The Role of Mitochondrial DNA Replication Fidelity in Toxicity Initiated by Drugs that Impair Mitochondrial Function

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Mitochondrial DNA (mtDNA) is crucial for proper mitochondrial function in part because it encodes for 13 subunits of the electron transport chain (ETC). mtDNA is replicated exclusively by DNA polymerase gamma (Polg), which has an exonuclease proof-reading domain critical for maintaining mtDNA replication fidelity. Drug-induced mitochondrial toxicity is a common problem that can occur by many mechanisms, including ETC inhibition and mtDNA damage. Gene-environment interactions can modulate mitochondrial toxicity, and individuals with POLG mutations are at risk for toxicity from valproic acid, a drug that impairs mitochondrial function. We hypothesize that mtDNA replication fidelity protects against toxicity initiated by drugs that impair mitochondrial function. I observed that mouse embryonic fibroblasts (MEFs) with impaired polymerase gamma exonuclease activity (Polg^{m/m}) and increased mtDNA mutation load have increased susceptibility to toxicity caused by drugs that impair mitochondrial function. The ETC complex I inhibitor rotenone caused increased apoptosis in Polg^{m/m} MEFs relative to wild type. This sensitivity was also observed for other ETC complex inhibitors and clinical drugs with off-target ETC complex I inhibition. A mitochondria-penetrating peptide (MPP) was used to target the alkylating agent chlorambucil (Cbl) specifically to mitochondria. Mitochondria-
targeted Cbl (mt-Cbl) caused bioenergetic differences between Polg<sup>m/m</sup> and wild type MEFs, but was not differentially toxic at the level of cell viability. Increasing mitochondrial autophagy with rapamycin attenuated rotenone toxicity in Polg<sup>m/m</sup> MEFs, while changes to mitochondrial fusion/fission dynamics did not alter toxicity. Overall, these data suggest that mtDNA replication fidelity maintained by polymerase gamma exonuclease activity protects against bioenergetic stress caused by ETC inhibition.
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<tbody>
<tr>
<td>2-DG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>8-oxodG</td>
<td>8-oxodeoxyguanosine</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcls-antagonistic/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl2 homology domain 3</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>Cbl</td>
<td>Chlorambucil</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>CPEO or PEO</td>
<td>Chronic progressive external ophthalmoplegia</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell penetrating peptides</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCF</td>
<td>Dichlorofluorescein</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>Dichlorofluoroscein diacetate</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-induc ing signaling complex</td>
</tr>
<tr>
<td>DLCs</td>
<td>Delocalized lipophilic cations</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>Drp1</td>
<td>Dynamin-related protein 1</td>
</tr>
<tr>
<td>dRPase</td>
<td>5’-deoxyribosephosphate lyase</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide</td>
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FAS/CD95  Transmembrane death receptors
FBS  Fetal bovine serum
FCCP  Carbonyl cyanide p-trifluoromethoxy-phenylhydrazone
FDA  Food and Drug Administration
FIP200  Family interacting protein of 200 kD
Fis1  Mitochondrial fission protein 1
FITC  Fluorescein isothiocyanate
FKBP12  FK506-binding protein 12
Fx  Cyclohexylalanine
GFP  Green fluorescent protein
GLUT1  Glucose transporter
GTP  Guanosine triphosphate
HAP1  Htt-associated protein 1
HIF-1α  Hypoxia-inducible transcription factor
HIV/AIDS  Human immunodeficiency virus / acquired immunodeficiency syndrome
HR  Heptad repeat
HTT  Huntingtin
IC50  Median inhibitory concentration
JC-1  5,5’,6,6’-tetrachloro-1,1’,3,3’ tetraethylbenzimidazolylcarbocyanine iodide
KSS  Kearns-Sayre syndrome
LC3  Microtubule associated protein 1 light chain 3
LD50  Median lethal dose
LHON  Leber’s hereditary optic neuropathy
MDIVI-1  Mitochondrial division inhibitor
MEFs  Mouse embryonic fibroblasts
MELAS  Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MEM  Minimum essential medium
MERRF  Myoclonus epilepsy and ragged red fibers
Mff  Mitochondrial fission factor
Mfn  Mitofusin
Miro1/2  Mitochondrial RHO GTPase
MNGLE  Neurogastrointestinal encephalomyopathy
MOMP  Mitochondrial outer membrane permeabilization
MPP  Mitochondria-penetrating peptide
MPP+  1-methyl-4-phenylpyridinium
MPTP  1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mPTP  Mitochondrial permeability transition pore
MPV17  Mitochondrial inner membrane protein
mt-Cbl  Mitochondria-targeted chlorambucil
mt-Dox  Mitochondria-targeted doxorubicin
mtDNA Mitochondrial DNA
mTERF Mitochondrial transcription termination factor
mtIF-2 Mitochondrial translation-initiation factor 2
mTOR Mammalian (or mechanistic) target of rapamycin
mt-Pt Mitochondria-targeted Pt-AcAc
MTS Mitochondrial targeting sequence
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC N-acetyl-cysteine
NADH Nicotinamide adenine dinucleotide (reduced)
NARP Neuropathy, ataxia and retinitis pigmentosa
ND mtDNA-encoded NADH-dehydrogenase
NDUFS NADH dehydrogenase (ubiquinone) Fe-S protein
NER Nucleotide excision repair
NRTI Nucleoside analog reverse transcriptase inhibitors
OCR Oxygen consumption rate
OMA1 Overlapping activity with M-AAA protease 1
OPA1 Optic atrophy 1
OXPHOS Oxidative phosphorylation
p53 Tumor protein 53
p62 Nucleoporin p62
PAM Translocase-associated motor
PARL Presenilin associated, rhomboid-like
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PFA Paraformaldehyde
Pi Organic phosphate
PINK1 PTEN-induced putative kinase 1
Polg DNA Polymerase Gamma
POLRMT Mitochondrial RNA polymerase
Polβ DNA polymerase beta
PPi Pyrophosphate
Pt-AcAc Acyl-acyl-cis-Diammineplatinum (II)-β-Diketonate
PVDF Polyvinylidene fluoride
Raptor Regulatory associated protein of mTOR
RMC Random mutation capture
RNA Ribonucleic acid
RNAi RNA interference
ROS Reactive oxygen species
rRNA Ribosomal RNA
SDS Sodium dodecyl sulfate
SDS-PAGE SDS-polyacrylamide gel electrophoresis
Smac/Diablo Second Mitochondrial Activator of Caspases/Direct IAP Binding protein
with Low pI

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SSBP1</td>
<td>Mitochondrial single stranded DNA binding protein 1</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>Tfam</td>
<td>Mitochondrial transcription factor</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocase of the mitochondrial inner membrane</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the mitochondrial outer membrane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TTFA</td>
<td>Thenoyltrifluoroacetone</td>
</tr>
<tr>
<td>UKL-1</td>
<td>UNC-51-like kinase 1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Voltage dependent ion channel 1</td>
</tr>
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</table>
1 Introduction

1.1 Mitochondrial structure and functions

Mitochondria are organelles that act as the main source of adenosine triphosphate (ATP) energy in the cell, and are involved in many other important cellular processes. These include reactive oxygen species (ROS) generation, fatty acid metabolism, apoptosis, and amino acid catabolism (Chan, 2006a). Most mammalian cells contain between a few hundred to several thousand mitochondria (Sawyer and Van Houten, 1999). These organelles are composed of a mitochondrial matrix enclosed by the inner mitochondrial membrane, and the inner membrane space enclosed by the outer mitochondrial membrane (Scheffler, 2001). The outer membrane is usually smooth and forms the boundary with the cytosol, while the inner mitochondrial membrane has multiple invaginations into the matrix that are called cristae (Perkins and Frey, 2000). The inner mitochondrial membrane is the site of the ETC which consists of four major multi-subunit protein complexes that transfer electrons in order of increasing redox potential from the original donor molecules (NADH and FADH$_2$) to molecular oxygen (Dykens and Will, 2008; Lenaz and Genova, 2010). The process of electron transfer is coupled to proton-pumping machinery, which translocates protons across the inner mitochondrial membrane into the inner membrane space and creates an electron potential gradient across the membrane of approximately 150-180 mV (Trifunovic and Larsson, 2008). This membrane potential is ultimately used to catalyze ATP formation (Scheffler, 2001). The protein complexes of the ETC are NADH-coenzyme Q reductase (Complex I), succinate-coenzyme Q reductase (Complex II), ubiquinolcytochrome C reductase (Complex III), cytochrome C oxidase (Complex IV), and ATP synthase (Complex V) (Figure 1) (Lenaz and Genova, 2010).
Figure 1. Mitochondrial Electron Transport Chain. Oxidative phosphorylation is carried out by five protein complexes (I-V) in the mitochondrial inner membrane. Complex I (NADH dehydrogenase-CoQ reductase), complex II (succinate dehydrogenase), complex III (ubiquinone-Cyt c oxidoreductase), and complex IV (COX) utilize electropotential energy to generate a proton gradient across the inner mitochondrial membrane. This proton gradient is then used by complex V (ATP synthase; F0 and F1) to generate ATP from ADP and organic phosphate. With the exception of complex II, these protein complexes are composed of nuclear-encoded (blue subunits), and mtDNA-encoded (red for complex I, orange for complex III, purple for complex IV, and yellow for complex V) subunits (Figure adapted from Schon 2012).
Complex I is responsible for the first step in the ETC by transferring electrons from NADH to Coenzyme Q (CoQ), and contributes to the membrane potential by translocating four protons from the matrix to the inner membrane space (Brandt, 2006). It is composed of 45 subunits, seven of which are encoded by mitochondrial DNA (Kato, 2001). Complex I activity can be inhibited by over 60 classes of compounds (Lummen, 1998). A classic example of a complex I inhibitor is the pesticide rotenone (Lummen, 1998). Complex II is unique among ETC complexes in the fact that none of its protein subunits are encoded by the mitochondrial DNA, but instead all originate from the nuclear genome (Chan, 2006a). Complex II does not directly contribute to the proton gradient across the membrane (Lancaster, 2002). It contributes to the ETC by transferring electrons from succinate to CoQ, which can then be used by complex III. Complex II consists of 4 subunits, all encoded by the nuclear genome (Ghezzi and Zeviani, 2012). Examples of classic complex II inhibitors include thenoyltrifluoroacetone (TTFA) and sodium malonate (Drose et al., 2011). Complex III is able to accept electrons from reduced CoQ, also known as ubiquinone, and transfer them to cytochrome c, translocating 4 protons into the inner membrane space (Berry et al., 2000). Complex III is composed of 11 subunits, only one of which, cytochrome b, is encoded by the mtDNA (Ghezzi and Zeviani, 2012). One example of a classic inhibitor is antimycin A, which inhibits electron transport between cytochromes b and c inside complex III (King and Radicchi-Mastroianni, 2002; You and Park, 2010). Complex IV is the final component of the ETC, where electrons from reduced cytochrome c are transferred to molecular oxygen and reduce it to water, translocating a maximum of two protons in the process (Belevich et al., 2007). Complex IV is composed of 13 subunits, of which 3 are encoded by the mtDNA and involved in the active site (Ghezzi and Zeviani, 2012; Lenaz and Genova, 2010). Complex IV activity can be effectively inhibited by cyanide, azide, or carbon monoxide through their affinity to the oxygen binding site (Cooper and Brown, 2008).

For each electron pair transferred from NADH to oxygen, a maximum of 10 protons can be translocated across the inner mitochondrial membrane into the inner membrane space (Dykens and Will, 2008). Complex V, also known as ATP synthetase, utilizes the proton electrochemical potential across the inner mitochondrial membrane to generate ATP. Complex V is composed of an integral membrane rotor-like structure, the F₀ particle, and the ATP synthase catalytic structure facing the matrix, the F₁ particle (Boyer, 1997). Protons follow the electrochemical gradient and flow from the inner membrane space back towards the matrix through a channel...
creating rotary motion to F₀. This motion is transmitted to the catalytic head of F₁, and with every rotation cycle, 3 ATP molecules are created from condensation of ADP and organic phosphate (Pi) (Devenish et al., 2008; Ghezzi and Zeviani, 2012). Two subunits of complex V are encoded by the mtDNA (both found in the F₀ particle) while the remaining 13 subunits are encoded by the nuclear genome (Ghezzi and Zeviani, 2012).

