E-07	3.218E-07	**1.156E-89**	
DmH1038A/ DmD263A F_0			1.409E-02	3.767E-01	4.987E-03	**1.174E-91**	
DmD263A/ DmH1038A F_1				1.776E-05	6.377E-02	**1.858E-54**	
DmH1038A/ DmD263A F_5					8.308E-05	**8.455E-119**	
DmD263A/ DmH1038A F_5						**1.632E-76**	
DmD263A/ DmH1038A $>F_{35}$							

ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorlegen hat, dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Nils-Göran Larsson betreut worden.

Köln, 20.11.2017

Timo Kauppila

Teilpublikationen

Increased total mtDNA copy number cures male infertility despite unaltered mtDNA mutation load. Jiang M, Kauppila TES, Motori E, Li X, Atanassov I, Folz-Donahue K, Bonekamp NA, Albarran-Gutierrez S, Stewart JB, Larsson NG. Cell Metabolism 2017 Aug 1;26(2):429-436.

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Weitere Publikationen

A multi-systemic mitochondrial disorder due to a dominant p.Y955H disease variant in DNA polymerase gamma. Siibak T, Clemente P, Bratic A, Bruhn H, Kauppila TES, Macao B, Schober FA, Lesko N, Wibom R, Naess K, Nennesmo I, Wedell A, Peter B, Freyer C, Falkenberg M, Wredenberg A. Human Molecular Genetics 2017 Jul 1;26(13):2515-2525.

Mammalian Mitochondria and Aging: An Update. Kauppila TES, Kauppila JHK, Larsson NG. Cell Metabolism 2017 Jan 10;25(1):57-71.

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Training

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Scientific presentation course	**2014, Cologne, DE**
Seahorse FX advanced training	**2011, Michigan, USA**
Mitochondria summer school	**2011, Tampere, FI**
FELASA category C course	**2011, Jyväskylä, FI**

Prices and awards

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Finnish Cultural Foundation's Grant	**2014-2015**
Biochemistry Research Trainee Program Position, MSU, USA	**2011**
Summer scholarship for B.Sc. student	**2010**

Peer review

PLOS Genetics	**2016**

Publications

Increased total mtDNA copy number cures male infertility despite unaltered mtDNA mutation load.
Jiang M, Kauppila TES, Motori E, Li X, Atanassov I, Folz-Donahue K, Bonekamp NA, Albarran-Gutierrez S, Stewart JB, Larsson NG.
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Identification of proprotein convertase substrates using genome-wide expression correlation analysis.
Hannu Turpeinen, Sampo Kukkurainen, Kati Pulkkinen, Timo Kauppila, Kalle Ojala, Vesa P. Hytönen, Marko Pesu
BMC Genomics. 2011 Dec 20;12(1):618.

Participation in conferences

Poster presentation	**EUROMIT** **2017**
Poster presentation	**Gordon Research Conference: Mitochondria & Chloroplasts** **2016**
Talk	**Mitochondrial Medicine: Developing New Treatments for Mitochondrial Disease** **2016**
Talk	**24th European Drosophila Research Conference** **2015**
Poster presentation	**ZING conferences. DNA Polymerases: Biology, Diseases and Biomedical Applications Conference** **2014**
Poster presentation	**Wenner-Gren Foundations Symposium: DNA Metabolism** **2014**
Poster presentation	**Recent Insights in Mitochondrial Evolution Applied to Health and Ageing** **2013**