1.1.1 Mitochondrial metabolism and biochemistry

Mitochondria play an integral role in several different cellular metabolic and biosynthesis pathways (Figure 2) (Galluzzi et al., 2012). The most important of these pathways are glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS). Glycolysis occurs in the cytosol, and converts glucose to two molecules of pyruvate while also generating two ATP and two NADH molecules (Fernie et al., 2004). Pyruvate can either undergo anaerobic metabolism and be converted into lactate, or enter the mitochondrial matrix and feed into the Krebs cycle. Also known as the TCA cycle, this pathway generates the high energy molecules NADH and FADH₂ that supply electrons to the ETC, and GTP, which can serve as an energy source for several enzymes. Pyruvate is imported into the mitochondrial matrix and converted to acetyl-Coenzyme A (acetyl-CoA), and one molecule of NADH is generated in this reaction. Acetyl-CoA is then fed into the Krebs cycle when the enzyme citrate synthase conjugates it with oxaloacetate to generate citrate. Citrate is further converted to cis-aconitate, D-isocitrate, and alpha-ketoglutarate, the latter reaction generating another NADH molecule (Fernie et al., 2004). In the next step of the pathway alpha-ketoglutarate is converted to succinyl-CoA, producing yet another NADH. Succinyl-CoA is converted to succinate in a reaction that produces guanosine triphosphate (GTP), a high energy triphosphate nucleotide that serves as substrate and energy source for many enzymatic reactions (Fernie et al., 2004). Succinate is converted to fumarate, and the electron donor FADH₂ is also generated in this step. Fumarate is converted to malate, which in turn is converted to oxaloacetate. Another NADH molecule is generated in this final step of the pathway, and the cycle can begin again with the newly generated oxaloacetate reacting with another acetyl-CoA (Fernie et al., 2004).
**Figure 2. Mitochondrial involvement in metabolic pathways.** Mitochondria are involved in several metabolic and bioenergetic pathways critical for proper cellular function and survival. These pathways may occur entirely or partially inside the mitochondria. Metabolic pathways with mitochondrial involvement include oxidative phosphorylation, glycolysis, the TCA cycle, urea cycle, beta-oxidation and ketogenesis. Red lines indicate boundary of mitochondrial membranes (Galluzzi 2012).
One important consequence of the Krebs cycle is the accumulation of NADH and FADH$_2$ in the mitochondrial matrix. These molecules act as electron donors to the ETC (Galluzzi et al., 2012). Another important metabolic pathway that occurs exclusively within the mitochondrial matrix is the process of $\beta$-oxidation. Along with glycolysis, $\beta$-oxidation is a crucial source of acetyl-CoA molecules that serve as substrates of the Krebs cycle. Fatty acids are imported into the mitochondrial matrix by fatty acyl-coenzyme A synthetase and the carnitine shuttle (Houten and Wanders, 2010). The $\beta$-oxidation cycle is composed of four enzymatic reaction steps. Each cycle completion degrades fatty acids by two carbons, consumes water and CoA-SH, and in turn generates FADH$_2$, NADH, and acetyl-CoA (Houten and Wanders, 2010). Mitochondria also play an important role in heme biosynthesis (Ajioka et al., 2006). Succinyl-CoA, an intermediate metabolite of the Krebs cycle, is used to generate the heme precursor $\delta$-aminolevulinic acid (Ajioka et al., 2006). Furthermore, three final steps of this pathway are also catalyzed by mitochondrial enzymes. Mitochondria also have a significant contribution to the urea cycle, steroidogenesis, cholesterol metabolism, amino acid metabolism, gluconeogenesis, ketogenesis (when acetyl-CoA is transformed to ketone bodies), calcium homeostasis, and the assembly of Fe/S clusters (Galluzzi et al., 2012).

1.1.2 Mitochondria in cancer metabolism – the Warburg effect

Compared to normal cells, cancer cells generally exhibit higher glucose consumption and higher lactate production even in the presence of sufficient oxygen. This suggests that cancer cells prefer to utilize anaerobic glycolytic metabolism for ATP production instead of the more efficient aerobic oxidative phosphorylation (de Moura et al., 2010). This preference is known as aerobic glycolysis, or the Warburg effect (Warburg, 1956). The observation of aerobic glycolysis in cancer cells led to the theory that changes to respiratory chain capacity caused by mitochondrial impairment may be the origin of cancer (de Moura et al., 2010). This theory states that a gradual and cumulative decrease in mitochondrial respiration leads to the transformation of healthy cells into malignant cancer cells (Samudio et al., 2009). This transformation was proposed to occur in two stages. First, the mitochondrial respiration system would be damaged to the point where it could no longer generate adequate ATP levels. Second, these cells would seek to compensate by generating ATP energy through glycolysis, and cells that survived this metabolic shift would eventually become cancer cells (Nakajima and Van Houten, 2012). More recent studies have demonstrated that cancer cell mitochondria are for the most part functional.
and able to carry out respiration and ATP production (Gogvadze et al., 2010). Despite this functionality, glycolysis remains a hallmark of many but not all cancers (de Moura et al., 2010). This hallmark is most likely due to alterations in signaling pathways that maintain mitochondrial activity and glucose uptake and utilization, rather than due to primary mitochondrial defects in cancer cells. However, there are examples of aggressive tumors which do display mitochondrial impairment and functional deterioration (Gogvadze et al., 2010). It is not completely clear why cancer cells preferentially use glycolysis for ATP production, considering it is a far less efficient process when compared to oxidative phosphorylation. While glycolysis produces 16-fold less ATP, it is a rapid process that can quickly generate ATP, and the consideration of speed becomes more important with higher rates of cellular growth and proliferation (de Moura et al., 2010). Glycolysis also allows cancer cells to use intermediates of the TCA cycle for macromolecular synthesis instead of energy production. For instance, citrate can be used for lipid synthesis and malate for nucleotide synthesis (de Moura et al., 2010).

Recently, new evidence suggests that aerobic glycolysis may not be as inefficient relative to oxidative phosphorylation as previously thought. Using a reduced flux balance model that takes into account the fact that ATP generation can be limited by glucose uptake and solvent capacity in the cellular cytosol (the maximum amount of macromolecules that can fit into the intracellular space), ATP generation by mitochondrial respiration is more efficient than glycolysis only under certain conditions (Vazquez et al., 2010). At low rates of glucose uptake, mitochondrial respiration is indeed far more efficient than glycolysis for the purpose of ATP generation. However, above a certain threshold of glucose uptake, complete oxidative phosphorylation is no longer the most efficient mechanism. Under these conditions, with increasing glucose uptake, activation of aerobic glycolysis and a gradual decrease of mitochondrial respiration becomes the most efficient way for cells to reach the highest rate of ATP production (Vazquez et al., 2010). This occurs because, while mitochondrial respiration is indeed far more efficient in terms of ATP generation per glucose uptake, aerobic glycolysis is the more efficient pathway in terms of required solvent capacity, which becomes rate limiting as glucose uptake increases above normal physiological levels (Vazquez et al., 2010).

Another reason for the predominance of aerobic glycolysis in cancer cells is that growing tumors are often not efficiently infused with blood vessels, and eventually outgrow their blood supply (Gillies and Gatenby, 2007). In the absence of angiogenesis or vasculogenesis, as the
oxygen availability decreases, cancer cells will still be able rely of glycolysis to generate ATP without a significant decrease in proliferation rate (Gillies and Gatenby, 2007). The hypoxic conditions within tumors can lead to the stabilization of the hypoxia-inducible transcription factor HIF-1α that helps cells adapt to stressful environments through alterations in transcription of key target genes (Hsu and Sabatini, 2008). HIF-1α can help cancer cells compensate for the low efficiency of glycolytic ATP generation by overexpressing the glucose transporter GLUT1 and increasing glucose uptake into the cell (Airley and Mobasher, 2007). Similarly, mutations in signaling kinases, oncogenes, or tumor suppressor genes can also help alter the metabolism of cancer cells (de Moura et al., 2010).

The tumor suppressor protein p53 plays a crucial role in protecting against cancer by inducing apoptosis, cell cycle arrest, DNA repair, and senescence in response to various forms of cellular stress (Bensaad and Vousden, 2007). p53 is mutated or deleted in up to 50% of solid tumors (Royds and Iacopetta, 2006). The loss of p53 provides a great proliferative advantage to cancer cells, especially in conditions of low oxygen. An interesting relationship has been observed between p53 and cellular metabolism, where loss of p53 seems to favor glycolysis over oxidative phosphorylation (Matoba et al., 2006). There was a proportional decrease in mitochondrial respiration in p53+/+, p53+-, and p53-/- cells (Matoba et al., 2006). One explanation for this observation is that p53 seems to induce the synthesis of cytochrome c oxidase 2, an important peptide in the assembly of the electron transport chain complex II. Through this mechanism, the lack of p53 may impair proper assembly of the electron transport chain, and promote glycolytic metabolism in cancer cells (de Moura et al., 2010).

Mitochondrial uncoupling (or the dissociation between mitochondrial membrane potential and ATP generation) is another important contributor to the reliance on aerobic glycolysis in cancer cells (Samudio et al., 2009). Mitochondrial uncoupling can be observed under physiological conditions, such as cold acclimation mediated by uncoupling proteins that dissipate mitochondrial membrane potential energy as heat in mammalian brown fat cells (Samudio et al., 2009). Uncoupling proteins are overexpressed in chemoresistant cancer cell lines, and since these proteins can increase the apoptotic threshold, their overexpression in cancer can provide a proliferative advantage both through shifting metabolism toward glycolysis, and by attenuating apoptosis (Samudio et al., 2009).
In addition to glucose, cancer cells can also accept many other forms of carbon sources for metabolism. These include lactate, pyruvate, glutamine, and fatty acid oxidation (Nakajima and Van Houten, 2012). A theory that suggests cancer metabolism is far more complex than just aerobic glycolysis is the concept of metabolic symbiosis (Nakajima and Van Houten, 2012). It states that within the growing tumor there exist two metabolically distinct cancer cell populations. One population relies on glycolysis and produces lactate as the end product. A second cancer cell population then uses this lactate as a metabolite for oxidative phosphorylation (Nakajima and Van Houten, 2012). A slight variation of the metabolism symbiosis theory is the reverse Warburg effect, where cancer-associated fibroblasts carry out aerobic glycolysis to provide cancer cells with metabolites for oxidative phosphorylation (Nakajima and Van Houten, 2012). This phenomenon is most likely to occur in tumors where limited oxygenation by blood vessels produces microenvironments that are hypoxic. The cancer cells in the hypoxic environments generate only two ATP per glucose molecule metabolized by glycolysis, while cells with access to oxygen can generate up to 29 ATP per two acetyl-CoA molecules. The hypoxic cancer cells import glucose through glucose transporters and generate two molecules of pyruvate by glycolysis. Under hypoxic conditions, the enzyme pyruvate dehydrogenase becomes inhibited, so instead of pyruvate being metabolized to acetyl-CoA, it is instead converted into lactate by lactate dehydrogenase-5. The lactate generated by hypoxic cells is transported out of the cell through monocarboxylate transporter-4 and is imported by oxygenated cells, which convert it back to pyruvate via lactate dehydrogenase-1. This pyruvate can then undergo metabolism through aerobic respiration within the oxygenated cells (Nakajima and Van Houten, 2012).

1.1.3 Mitochondrial respiration in cell culture and consequences for drug toxicity

A variation of the Warburg effect can be observed in fast growing cells in culture. Standard cell culture media typically contain glucose concentrations at least five times higher than physiological levels (Marroquin et al., 2007). This excess glucose allows cells to generate most of their ATP via glycolysis and not mitochondrial respiration, even though mitochondrial activity is functional in these cells (Rodriguez-Enriquez et al., 2001). This phenomenon is known as the Crabtree effect (Rodriguez-Enriquez et al., 2001). The Crabtree effect allows cells cultured under routine glucose media conditions to gain increased resistance to drugs and xenobiotics that
impair mitochondrial function (Marroquin et al., 2007). This artificial resistance to mitochondrial damage can be reversed if galactose instead of glucose is the only carbon source available. Cells will be forced to first convert galactose to galactose-1-phosphate by the enzyme galactokinase, which consumes ATP (Frey, 1996). Galactose-1-phosphate and the cofactor uridine-diphosphoglucone (UDP-glucose) are then converted to glucose-1-phosphate and UDP-galactose in an epimerization reaction carried out by galactose-1-Phosphate uridylyltransferase (Frey, 1996). UDP-galactose is converted back to UDP-glucose by the enzyme UDP-galactose-4-epimerase. Generation of the UDP-glucose cofactor by the enzyme UDP-glucose pyrophosphorylase consumes UTP and transfers uridine monophosphate (UMP) to glucose-1-phosphate and produces pyrophosphate (PPi) (Frey, 1996). The UTP consumed in this reaction has two high-energy phosphate bonds that must be regenerated by reactions equivalent to the formation of two ATP molecules from two ADP molecules. Since glycolytic metabolism from glucose to pyruvate generates two net ATP molecules, and the entry of galactose into glycolysis also consumes two additional ATP-equivalents, glycolytic metabolism of galactose generates no net ATP (Marroquin et al., 2007) (Aguer et al., 2011). The energetic futility of galactose conversion can force cells to once again rely on mitochondrial respiration for ATP energy production. As a consequence, sensitivity to xenobiotics that impair mitochondrial function is restored (Marroquin et al., 2007).

1.1.4 Role of mitochondria in reactive oxygen species generation

Reactive oxygen species (ROS) are a normal side product of the electron transport chain, and can be generated at several different points within the ETC. Not all electrons are transferred along the redox path mentioned above. During the ETC electrons may contribute to the partial reduction of oxygen, generating ROS (Lenaz, 2001). Complex I is considered one of the main sites of ROS production through electron leak that releases single electrons to molecular oxygen and generates superoxide anions from one of the iron-sulfur clusters in the complex (Hirst et al., 2008; Turrens and Boveris, 1980). Complex I can also generate ROS through the redox cycling of redox-active compounds. One example of redox-active drugs is doxorubicin, which is initially reduced to a semiquinone radical by a one-electron transfer, then reacts with oxygen to regenerate the parent compound and produces superoxide in the process (Sun et al., 2001). Futile redox cycles are initiated with a one-electron reduction of compounds such as quinones, which leads to the generation of a reactive semiquinone (Hochstein, 1983). This short-lived
intermediate quickly reacts with molecular oxygen to generate the parent compound as well as a superoxide anion. The parent compound can undergo another round of reduction and oxidation, and continually generate superoxide in a futile redox cycle (Hochstein, 1983; Lenaz and Genova, 2010). In addition to generating harmful ROS, futile redox cycles are also harmful to the mitochondria because they divert electrons away from oxidative phosphorylation and energy metabolism (Hochstein, 1983). In complex III, another major site of superoxide generation, the mechanism occurs by CoQ cycling where ubisemiquinone functions as a redox intermediate (Dykens and Will, 2008). It is estimated that approximately 1% of total oxygen uptake in mammalian tissues is converted to superoxide (Dykens and Will, 2008). Superoxide can damage proteins or nucleic acids, or react with lipids by attacking double bonds of unsaturated fatty acids. The peroxides of fatty acids can in turn initiate chain reactions propagating to other acyl chains, which multiplies the initial damage and destabilizes membranes (Dykens and Will, 2008). Since superoxide is a highly unstable, short-lived molecule, its release from the mitochondria is rare, but highly dependent on mitochondrial membrane potential, such that a change in membrane potential from 170 to 190 mV will cause a 15-fold increased superoxide release from the mitochondria (Bartosz, 2009).

The superoxide anion can be further converted to hydrogen peroxide in a reaction catalyzed by the enzyme superoxide dismutase (Dykens and Will, 2008). Hydrogen peroxide can be safely eliminated from the cells by catalases or peroxidases, which convert hydrogen peroxide to water and oxygen. Another important mitochondrial antioxidant is glutathione, a tripeptide that can be recycled by the enzyme glutathione reductase. Glutathione peroxidase can also use glutathione-reducing activity to convert H₂O₂ into H₂O. Mitochondrial thiol-containing proteins can also serve as antioxidants by buffering ROS (Balaban et al., 2005). However, hydrogen peroxide is able to easily cross biological membranes, and is stable enough to exit the mitochondria and cause oxidative damage elsewhere in the cell. It can react with metal cations (Fe²⁺ and Cu⁺) nonenzymatically in the Fenton reaction and generate hydroxyl radicals which are extremely reactive and can damage virtually any nearby compound (Dykens and Will, 2008). ROS damage can have a wide range of consequences within mitochondria. The generated ROS can then damage ETC proteins, or cause mtDNA damage ultimately leading to loss of ETC machinery and mitochondrial dysfunction (Lenaz and Genova, 2010). ROS damage to mitochondrial thiols can impair the activity of enzymes such as the adenine nucleotide
translocase, which transports ADP and ATP between the cytosol and the mitochondrial matrix (Costantini et al., 2000). Impairment of this enzyme can result in a shortage of ADP in the mitochondrial matrix, which will inhibit ATP synthase and cause ATP depletion (Costantini et al., 2000).

### 1.1.5 Mitochondrial biogenesis

All of the proteins involved in the transcription and translation of ETC components are of nuclear origin. Replication, transcription, and repair of mtDNA are also carried out by nuclear-encoded enzymes (Herrmann et al., 2012). Crucial mitochondrial enzymes encoded by the nuclear genome include: DNA polymerase gamma (Polg), mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A, B1 and B2, mitochondrial transcription termination factor, and mitochondrial translation-initiation factor 2 (Herrmann et al., 2012). In addition to nuclear-encoded ETC proteins, the mitochondria also receive shuttle and transporter proteins, heme-biosynthesis machinery, mtDNA replication and transcription machinery, and mtRNA-translation proteins from nuclear genes (Herrmann et al., 2012). All these mitochondrial proteins of nuclear origin must be transported across the mitochondrial outer membrane, inner-membrane space, and inner membrane. In many cases this transport relies on the mitochondrial targeting sequence (MTS), an amino-terminal presequence. However, many other proteins lack this classic MTS, but instead have an internal mitochondrial targeting signal (Herrmann et al., 2012). Both the peptides with and without a MTS are recognized by the TOM-complex. Peptides with a MTS go through the translocase of the mitochondrial inner membrane (TIM) complex and the presequence translocase-associated motor complex, into the mitochondrial matrix, where the MTS is cleaved by mitochondrial processing peptidase (Herrmann et al., 2012). Peptides lacking the classic MTS must bind to chaperone proteins Tim9 and Tim10 before being translocated by the TOM-complex (translocase of the mitochondrial outer membrane). When necessary, the TIM22 complex inserts proteins into the inner mitochondrial membrane (Herrmann et al., 2012).

### 1.1.6 The role of mitochondria in cell death

Programmed cell death encompasses several different mechanisms carried out by different signaling pathways. The three most important such pathways are extrinsic apoptosis, intrinsic apoptosis, and regulated necrosis (Figure 3) (Galluzzi et al., 2012). Apoptosis is the programmed death of cells that are damaged, inappropriate, or no longer needed, and also occurs during
embryonic development (Ethell and Fei, 2009). As a cell undergoes apoptosis, characteristic morphological changes occur. The plasma membrane remains intact while the organelles are degraded and cellular compartments are repackaged to facilitate phagocytosis by neighboring cells or macrophages (Ethell and Fei, 2009). Cellular degradation is ultimately carried out by a family of enzymes termed caspases, which must themselves be activated through a tightly controlled caspase cascade (Ethell and Fei, 2009).
Figure 3. Role of mitochondria in cell death. Mitochondria play a crucial role in regulating apoptosis and cell death. This can occur through extrinsic apoptosis (A), intrinsic apoptosis (B), or regulated necrosis (C). In extrinsic apoptosis, activation of death receptors at the cellular surface initiate caspase cascades and induce apoptosis. Intrinsic apoptosis is initiated by the mitochondria. Members of the Bcl-2 protein family permeabilize the inner mitochondrial membrane and release mitochondrial pro-apoptotic factors. These factors organize the assembly of a pro-apoptotic protein complex known as the apoptosome, which initiates a caspase cascade to induce apoptosis and cell death. Regulated necrosis can also be initiated by activation of death receptors in the cell surface. The downstream cascade causes mitochondrial fragmentation and cell death (Galluzzi 2012).
The extrinsic apoptosis pathways can be initiated when a ligand activates transmembrane death receptors such as FAS/CD95 and tumor necrosis factor receptor 1 (Wajant, 2002). Alternatively, this pathway can be initiated if the extracellular concentration of certain trophic factors such as netrin-1 falls below a certain threshold, activating the transduction of lethal signals (Mehlen, 2010). Following their activation, these death receptors assemble the death-inducing signaling complex (DISC), which leads to the activation of pro-apoptotic caspase-8 and caspase-3 cascades (Ethell and Fei, 2009; Galluzzi et al., 2012). Mitochondria have limited involvement in extrinsic apoptosis, and serve only to amplify the cascade of caspase activation once the process has already been initiated (Galluzzi et al., 2012).

By contrast, mitochondria are heavily involved in the intrinsic pathway, which can be initiated by many different intracellular events such as DNA damage, oxidative stress, calcium imbalance, and mitochondrial stress (Galluzzi et al., 2012; Kroemer et al., 2007; Ravagnan et al., 2002). In the presence of pro-apoptotic signals, mitochondrial outer membrane permeabilization (MOMP) occurs, which immediately disrupts mitochondrial ATP synthesis (Li et al., 1997). MOMP can initiate apoptosis by the release of several proteins from the inner membrane space. These death-inducing factors include: cytochrome c, SMAC/Diablo (Second Mitochondrial Activator of Caspases/Direct IAP Binding protein with Low pI), and apoptosis inducing factor (AIF) (Ethell and Fei, 2009). Their release is largely controlled by members of the Bcl-2 protein family, which regulate cell death by controlling the permeability of the outer mitochondrial membrane. Bcl-2-associated X protein (Bax) and Bcl-antagonistic/killer (Bak) are pro-apoptotic members of the Bcl-2 family, and are able to form pores that enable MOMP (Ethell and Fei, 2009; Ravagnan et al., 2002). Bcl2 and BclxL are anti-apoptotic factors, that can bind directly to Bak and Bax to prevent MOMP (Ethell and Fei, 2009). Another subgroup of the Bcl-2 family is the BH3-only proteins, which have a common motif called the BH3 domain. BH3-only proteins are pro-apoptotic because they inhibit Bcl-2 and BclXL activity and therefore activate Bak/Bax (Garcia-Saez, 2012). Under normal conditions the anti-apoptotic factors Bcl-2 and BclXL bind and inactivate Bak/Bax. In the presence of pro-apoptotic signals, BH3-only proteins become activated by post-translational modification and increased expression, and antagonize the activity of pro-survival factors such as Bcl-2 and BclXL. This allows Bax and Bak to become activated, causing a conformational change where these proteins oligomerize and insert into the outer
mitochondrial membrane, causing its permeabilization and release of pro-apoptotic factors such as cytochrome c, SMAC/Diablo, and AIF (Garcia-Saez, 2012).

Cytochrome c is found in the mitochondrial intermembrane space, where it serves as an electron shuttle between complexes III and IV. Once released to the cytosol by MOMP, cytochrome c binds Apaf-1 and assembles into a complex called the apoptosome, which activates caspase-9/ caspase-3 and initiates a cascade leading to apoptosis (Li et al., 1997). In the absence of apoptotic signals, inhibitor of apoptosis proteins (IAP) binds to and inhibits caspases 3 and 9. During apoptosis induction, the SMAC/Diablo protein is released into the cytosol and binds IAP to prevent it from inhibiting caspases (Ravagnan et al., 2002). Apoptosis inducing factor (AIF) also localizes to the mitochondria but plays a role in nuclear apoptosis. Following apoptosis initiation, AIF translocates to the cytosol and the nucleus where it induces chromatin condensation and DNA fragmentation (Ravagnan et al., 2002). The tumor suppressor protein p53 can regulate both apoptosis and autophagy. In response to stress, p53 in the nucleus transactivates several pro-apoptotic genes, including Bax and Bak. p53 also translocates to the mitochondria and promotes membrane permeabilization. p53 has been reported to directly bind and activate Bax and Bak (Kubli and Gustafsson, 2012).

Regulated necrosis, also known as necroptosis, can be regulated by necrostatin-1, a necrosis inhibitor that selectively inhibits the receptor-interacting protein kinase 1 (Degterev et al., 2008). Permeabilization of the inner mitochondrial membrane by the opening of the mitochondrial permeability transition pore (mPTP) causes the influx of water into the matrix, eliminates the proton gradient, and eventually ruptures the outer mitochondrial membrane leading to necrotic cell death (Kubli and Gustafsson, 2012). Even though apoptosis and necrosis are distinct death pathways, mitochondria allow for cross-talk and some overlap in regulation and mechanism of action of these two pathways. Bcl-2, the anti-apoptotic protein that inhibits Bax, can block the opening of the mPTP by increasing its calcium threshold. It has also been shown that cells deficient for both Bax and Bak are resistant to mPTP opening and necrotic cell death (Kubli and Gustafsson, 2012).

In addition to cell death, proliferating cells also have the option to undergo cellular senescence in response to several stresses including telomerase shortening, oxidative stress, DNA damage, and expression of oncogenes (Campisi, 2011). Senescence is a prolonged and
progressive process that can be defined as an irreversible growth arrest (Young and Narita, 2013). Senescent cells tend to present with enlarged morphology and express beta-galactosidase activity (Campisi, 2011; Young and Narita, 2013). Senescence is strongly associated with aging because the number of senescent cells increases with age, and senescence of crucial stem cell populations may contribute to the decrease of tissue repair and regeneration that occurs with aging (Campisi, 2011). Senescence and apoptosis are similar in that both can serve as potent mechanisms of tumor suppression (Campisi, 2011). While apoptotic triggers converge in the activation of caspases, senescence involves different mechanisms that collectively contribute to senescence (Young and Narita, 2013). p53 is crucial for senescence response, and inhibition of p53 can impair senescence and accelerate the development of malignant tumors (Campisi, 2011). Oxidative stress caused by sub-lethal H$_2$O$_2$ exposure can induce premature senescence in human fibroblasts, leaving the surviving cells unable to proliferate or synthesize DNA, and causing an increase in cell surface area and morphological changes consistent with replicative senescence (Chen, 2000).

1.2 Drug-induced mitochondrial toxicity

Drug-induced mitochondrial toxicity can be caused by a wide range of different drug classes. Analgesics, antibiotics, HIV/AIDS drugs, and chemotherapy medications are among the drug classes where mitochondrial toxicity has been observed (Neustadt and Pieczenik, 2008). Different xenobiotics can damage mitochondria through a variety of mechanisms (Table 1) (Cohen, 2010). Mitochondrial processes implicated in drug toxicity include but are not limited to mitochondrial DNA replication, oxidative phosphorylation, membrane potential, lipid biosynthesis, and free radical production (Cohen, 2010). The mode of mitochondrial toxicity can be very similar to, or completely different from the mechanism of its intended pharmacological effect. For example, antibiotics such as aminoglycosides and chloramphenicol bind to bacterial ribosomes, but can also inhibit mitochondrial protein synthesis due to the structural similarity between bacterial and mitochondrial ribosomes (Martin et al., 2001). Similarly, nucleoside analog reverse transcriptase inhibitors (NRTI) are used for the treatment of HIV/AIDS by inhibiting the HIV reverse transcriptase, but they will also be incorporated by the mitochondrial DNA polymerase gamma into the nascent mitochondrial genome (White, 2001). By contrast, the broad-spectrum antineoplastic agent doxorubicin has a planar anthacycline ring that intercalates the double helix of the cancer cell’s nuclear DNA (Oliveira et al., 2004). However, it also causes
mitochondrial toxicity through a completely unrelated mechanism. Doxorubicin has electrochemical properties that allow it to accumulate within the inner mitochondrial membrane, where its redox potential allows it to act as an alternate electron acceptor at complex I, causing electron transport chain (ETC) inhibition and a massive increase in free-radical production (Cohen, 2010).
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Xenobiotics can also indirectly cause mitochondrial damage by depleting or sequestering important antioxidants, nutrients, or cofactors that are crucial for proper mitochondrial function. For example, acetaminophen is metabolized by CYP2E1 to a toxic intermediate that becomes conjugated with glutathione, an important mitochondrial antioxidant. Acetaminophen toxicity can therefore lead to glutathione depletion, free-radical accumulation, and mitochondrial dysfunction (Jaeschke and Bajt, 2006). Similarly, aspirin can sequester Coenzyme A, while the anti-epileptic drug valproic acid can sequester Coenzyme A and carnitine, which will lead to the inhibition of lipid biosynthesis (Fromenty and Pessayre, 1997). Some xenobiotics will trigger mitochondrial toxicity in any individual regardless of genetic background. One example is MPTP, a contaminant generated during the production of a synthetic narcotic (Annepu and Ravindranath, 2000). MPTP is oxidised to MPP+, a powerful inhibitor of ETC complex I, which causes increased free-radical generation and death of dopaminergic neurons, ultimately leading to parkinsonism (Neustadt and Pieczenik, 2008). By contrast, other drugs may only affect a susceptible population or exacerbate pre-existing symptoms (Cohen, 2010). For example, a rare side effect of valproic acid is a rapid-onset life-threatening hepatotoxicity, which occurs predominantly in patients with mutations in \textit{POLG}, the gene encoding for the mitochondrial DNA polymerase gamma (McFarland et al., 2008; Saneto et al., 2010).

1.2.1 Rotenone

Rotenone is an inhibitor of complex I of the ETC. It acts at the site of electron donation to inhibit CoQ reduction in an inhibitor binding site composed of the protein subunits NDUFS2, NDUFS7, ND1, and ND5 (Koopman et al., 2010). Rotenone is a naturally occurring ketone made by tropical plants to ward off insects (Ethell and Fei, 2009). This compound has been used in agriculture as an insecticide to kill mites and spiders, and also as a fish poison (Koopman et al., 2010). Rotenone is considered moderately to highly lipophilic, with an octanol to water partition coefficient of 4.1 (Koopman et al., 2010). An indirect consequence of rotenone inhibition of complex I is that ATP depletion can lead to a metabolic shift towards anaerobic respiration (Xiong et al., 2012). Inhibition of complex I by rotenone causes an increase in reactive oxygen species and reactive nitrogen species generation, which can damage lipids and proteins, causing lipid peroxidation and protein misfolding (Xiong et al., 2012). Rotenone has also been shown to increase Ca\textsuperscript{2+} levels in the mitochondria through the opening of voltage-gated Ca\textsuperscript{2+} channels. Excessive Ca\textsuperscript{2+} levels in the mitochondria can lead to cytochrome c release and
apoptosis (Xiong et al., 2012). Ultimately, rotenone induces apoptosis through members of the Bcl-2 family of proteins. This leads to mitochondrial membrane depolarization, Bax expression, release of cytochrome c, and activation of caspases 3 and 9 (Xiong et al., 2012).

### 1.2.2 Assessment of mitochondrial function

Currently there are several different methods of measuring overall mitochondrial health and toxicity. The enzymatic activities of individual ETC complexes can be measured spectrophotometrically, relying on changes in absorbance that occur during redox reactions catalyzed by the ETC. For example, complex I activity can be measured by following the decrease in absorbance at 340 nm that occurs during NADH oxidation. Complex II activity can be measured at 600 nm by following the reduction of 2,6-dichlorophenolindophenol. Complex III activity can be measured by the reduction of cytochrome C at 550 nm, and complex IV activity can be measured by following the oxidation of cytochrome C at 550 nm (Birch-Machin et al., 1994). Some disadvantages of these classic techniques are that mitochondria must be isolated from large amounts of tissue, which is not always feasible with human tissue samples.

Another way to measure activity of individual ETC complexes is with immunocapture-based assays for OXPHOS activity (Nadanaciva et al., 2007). Homogenized tissue samples are transferred to wells coated with monoclonal antibodies specific against ETC complexes. Once the desired complexes have been isolated, traditional spectrophotometric assays can be carried out. The initial immunocapture step can increase the sensitivity of the assays and requires less tissue, but limitations remain because this technique cannot be utilized with intact cells (Dykens and Will, 2008).

Another very traditional method involves the indirect measurement of mitochondrial respiration by following oxygen consumption through polarographic analysis with sensors for oxygen (such as the Clark electrode) and protons (by measuring the pH) (Gnaiger et al., 1995; Hofhaus et al., 1996). As the sample respires aerobically, oxygen will be consumed and oxygen levels will drop. Alternatively, when anaerobic respiration occurs, lactic acid production may be detected by measuring changes in pH. Coupled with the titration of multiple substrates, uncouplers and inhibitors, these methods can be customized to analyze various mitochondrial pathways (Wenchich et al., 2003). Alternatively, another way to indirectly measure mitochondrial function through oxygen consumption is with oxygen-sensitive fluorescent probes,
able to detect the concentration of dissolved oxygen based on its ability to quench certain fluorescent luminophores (Papkovsky, 2004). This technique has an advantage over polarographic measurements since it can be adapted into a high-throughput, plate-based assay (Weiss et al., 2002). Fluorescent reporter molecules can also be used to measure the mitochondrial membrane potential, a very important marker of mitochondrial health because it often dissipates in response to drug damage and occurs during mitochondrial dysfunction. Fluorescent probes with a delocalized positive charge are able to accumulate within the mitochondria in response to the electrical potential, such that fluorescence will be proportional to the membrane potential (Reers et al., 1995; Scaduto and Grotyohann, 1999). Other fluorescent probes are able to detect mitochondrial ROS generation, an important molecular marker of mitochondrial toxicity because many mitochondrial damaging xenobiotics cause an increase in mitochondrial ROS production (Ong et al., 2007). These techniques are advantageous because they can be used both with isolated mitochondria and with intact cells. One limitation is that these probes can often interfere with mitochondrial function and cause phototoxicity (Dykens and Will, 2008).

A novel method that functions under the same principles mentioned above is the extracellular flux analysis using the XF24 analyzer, a plate-based high-throughput instrument able to simultaneously measure oxygen consumption rate (a measure of mitochondrial respiration) and extracellular acidification rate (a measure of glycolysis) in intact cells using highly sensitive probes (Wu et al., 2007). The ability to measure mitochondrial activity in intact cells is superior over measurements in isolated mitochondria because it allows for mitochondrial function to be examined under more physiological conditions, and allows for the evaluation of drug effects on complex cellular signaling pathways (Dykens and Will, 2008). By sequentially injecting inhibitors and uncouplers into the cells it is possible to interrogate different components of mitochondrial respiration and obtain a profile of mitochondrial bioenergetics (Ferrick et al., 2008). The total reserve capacity, a measure of the maximal oxygen consumption that mitochondria are able to tolerate, is an extremely sensitive measure of mitochondrial health, and can be stimulated in this system following administration of oligomycin, FCCP, and 2-DG (Qian and Van Houten, 2010).
1.3 Mitochondrial DNA

The mitochondrial DNA (mtDNA) is a small (16.5kb) circular molecule that includes 37 genes, of which 13 encode essential proteins of the respiratory chain complexes I, III, IV and V (Figure 4) (Clay Montier et al., 2009). mtDNA also encodes 22 transfer RNAs and 2 ribosomal RNAs, all of which are important for mitochondrial function and ATP generation (Van Houten et al., 2006). The remainder of the approximately 900 proteins that make up the mitochondrial proteome are of nuclear origin and are imported to the mitochondria (Chan, 2006a). Each mitochondrion contains approximately 10-15 (Van Houten et al., 2006) copies of mtDNA, and each cell contains approximately 1000-5000 mtDNA copies (Clay Montier et al., 2009; Moraes, 2001). mtDNA is a very important target for oxidative stress, as damage to this molecule can lead to impaired electron transport, membrane potential, and ATP production (Van Houten et al., 2006).
Figure 4. Mitochondrial DNA. The mitochondrial genome is a circular molecule 16.5 kb in length and encodes for 37 genes: two ribosomal RNAs (12S and 16S), 22 transfer RNAs, and 13 protein subunits of the electron transport chain. Among these protein subunits, 7 belong to complex I (ND1, 2, 3, 4, 4L, 5 and 6), 1 belongs to complex III (cytochrome b), three belong to complex IV (cytochrome c oxidase I, II, and III), and two belong to complex V (A6 and A8) (adapted from Schon 2012).
Within the mitochondria, mtDNA are organized into nucleoid structures, made up of several mtDNA genomes (usually around 5-7) and protein factors (Gilkerson, 2009). Nucleoids are distributed at regular spatial intervals throughout the mitochondria and are anchored to the inside of the inner mitochondrial membrane. Since the nucleoids are adjacent to the inner mitochondrial membrane that is the site of the ETC, mtDNA is located near the main source of ROS within the cell, and as such is exposed to high amounts of oxidative stress (Gilkerson, 2009). While nucleoids provide some degree of protection to mtDNA, mtDNA is much more vulnerable than the nuclear genome, which receives protection from histones (Liu and Demple, 2010). As a result, mtDNA accumulates mutations at a 10-50 –fold higher rate than the nuclear genome (Gilkerson, 2009). mtDNA also suffers 3-10 –fold higher damage than nuclear DNA when exposed to oxidative stress (Van Houten et al., 2006). Another reason for the relatively higher susceptibility to oxidative stress in mtDNA compared to nuclear DNA is the high iron content in mitochondria that can mediate ROS generation by Fenton chemistry (Van Houten et al., 2006). Persistent damage to mtDNA can result in cell death despite full repair of the nuclear DNA (Sawyer and Van Houten, 1999).

Oxidative damage to DNA can result in modifications to the DNA base or sugar, single or double stranded breaks to the DNA backbone, or crosslinks to other macromolecules. Large deletions are also common, and mtDNA contains hot-spot regions where large deletions commonly occur (Sawyer and Van Houten, 1999). It has also been shown that mtDNA contains hot-spots for DNA damage and induction of mutations (Sawyer and Van Houten, 1999). The result of these lesions can be impairment of DNA replication and transcription, or mutagenesis (Stuart and Brown, 2006). An example of a common hydroxyl radical DNA lesion is 8-oxo-2’deoxyguanosine (8-oxodG), formed by the addition of a hydroxyl group to the C8 of guanine. 8-oxodG can be mutagenic by causing CG → TA transversions during DNA replication (Stuart and Brown, 2006).

1.3.1 mtDNA replication

There are currently two models of mtDNA replication: the asynchronous strand displacement model and the strand-coupled bidirectional replication model (Chan and Copeland, 2009). mtDNA replication is coupled to mitochondrial RNA transcription. RNA transcripts initiated near the heavy chain (guanine rich, heavy - H) origin of replication can serve as primers
for the start of mtDNA replication of the heavy chain (Graziewicz et al., 2006). In the strand displacement model, replication occurs in an asymmetric manner. The leading strand synthesis starts at the H strand origin of replication in the D-loop, and occurs in the absence of lagging-strand DNA replication. The leading strand advances to approximately two thirds of the genome before the L strand (cytosine rich, light – L) origin of replication becomes exposed by the replication fork, and the second strand synthesis is initiated in the opposite direction. In this model, mtDNA synthesis is continuous but asynchronous on opposing strands (Chan and Copeland, 2009; Graziewicz et al., 2006; Holt, 2009). In the strand-coupled model, bidirectional replication of the leading and lagging strand occurs simultaneously in a symmetric, semidiscontinuous way. The lagging strand is composed of short okazaki fragments, and replication following the progression of the two replication forks around the mtDNA molecule (Graziewicz et al., 2006; Holt, 2009). While all mitochondria have the machinery necessary to carry out mtDNA replication, synthesis of mtDNA seems to occur preferentially in mitochondria located near the nucleus (Graziewicz et al., 2006).

The rates of mtDNA degradation and synthesis are coordinated to maintain copy number. Constant mtDNA turnover means that this genome is constantly being replicated, even in post-mitotic cells such as neurons (Clay Montier et al., 2009). In order to control mtDNA abundance and maintain mtDNA steady-state levels, mammalian cells maintain constant mtDNA mass, as opposed to a constant number of mtDNA molecules. Evidence for this comes from studies where in cell lines containing either wild type, duplicated, or deleted mtDNA genomes, the mtDNA copy number was inversely proportional to the size of their respective mtDNA molecules (Moraes, 2001).

1.3.2 mtDNA repair

Mitochondrial DNA repair is very limited compared to nuclear DNA repair (Van Houten et al., 2006). All mtDNA repair machinery proteins are encoded by the nuclear genome and all gene products must be transported to the mitochondria, generally via a mitochondrial targeting sequence comprising the first 20-25 N-terminal amino acids which are cleaved off during transport (Van Houten et al., 2006). UV-induced lesions and large, bulky lesions do not appear to be repaired in mtDNA (Sawyer and Van Houten, 1999). This suggests that mitochondria lack nucleotide excision repair (NER), the major repair pathway responsible for the removal of DNA.
adducts typically caused by UV-irradiation, polyaromatic hydrocarbons and chemotherapeutic agents (Liu and Demple, 2010). Mitochondrial DNA repair consists mostly of base excision repair (BER), the repair pathway responsible for the removal of oxidative DNA damage (Van Houten et al., 2006). BER in mitochondria replaces a single nucleotide, and requires the activity of DNA glycosylases, AP endonuclease 1, DNA polymerase gamma, and DNA ligase III (Liu and Demple, 2010). DNA glycosylases remove the damaged base creating an abasic site, which AP endonuclease 1 recognizes and processes into a 3’OH and 5’phosphate single-stranded break. In the next step, DNA polymerase gamma replaces the damaged base, and DNA ligase seals the phosphodiester bond of the DNA backbone (Stuart and Brown, 2006). Low levels of mismatch repair (MMR) activity have also been identified in mitochondria. This repair pathway is able to proofread replication errors and eliminate premutagenic dNTPs to prevent mis-incorporation and mutagenesis (Liu and Demple, 2010). Homologous recombination can also occur in the mitochondria to repair low levels of double stranded breaks (Liu and Demple, 2010).

Due to the multi-copy nature of the mitochondrial genome, another option aside from repair is to simply target damaged mtDNA molecules for degradation, leaving the remaining undamaged molecules to repopulate the mtDNA pool. Under this mechanism, mtDNA containing large amounts of damage, or persistent or irreparable lesions will be targeted for degradation. The signals that trigger mtDNA degradation are thought to include double stranded breaks and stalled DNA or RNA polymerases (Liu and Demple, 2010).

The tumor suppressor p53 plays a crucial role in maintaining genomic integrity in the nucleus, where it responds to DNA damage to induce cell cycle arrest and allow DNA repair, or alternatively activates programmed cell death if the damage is extensive (Holt, 2010). It has been proposed that p53 plays a similar role in the mitochondria. Cellular stresses including oxidative stress can recruit p53 to mitochondria, where p53 has been shown to interact with mtDNA, POLG1, and other mtDNA binding proteins, including the mtDNA maintenance protein TFAM and the mitochondrial single stranded DNA binding protein SSBP1 (Holt, 2010). Oxidative stress conditions that cause p53 trafficking to the mitochondria are accompanied by reductions in mtDNA, suggesting that p53 might play a role in the choice between mtDNA repair and mtDNA depletion (Holt, 2010).
1.3.3 mtDNA mutations and deletions

Mitochondrial DNA molecules often exist in a state of heteroplasmy, where mtDNA molecules containing pathogenic mutations co-exist with wild type mtDNA molecules within the same pool. In many diseases associated with mtDNA mutations, the disease phenotype will only occur when the proportion of mutated mtDNA copies exceeds a certain threshold. Depending on mutation and cell type, this threshold can range between 50-95% (Smith and Lightowlers, 2011).

Several mechanisms of selection and depletion of mtDNA molecules have been identified. mtDNA molecules with duplicate or triplicate regions could have a selection advantage if these regions include additional origins of replication. Conversely, mtDNA molecules with deletions may also receive a selection advantage since they will complete the replication cycle faster than wild type mtDNA, and can therefore be replicated more often than wild type copies (Holt, 2010). Another mechanism has recently been identified that could confer a selection advantage to mtDNA mutations that generate ROS. mtDNA exists in a supercoiled state, and so nicking is necessary to initiate replication. ROS stimulates the protein-mediated nicking of mtDNA before the initiation of rolling-circle replication, and so through this process, ROS is implicated in the maintenance of mtDNA copy number.

Cancer cells are especially prone to select for ROS generating mutations (Holt, 2010). mtDNA mutations are frequently found in cancer cells at higher rates than in the surrounding tissue for two reasons. Firstly, cancer cells tend to rely on glycolysis as opposed to oxidative phosphorylation for ATP energy production (the Warburg effect), which diminishes the selective pressure for proper mitochondrial function. In addition, ROS has a role in signal transduction that stimulates cell proliferation, giving cancer cells another advantage over surrounding cells (Holt, 2010).

Epidemiological studies have identified mitochondrial disease in approximately 1 in 5000 adults, while pathogenic mtDNA mutations are present at a much higher incidence of 1 in 200 individuals (Schon et al., 2012). The number of human mtDNA mutations identified is over 300 (Greaves et al., 2012), and these affect every gene within the mitochondrial genome. An unexpected observation is that over half of these mutations were identified in tRNA genes even though tRNAs only make up 10% of the mtDNA coding capacity (Schon et al., 2012). Mutations in peptide–coding regions only account for 40% of the mutations identified, even though these
regions make up 70% of the mtDNA. This over-representation of tRNA genes is likely due to the fact that tRNA mutations have a very high threshold for mutation load, usually requiring >90% mutant copies for disease to occur. By contrast, protein-coding genes of the mtDNA tend to have a lower threshold for mutation load, with disease occurring in some cases where 70% of mtDNA copies carry the mutant allele (Schon et al., 2012). Therefore tRNA mutations are tolerated at a wider range of mutation load than mutations in protein-coding genes (Schon et al., 2012). In the other extreme, mtDNA deletions seem to be very poorly tolerated by the mitochondria. A threshold of 50-60% mtDNA copies harboring deletions is often enough to cause a disease phenotype (Greaves et al., 2012). mtDNA deletions tend to occur in regions of the mtDNA that are flanked by tandem repeat sequences, and often arise randomly during repair of damaged mtDNA.

1.3.4 mtDNA depletion

mtDNA depletion refers to a significant decrease in mtDNA copy number within the cell. A pathogenic decrease in mtDNA abundance is generally considered to be anywhere from 20-50% relative to the normal range (Holt, 2010). mtDNA depletion can occur if damaged mtDNA copies are targeted for destruction, or if they are unable to undergo replication. Defects that precipitate depletion may be point mutations or deletions. Increased ROS generation may be a regulator in the decision to degrade mtDNA, serving to eliminate copies encoding for defective proteins that cause excessive ROS generation (Holt, 2010). mtDNA deletions where an origin of replication is missing could also lead to depletion. Depletion may also occur when the materials for mtDNA replication or mitochondrial biogenesis are not available. While mammalian cells with very little or no mtDNA can persist both in culture and in vivo, complete mtDNA loss will lead to cell death by apoptosis in vivo (Holt, 2010).

Mutations in nine different genes have been identified as causing mtDNA depletion autosomal recessive syndromes. These genes are: DNA polymerase gamma (POLG), deoxyguanosine kinase, thymidine kinase, mitochondrial thymidine phosphorylase, mitochondrial inner membrane protein (MPV17), succinate-CoA ligase alpha and beta subunits, ribonucleotide reductase, and Twinkle (Dimmock et al., 2010). mtDNA depletion tends to be associated with infantile neurogenetic disorders, hypotonia, and developmental delays in early childhood (Clay Montier et al., 2009). Depending on the gene affected, these disorders may be
tissue specific or affect multiple systems. For example, MPV17 mutations cause hepatic failure during infancy, while certain POLG mutations can cause encephalopathy or hepatic impairment that may present at any age (Dimmock et al., 2010).

1.3.5 **Rho0 cells**

Rho0 cells are completely depleted of mtDNA (Chandel and Schumacker, 1999). These cells can serve as very useful tools for studying the effects of mtDNA mutations on mitochondrial function and other cellular processes (Hashiguchi and Zhang-Akiyama, 2009). Rho0 cells can be generated by chronic treatment with low doses of a DNA intercalator such as ethidium bromide (EtBr). At low doses EtBr binds preferentially to mtDNA and is able to inhibit the replication of mtDNA without significantly affecting the nuclear genome. Over time this will lead to the complete depletion of mtDNA from the cell population (Hashiguchi and Zhang-Akiyama, 2009). The consequences of this complete mtDNA depletion is that oxidative phosphorylation is severely impaired in Rho0 cells, such that they must rely completely on glycolysis for ATP generation. As a result Rho0 cells require high glucose, pyruvate, and uridine supplementation in order to survive (Hashiguchi and Zhang-Akiyama, 2009). Rho0 cells also have mitochondrial fragmentation (Qian and Van Houten, 2010), swollen mitochondria, disorganized cristae structure, and relatively empty mitochondrial matrix (Kukat et al., 2008). Rho0 cells are able to maintain a membrane potential across the inner mitochondrial membrane by consuming ATP through the reverse action of the ATP synthase. This process is extremely energy inefficient, and further adds to their bioenergetics insufficiency (Hashiguchi and Zhang-Akiyama, 2009).

Once the cells are completely void of mtDNA, they may be used in cytoplasmic transfer experiments to introduce mtDNA with a particular mutation of interest (Kukat et al., 2008). Rho0 cells repopulated with mtDNA harboring pathogenic mutations are able to replicate the underlying biochemical phenotype of the disease (Raha et al., 1999). The repopulated cells, called Rho0 fusion cybrids (cytoplasmic hybrids), have been used to identify several mtDNA mutations responsible for human diseases including: mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), and Leigh’s disease (Raha et al., 1999). Alternatively, repopulating patient Rho0 cells with wild type mtDNA is able to rescue the biochemical disease phenotype of MERRF cells.
(Chang et al., 2012). This indicates as a proof of principle, that mitochondrial therapy through the transfer of healthy mtDNA into cells with mtDNA disease is feasible, so long as the wild type mtDNA copies are able to push pathogenic mtDNA below the disease threshold.

1.3.6 mtDNA inheritance

mtDNA is transmitted directly from the mother to the offspring (St John et al., 2010). Since mtDNA mutations accumulate with aging, the implication is that higher mtDNA mutation rates would be expected to accumulate irreversibly over generations (Zhou et al., 2010). However, deleterious mtDNA mutations are eliminated from the maternal germ line through a selection mechanism that greatly decreases mtDNA genotypic variance (Poulton et al., 2009). This selection occurs through a mechanism of mtDNA genetic bottleneck, where only a limited number of mtDNA template molecules are selected for replication (Zhou et al., 2010). The fertilized oocyte initially contains a very high mtDNA copy number, approximately 175,000 copies. As cell division of the early embryo begins, mitochondrial replication is arrested and mtDNA copy number drops dramatically (Wai et al., 2008). At this stage, primordial germ cells are present in small numbers and have less than 10 mitochondria per cell (Zhou et al., 2010). This allows for the selection against detrimental mtDNA mutations to occur during the extension and maturation of the oocyte population. Once mitochondrial replication resumes again, the mtDNA copy number increases 10-20-fold, but only a specific subpopulation of mtDNA will be selected for replication. Once the oocytes become mature only a single predominant mtDNA genotype is found in each mature oocyte (Zhou et al., 2010).

The mtDNA selection process appears to be dependent on mitochondrial function, but the exact mechanism of this selection is not yet fully understood (Poulton et al., 2010). One proposed mechanism is that increased ROS generation by dysfunctional mitochondria serves as a signal for the removal of oocytes with high mtDNA mutation load. Approximately 70% of oocytes are eliminated by apoptosis and do not reach maturity (Poulton et al., 2010). Therefore it is possible that only the oocytes with the highest mitochondrial function are allowed to reach the mature state. Another possible mechanism for the selective pressure may occur at the level of the organelle. Germ cells have a very low number of mtDNA copies per mitochondria relative to somatic cells, which may reduce the protective effect of heteroplasmy and cause mtDNA mutations to induce mitochondrial dysfunction. The individual organelles that display decreased
respiration activity would then be targeted for degradation by autophagy processes (Poulton et al., 2010). Another proposed selection mechanism involves a structure called the mitochondrial cloud (Wai et al., 2008). Mitochondrial cloud is composed of mitochondria and endoplasmic reticulum organized around golgi elements that are rich in germline-determining mRNAs (Zhou et al., 2010). Mitochondria contained within the mitochondrial cloud are specifically inherited by progenitor germ cells. Mitochondria with the highest membrane potentials are found in the mitochondrial cloud, suggesting that only the highest quality organelles are assembled into this structure in order to be passed to the progenitor germ cells and along to the next generation (Poulton et al., 2010). mtDNA deletions are not passed down to the offspring (Greaves et al., 2012). However, point mutations in tRNA, rRNA or protein-coding genes are not selected against in this process, and can be passed maternally to the offspring (Greaves et al., 2012).

1.3.7 mtDNA diseases

Several human diseases are caused by mtDNA mutations. mtDNA deletions, duplications or rearrangements can also be pathogenic. Leber’s hereditary optic neuropathy (LHON) is one of the most common mtDNA-related disorders, and causes loss of vision predominantly in men (Schon et al., 2012). LHON is most often caused by mutations in genes that encode complex I proteins, such as ND4, ND1, and ND6. While these mutations are found in virtually all cell types, retinal ganglion cells seem to be selectively affected by this disease (Hudson et al., 2007).

Leigh’s disease is more commonly due to mutations in nuclear-encoded mitochondrial genes, but can also occur due to mtDNA mutations. The affected ETC complexes tend to be complex I and assembly factors of complex IV. The clinical features of Leigh’s disease range from developmental delays, respiratory abnormalities, ataxia, dystonia, and potentially early death. The tissues most affected are the basal ganglia and the brainstem (Santorelli et al., 1993).

Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) affects several different organ systems, including the brain, muscles, and the endocrine system. Over a dozen mutations are associated with MELAS, mostly in tRNA and protein coding genes. The most common mutation is to the tRNA^{Leu(UUR)} (Kaufmann et al., 2011). This disease is often fatal in childhood or early adulthood. Affected individuals have stroke-like episodes that are caused by infarcts in the temporal and occipital lobes.
Myoclonus epilepsy and ragged red fibers (MERRF) leads to cervical lipomas, myoclonus, and epilepsy (Chong et al., 2003). The muscle ragged red fibres of patients are negative for cytochrome c oxidase activity. MERRF is caused almost exclusively by mutations to tRNA\textsuperscript{Lys}. As with MELAS, mutations to mitochondrial tRNAs cause impairment of post-transcriptional modification of tRNAs, which affects translational efficiency of mitochondrial transcripts (Suzuki and Nagao, 2011).

Neuropathy, ataxia and retinitis pigmentosa (NARP) and maternally inherited Leigh’s syndrome (MILS) are two separate diseases that arise from mutations in the same nucleotide (m8993T). The onset, severity, and presentation of these diseases depends on mutation load. A mutation load of approximately 70% will present as NARP, while >90% will lead to MILS (Holt et al., 1990). This exemplifies the concept of mitochondrial heteroplasmy, in which each cell contains multiple copies of mtDNA, and disease only occurs once a certain threshold of mutation load is reached. In this example, there exist two separate thresholds where two separate diseases can arise from the same mtDNA mutation (Schon et al., 2012). Reversible respiratory chain deficiency is a rare disease, where a mutation in tRNA\textsuperscript{Glu}\textsuperscript{Glu} causes a deficiency in oxidative phosphorylation. Infants affected with this disorder are severely ill, but can recover full mitochondrial function spontaneously within two years if they are sustained vigorously during the perinatal period (Schon et al., 2012).

Kearns-Sayre syndrome (KSS) is caused not by mtDNA mutations, but by sporadic deletions of mtDNA (Kearns and Sayre, 1958). KSS has an onset before the age of 20, and causes multiple problems, including deafness, short stature, ophthalmoplegia, ptosis, pigmentary retinopathy, heart block, high levels of protein in the cerebrospinal fluid, and neurological symptoms such as cognitive impairment and cerebellar ataxia (Greaves et al., 2012; Schon et al., 2012). Patients have mtDNA deletions in all tissues examined, which suggests that the deletion occurred in the germ line or soon after fertilization. The deletion likely occurred randomly in a single mtDNA molecule that was repeatedly amplified during development (Schon et al., 2012). Chronic progressive external ophthalmoplegia (CPEO or PEO) leads to progressive paralysis of the eye muscles, myopathy, ophthalmoplegia and ptosis. CPEO is caused by mtDNA deletions that occur only in muscle tissues, suggesting that these deletion events occurred after fertilization in the muscle lineage of the mesoderm (Moraes et al., 1989). Pearson’s syndrome is a severe disorder that presents in early childhood (Pearson et al., 1979). It leads to anemia and exocrine
pancreas dysfunction, and is caused by mtDNA deletions that are abundant in haematopoetic
cells. In patients with Pearson’s syndrome who survive anemia, the load of mtDNA deletion will
decrease in blood, but increase in terminally differentiated tissues such as muscle and brain.
Interestingly, these patients will often develop KSS later in life (Schon et al., 2012).

Secondary mtDNA-related diseases are those caused not by direct damage or alterations to
the mtDNA itself. Rather the mtDNA is affected indirectly through mutations in nuclear encoded
genes required for proper replication and maintenance of mtDNA (Schon et al., 2012).
Depending on the nuclear gene involved, secondary mtDNA-related diseases can cause mtDNA
point mutations, large scale deletions, or depletion of the mtDNA pool. Vulnerable processes
include the maintenance of mitochondrial nucleotide pools, nucleotide transport, mtDNA
replication, mitochondrial RNA transcription, and mitochondrial dynamics (Greaves et al.,
2012). Examples of mitochondrial diseases caused by nuclear DNA mutations include
neurogastrointestinal encephalomyopathy (MNGLE), Alper’s syndrome, and CPEO (Schon et
al., 2012).

1.3.8 mtDNA in the mitochondrial theory of aging

Progressive decrease in oxidative phosphorylation frequently accompanies aging. The
observed mitochondrial defects in aging tissues strongly resemble the pathology of some mtDNA
diseases, with the only difference being that in mtDNA disease, a much higher proportion of
cells are affected (Greaves et al., 2012). This observation has lead to the mitochondrial theory of
aging, which states that during life, a progressive accumulation of somatic mtDNA mutations
leads to a decline in mitochondrial function which is a driving force in the aging process
(Greaves et al., 2012). It was initially proposed that the accumulation of mtDNA mutations
impairs oxidative phosphorylation and increase ROS generation, which in turn causes more
mtDNA mutations in a vicious cycle of exponentially increasing ROS generation of mtDNA
mutations. However, it is more likely that instead of the vicious cycle mechanism, mtDNA
mutations lead to impaired mitochondrial activity and induce cell death by apoptosis (Greaves et
al., 2012).

The type of mtDNA lesions that accumulate with age seems to be highly tissue-specific. In
aging post-mitotic tissues such as brain and muscle, mtDNA deletions are predominant, while
few mtDNA point mutations are observed (Greaves et al., 2012). In mitotic tissues, mtDNA
point mutations are much more common, while mtDNA deletions are relatively infrequent (Greaves et al., 2012).

1.4 DNA polymerase gamma (Polg)

DNA polymerase gamma (Polg) is the only DNA polymerase to replicate mtDNA. In addition to its polymerase activity, Polg also has 3’ to 5’ exonuclease activity and 5’ dRPlyase activity. The human enzyme is composed of a catalytic subunit of 140 kDa and a homodimeric form of its accessory subunit of 55 kDa that allows for DNA binding. The catalytic subunit is the location of polymerase, exonuclease, and lyase activity, and is highly conserved across species. The 3’ to 5’ exonuclease domain comprises the first 418 amino acids, and is encoded by three conserved motifs (I, II, and III). The DNA polymerase domain comprises amino acids 756-1239, and is encoded by three conserved motifs (A, B, and C) (Chan and Copeland, 2009; Hudson and Chinnery, 2006).

Polg also has two important functions relevant to BER. In addition to polymerase activity, the 5’-deoxyribosephosphate lyase (dRPase) activity of Polg may be rate limiting for BER. This suggests that Polg may be an important factor in controlling the BER pathway (Stuart and Brown, 2006). Polg expression has been shown to be strongly up-regulated by oxidative stress. Doxorubicin, a cancer drug that produces superoxide by interacting with complex I, increases Polg expression, as does gamma-irradiation and LPS, which generates oxidative stress through the necrosis factor (TNF)-stimulated pathway (Stuart and Brown, 2006). Since it is not clear whether repair and replication of mtDNA by Polg can be separately regulated, this upregulation of mtDNA BER repair in response to oxidative stress may also lead to increased mtDNA replication (Stuart and Brown, 2006).

Polymerase fidelity is the ability to identify the incorporation of the wrong base pair and correct the error (Saneto et al., 2010). Polg has a high base-substitution fidelity of $<2.0 \times 10^{-6}$ errors per nucleotide (Hudson and Chinnery, 2006). Active site mutations in three exonuclease motifs of the yeast Polg homolog generated a mutant polymerase with a several hundred-fold increase in the frequency of de novo mutations (Graziewicz et al., 2006). Similar studies in human Polg substituted Asp198 and Glu200 with alanine. In this model 3’ to 5’ exonuclease activity in vitro was eliminated, and resulting in a 20-fold increase in de novo mutations (Graziewicz et al., 2006; Kujoth et al., 2005; Trifunovic et al., 2004).
1.4.1 Diseases involving mutations in \textit{POLG}

Approximately 150 pathogenic mutations have been identified in the \textit{POLG} gene, and are found in the polymerase and the exonuclease domains as well as the linker region between the two (Figure 5). Pathogenic mutations include missense, nonsense mutations, and splice site variants (Hudson and Chinnery, 2006). Most dominant mutations are present in the polymerase domain, while recessive mutations and single nucleotide polymorphisms are found throughout the gene, including the exonuclease domain (Hudson and Chinnery, 2006). These mutations cause a wide range of disease phenotypes mainly in the central nervous system, ranging from migraines to epilepsy to Parkinsonism (Neustadt and Pieczenik, 2008). \textit{Polg} mutations are associated with diseases such as: progressive external ophthalmoplegia, Alpers syndrome, infantile hepatocerebral syndromes, ataxia-neuropathy syndrome, Charcot-Marie-Tooth disease, idiopathic parkinsonism, nucleoside reverse-transcriptase inhibitor (NRTI) toxicity, and valproic acid hepatotoxicity (Chan and Copeland, 2009). Non-pathogenic but functional genetic variants of \textit{POLG} have been shown to be present in approximately 1 in 200 individuals within the general population (Hudson and Chinnery, 2006).
Figure 5. DNA Polymerase Gamma mutations. Pathogenic mutations in the human POLG gene have been identified in all the domains of the enzyme, including the exonuclease domain, the linker region, and the polymerase domain. Known pathogenicities caused by POLG mutations include progressive external ophthalmoplegia, Alpers syndrome, ataxia neuropathy syndrome, male infertility, Nucleotide reverse transcriptase inhibitor toxicity, Charcot-Marie Tooth disease, and Leigh’s syndrome. (Chan 2009).
1.4.2 Polg\(^{m/m}\) Mouse Model

A homozygous knock-in mouse model of proof-reading deficient Polg has been generated by the substitution of a critical aspartate amino acid with alanine (D257A) in the second exonuclease domain of the PolgA gene (Graziewicz et al., 2006; Kujoth et al., 2005; Trifunovic et al., 2004). This mutation caused a profound decrease in exonuclease activity without affecting polymerase activity (Trifunovic, 2006). These Polg\(^{m/m}\) mice, also known as the “mtDNA mutator mice” have been independently generated by two separate groups (Kujoth et al., 2005; Trifunovic et al., 2004). Both models were generated by injection of 129R1 stem cells containing targeting vector into blastocyst of C57Bl/6 mice. Chimeras with germline transmission were backcrossed in a C57Bl/6 mouse line (Kujoth et al., 2005; Trifunovic et al., 2004).

The knock-in mouse developed by Kujoth et al. presented with a premature aging phenotype beginning at approximately 9 months of age, consisting of hair loss, graying and kyphosis, thymic involution, testicular atrophy, infertility, loss of bone mass, loss of skeletal muscle, progressive decrease in circulating red blood cells, and age-related hearing loss. The median survival was 416 days and the maximal survival of 460 days (Kujoth et al., 2005; Vermulst et al., 2007). The knock-in mouse developed by Trifunovic et al. also presented with a premature aging phenotype, although the observed symptoms were slightly different. Presentation of symptoms began sooner for the Trifunovic et al. model, at 25 weeks of age, and consisted of osteoporosis, kyphosis, reduced bone mineral content, reduced body size, reduced hair density, alopecia, lower hemoglobin in peripheral blood, enlargement of the heart, reduced fertility, and testicular atrophy. The median lifespan was 336 days and the maximal lifespan was 427 days (Trifunovic et al., 2005). Neurological defects have also been identified in both mouse models, with progressive loss of spinal ganglion neurons in the Trifunovic et al. model (Niu et al., 2007), and increased TUNEL and caspase-3 staining in spinal ganglion neurons in Polg\(^{m/m}\) mice from both mouse models (Niu et al., 2007; Someya et al., 2008).

At the molecular level, both models reported a 3 to 5-fold increase in mtDNA point mutations compared to wild type as measured through a cloning and sequencing method (Kujoth et al., 2005; Trifunovic et al., 2004). Trifunovic et al. also observed an increased number of linear deletions in mtDNA, with mtDNA deletions potentially accounting for 25-30% of mtDNA molecules in this model (Trifunovic et al., 2005). Additional observations of decreased mtDNA
integrity were also made in the Kujoth et al. model, with increased mtDNA replication pausing and chromosomal breakage identified in the mtDNA of Polg\textsuperscript{m/m} mice (Bailey et al., 2009). While levels of mtDNA deletions did not change over time, the random point mutations to mtDNA accumulate in a linear manner, and were not associated with an increase of ROS generation or an increased sensitivity to cell death by ROS generation (Trifunovic et al., 2005). Kujoth et al. also observed no increase in oxidative stress markers in Polg\textsuperscript{m/m} mice relative to wild type (Kujoth et al., 2005). The accumulation of mtDNA point mutations was similar among different tissues, and much of the accumulation occurred early in embryonic development, being already substantial as early as mid-gestation (Embryonic day 13.5) (Trifunovic, 2006). These observations were especially interesting because they contradicted the ROS theory of aging. This theory stated that respiratory chain dysfunction would increase ROS production, which would in turn cause mtDNA mutations, leading to additional respiratory chain dysfunction and ultimately a vicious cycle of increasing ROS production, mtDNA mutations, and mitochondrial dysfunction (Trifunovic et al., 2005). The lack of ROS increase in the Polg\textsuperscript{m/m} model, coupled with linear (not exponential) mtDNA accumulation suggest that instead of the vicious cycle of ever increasing mitochondrial damage, respiratory chain dysfunction may instead induce a bioenergetic deficit that lowers the threshold for cell death and induces apoptosis (Trifunovic, 2006).

There is evidence to suggest that increased apoptosis occurs in various tissues of the Polg\textsuperscript{m/m} mice. In the Kujoth et al. model, there are reports of increased TUNEL staining in the thymus, intestine, and testis (Kujoth et al., 2005), increased caspase-3 and caspase-9 activity in skeletal muscle (Hiona et al., 2010), and increased nuclear DNA fragmentation in skeletal muscle, heart, and liver of Polg\textsuperscript{m/m} mice relative to wild type (Safdar et al., 2011). Increased rates of apoptosis and cell death in physiologically crucial cell populations such as somatic stem cells has been proposed as a cellular cause of the premature aging observed in these animals (Trifunovic et al., 2005). Evidence for stem cell involvement in the premature aging phenotype comes from observations that the Trifunovic et al. mouse model shows dysfunctional neural stem cells with decreased self-renewal, along with hematopoietic progenitors that demonstrate abnormal lineage differentiation leading to anemia (Ahlgqvist et al., 2012). Stem cell defects have also been identified in the Kujoth et al. model, with disrupted stem/progenitor cell cycling in the small intestine of Polg\textsuperscript{m/m} mice (Fox et al., 2012).
Both groups have also identified severe mitochondrial dysfunction at the biochemical level in the Polg\textsuperscript{m/m} mice. Respiratory chain dysfunction, decreased activity of ETC complex I, III, and IV, and decreased mitochondrial membrane potential have all been reported in the Kujoth et al. model (Hiona et al., 2010). Similarly, the Trifunovic et al. model also presents with severe respiratory chain dysfunction (Trifunovic et al., 2005). The main reason for the respiratory dysfunction in the Polg\textsuperscript{m/m} model seems to be random point mutations within protein-coding regions of mtDNA that lead to synthesis of respiratory chain subunits with amino acid substitutions. As a result of these residue substitutions, mtDNA-encoded proteins are incorrectly folded and fail to form stable complexes with their nuclear-encoded mitochondrial protein counterparts, leading to instability of ETC complexes. The result is a high turnover of mitochondrial proteins, reduced amounts of assembled complexes I, III, and IV, and a functional deficiency in these complexes that leads to impaired mitochondrial respiration (Edgar et al., 2009). The ultimate molecular cause of this biochemical phenotype in the Polg\textsuperscript{m/m} model is a topic of current debate, with some groups supporting mtDNA point mutations while others suggest that either linear or circular mtDNA deletions may be the underlying reason for the premature aging phenotype (Edgar and Trifunovic, 2009).

Mouse embryonic fibroblasts (MEFs) have been generated from Polg\textsuperscript{m/m} mice in both models. Kujoth et al. observed no differences in replicative senescence between wild type and Polg\textsuperscript{m/m} primary MEFs, with both genotypes undergoing senescence under conditions of routine culture oxygen (20% O\textsubscript{2}), but continuing to proliferate under physiological oxygen levels (2% O\textsubscript{2}) (Kujoth et al., 2005). By contrast, genotype differences were observed under both conditions in primary MEFs from the Trifunovic et al. model. Polg\textsuperscript{m/m} primary MEFs proliferated slower than wild type under physiological oxygen levels (3% O\textsubscript{2}). However, under conditions of routine culture oxygen (20% O\textsubscript{2}) wild type MEFs exhibited senescence and ceased to proliferate, while Polg\textsuperscript{m/m} MEFs underwent spontaneous immortalization and continued to proliferate (Kukat et al., 2011). The proposed interpretation for this paradoxical observation of senescence resistance in Polg\textsuperscript{m/m} primary MEFs was that the decreased oxidative phosphorylation in the Polg\textsuperscript{m/m} model causes an increase in aerobic glycolysis, which increases resistance to oxidative stress. Since oxidative stress is a trigger for senescence, Polg\textsuperscript{m/m} primary MEFs will also have increased resistance to senescence. However, decreased oxidative phosphorylation could also lead to
decreased ATP production, limiting resources available for DNA repair, and leading to spontaneous mutations that induce immortalization (Kukat et al., 2011).

While the premature aging and mitochondrial dysfunction phenotype of the Polg\textsuperscript{m/m} model is very severe it can still be modulated. Endurance exercise has been successfully employed as a rescue strategy against several Polg\textsuperscript{m/m} phenotypes (Safdar et al., 2011). Starting at 3 months of age, Polg\textsuperscript{m/m} mice were subjected to 5 months of forced endurance exercise (15 meters/min for 45 min, 3 times/week). At 8 months of age, Polg\textsuperscript{m/m} mice subjected to endurance exercise no longer presented any signs of premature aging phenotype, and were visually indistinguishable from wild type littermate controls (Safdar et al., 2011). Endurance exercise caused complete attenuation of the decreased lifespan and the decreased body weight normally observed as part of the Polg\textsuperscript{m/m} phenotype (Safdar et al., 2011). At the molecular level, endurance exercise completely rescued the age-related decline in mtDNA copy number, and was able to attenuate the increased mtDNA point mutation load in Polg\textsuperscript{m/m} mice, preventing it from reaching a critical threshold above which premature aging occurs (Safdar et al., 2011). It has been proposed that endurance exercise rescues the premature aging phenotype in Polg\textsuperscript{m/m} mice through a mechanism of selective mitochondrial biogenesis, where healthy mitochondria are targeted for biogenesis via modulation of mitochondrial fusion/fission, while mitochondria with a high mtDNA mutation load are targeted for autophagy (Safdar et al., 2011).

1.5 Mitochondrial stress responses

1.5.1 Mitochondrial fusion/fission

Mitochondria are highly dynamic organelles with morphology and distribution at constant flux, regulated by mitochondrial fission and fusion processes (Figure 6) (McBride et al., 2006). These opposing processes are balanced in order to maintain the overall shape and morphology of mitochondria (Chan, 2006b). Mitochondrial fusion and fission are crucial for normal cell function and mammalian development, and are involved in human diseases. Through fusion and fission, mitochondria are able to interact with each other and function not as a population of autonomous organelles, but rather as a network better able to respond to both endogenous and exogenous stresses (Chan, 2006b).
Figure 6. Mitochondrial Dynamics. Mitochondrial fusion and fission. Mitofusins 1/2 are responsible for fusion of the outer mitochondrial membrane and OPA1 promotes fusion of the inner mitochondrial membrane. Drp1 is responsible for fission (Chen and Chan 2005).
Mitochondrial morphology may exist in a variety of forms, ranging from long interconnected networks to individual small spheres. These mitochondrial tubules are able to migrate through the cell along microtubule tracks. When two mitochondria encounter each other they may fuse together. This process is initiated by the fusion of the mitochondrial outer membrane through the action of mitofusin (Mfn) proteins that are large conserved GTPases located in the outer membrane. In mammals there are two mitofusin homologs, Mfn1 and Mfn2. The absence of both mitofusins causes a complete stop to mitochondrial fusion, followed by mitochondrial fragmentation and impaired mitochondrial function (Chan, 2006b). Mfn1 and Mfn2 have very similar activity and seem to perform similar function in mitochondrial fusion. Overexpression of either mitofusin can rescue Mfn-null cells (Chen et al., 2003). However, in some cell types one mitofusin can play a more active role than the other. Fibroblasts null for Mfn1 are more severely affected by mitochondrial fragmentation than those null for Mfn2. Both Mfn proteins are located in the mitochondrial outer membrane with most of the protein facing towards the cytosol (Chan, 2006b). In order to initiate mitochondrial fusion, homotypic interactions between mitofusins mediated by hydrophobic heptad repeat regions (HR) cause a tethering together of the opposing mitochondrial outer membranes (Chan, 2006b). Another important protein in the mitochondrial fusion machinery is optic atrophy 1 (OPA1) (Tolkovsky, 2009), a GTPase located in the inner membrane space that is crucial for fusion, because it promotes inner mitochondrial membrane fusion (Green and Van Houten, 2011; Youle and van der Bliek, 2012), and also plays a role in maintaining proper cristae structure (Griparic et al., 2004). Since fusion involves the mixing of matrix contents, it requires that both the outer and inner membrane fuse in coordination while remaining mechanistically distinct. Hydrogen/potassium ionophores that decrease the mitochondrial membrane potential can disrupt mitochondrial fusion by preventing fusion of the inner membrane. This suggests that following outer membrane fusion progression to fusion of the inner membrane requires the electrical gradient across that membrane to remain intact (Chan, 2006b).

Mitochondrial fission is the opposing process, where a single mitochondrion is cleaved into separate organelles. This is also the process by which biogenesis, or budding of new mitochondria occur (Green and Van Houten, 2011). A crucial protein in mitochondrial fission is dynamin-related protein 1 (Drp1). Drp1 is mainly located in the cytosol, but a subpool is localized to spots in the mitochondrial tubules which mark future sites of fission (Smirnova et
In order to initiate mitochondrial fission, Drp1 is recruited to the mitochondria by Mid49, Mid51, Fis1 and Mff (Youle and van der Bliek, 2012). Fis1 and Mff can be found in protein complexes of different sizes and form different machineries for mitochondrial fission (Chen and Chan, 2009). This indicates that there are different mechanisms of mitochondrial fission, and while Drp1 recruitment is a common step in many of these pathways, the downstream process may be quite different depending on which protein machinery initiated the process. Following recruitment to the mitochondria, Drp1 uses GTP hydrolysis to drive constriction at fission sites of the mitochondrial tubule and mediate membrane fission (Chan, 2006b; Green and Van Houten, 2011). If Drp1 is inhibited either by expression of a dominant-negative mutant, or by RNAi, mitochondrial fission becomes impaired, causing length and interconnectivity of mitochondrial tubule networks to greatly increase (Lee et al., 2004; Smirnova et al., 2001). It has been noted that even in highly fragmented mitochondria, fission generally results in mitochondria that contain at least at least one nucleoid. This suggests that fission sites may be linked to nucleoid positioning (Chan, 2006b). Mutations in Drp1 can be especially detrimental to neurons because they reduce the number of mitochondria capable of reaching nerve terminals, which reduces the amount of ATP available at these terminals, and can lead to structural and functional defects at the synapse (Chan, 2006b). MEFs with impaired Drp1 activity are resistant to mitochondrial fragmentation caused by the ionophore carbonyl cyanide m-chlorophenylhydrazone that destroys mitochondrial membrane potential, but these MEFs are not resistant to fragmentation caused by etoposide-induced apoptosis. The latter observation indicates that Drp1-independent mechanisms of mitochondrial fission also occur (Chan, 2012).

While Drp1 appears to be crucial for fission initiation, there are observations to suggest that other important players remain undiscovered. Fission sites in the mitochondria are often associated with the endoplasmic reticulum even before Drp1 recruitment. This indicates the existence of an early mechanism to determine fission sites that is not yet well understood (Chan, 2012). Another unexplained observation is separation of the mitochondrial matrix in the absence of outer mitochondrial membrane fission. The only explanation for this is a yet undiscovered mechanism of inner membrane fission independent of Drp1 (Chan, 2012).

The overall morphology of a mitochondrial population depends on the balance between the opposing actions of fusion and fission. Increased fission will lead to overall fragmentation, while increased fusion will lead to elongation. Both these extremes can be detrimental to mitochondria.
function. Extremely long mitochondria can entangle and collapse (Chan, 2012). It is possible to counteract the negative effects of one process by manipulating the other, either pharmacologically or genetically (Chen et al., 2003; Szabadkai et al., 2004). Through constant cycles of fusion and fission, the boundaries of each individual mitochondrion are being constantly redefined, and this allows defects in dysfunctional mitochondria to be complemented by fusion with healthy mitochondria (Youle and van der Bliek, 2012). Wild type mtDNA, RNA or proteins can compensate for defects in mitochondria with mutant mtDNA as long as the total mutation load in the cell remains below a certain threshold (80 to 90%) (Youle and van der Bliek, 2012). Fusion between mitochondria with mutations in different genes can also cause a rescue through cross-complementation. Fusion can also relieve the effects of toxicity by the exchange of proteins and lipids among several mitochondria (Youle and van der Bliek, 2012).

Overall, mitochondrial fusion and fission serve as a stress response mechanism. Fusion helps sustain healthy mitochondrial function by mixing mitochondrial components across many organelles as a form of complementation (Chan, 2006b; Green and Van Houten, 2011). Fission has a quality control role as it allows for the removal of damaged mitochondria (Youle and van der Bliek, 2012). Mitochondrial fusion plays a protective role in cells with mtDNA mutations by allowing functional complementation of mtDNA gene products from healthy mtDNA. It has been shown that mitofusin over-expression can reduce apoptosis (Chan, 2006b). By contrast, mitochondrial fission is considered to have pro-apoptotic effects, as mitochondrial fission and fragmentation often occur during apoptotic cell death (Chan, 2006b), and the inhibition of fission results in reduced cellular sensitivity to apoptosis (Frank, 2006). Mitochondrial fusion and fission are also responsive to changes in metabolism. Cells increase fusion when forced to rely on oxidative phosphorylation for energy production (in the absence of glucose as a carbon source) (Youle and van der Bliek, 2012).

Another important aspect of mitochondrial dynamics that works together with fusion and fission is mitochondrial motility (Guo et al., 2005). This is the movement of mitochondria through the cell along microtubule tracks, driven by the kinestin 1 motor that interacts with the mitochondrial outer proteins Miro1 and Miro2 (Chen and Chan, 2009). This process is very important in all cells, but is especially crucial in highly polarized, branched, or long cells such as neurons (Guo et al., 2005). Proper motility is highly dependent on proper fusion and fission. In the absence of proper fission, long interconnected mitochondria tubules can form large tangles
that block efficient movement into small cellular extensions such as axons and dendrites. It is less clear how improper fusion interferes with motility, but it has been observed that small fragmented mitochondria lack directed movement and instead simply move over the same general area (Chen and Chan, 2009).

### 1.5.2 Mitochondrial autophagy

Mitochondrial fusion and fission allow mitochondria to compensate for defects by sharing various components. However, if damage becomes too great and reaches a certain threshold, these damaged mitochondria must be eliminated by autophagy. Mitophagy is the active elimination of defective mitochondria by autophagy (Figure 7A) (Youle and van der Bliek, 2012). Following fission, a stress test occurs where one daughter mitochondrion becomes briefly hyperpolarized while the other becomes hypopolarized. If the latter becomes completely depolarized after fission, it will be targeted for mitophagy and be eliminated. Mitophagy can be separated into two categories: stress-induced mitophagy and maintenance mitophagy (Goldman et al., 2010). Stress-induced mitophagy involves the rapid degradation of a large number of healthy mitochondria and is required in order to adapt to different metabolic conditions. This form of mitophagy often occurs in response to nutrient starvation (Bess et al., 2012). This process is non-selective, causing degradation in bulk so the materials can be re-used elsewhere in the cell (Goldman et al., 2010). Maintenance mitophagy is considered to be a more specific and more selective process, where highly damaged mitochondria are targeted for destruction. This process allows the cell to reduce cellular exposure to ROS (generated by dysfunctional mitochondria), and to preserve cellular energy by eliminating ineffective organelles.
Figure 7. Mitochondrial Autophagy. (A) Overview of mitochondrial autophagy. Autophagy is initiated by a cascade of activation of autophagy proteins (Atg proteins). This activation cascade leads to the lipidation of LC3 (conversion of LC3-I to LC3-II) and the generation of a membrane vesicle called the phagophore. The phagophore elongates and engulfs organelles targeted for degradation. Phagophore elongation also requires the assembly of Beclin-1 and p62. Mitochondria can be targeted for autophagy by polyubiquitination. Once the phagophore fully engulfs its target organelles it matures into an autophagosome. The autophagosome fuses with lysosomes to become an autophagolysosome and degrade its contents (Gump and Thorburn 2011). (B) Targeted mitochondrial autophagy. PINK1 constantly localizes to the mitochondria but becomes degraded by protease activity. Damaged mitochondria (defined as depolarized mitochondria) cannot degrade PINK1, which leads to its accumulation. PINK1 recruits Parkin to the outer mitochondrial membrane, and Parkin ubiquinates mitochondrial proteins such as Mfn1/2, which has an added effect of preventing mitochondrial fusion. Polyubiquitination of mitochondrial outer membrane proteins is a recognized by phagophore proteins as a signal for mitochondrial degradation (Youle and Narendra 2011). (C) mTOR regulation of autophagy, and rapamycin inhibition of mTORC1. mTOR signalling functions downstream of the phosphatidylinositol 3-kinase (PI3K) pathway. The kinase Akt phosphorylates TSC2, inhibit the activity of the TSC1/2 heterodimer. TSC1/2 inhibits Rheb, which activates mTORC1. mTORC1 suppresses autophagy by interacting with the ULK1-Atg13-FIP200 complex and phosphorylating ULK1 and Atg13, which inhibits the ULK1-Atg13-FIP200 complex and prevents autophagy. Rapamycin binds to the immunophilin FKBP12, and the rapamycin-FKBP12 complex inhibits mTOR kinase activity by preventing mTORC1 from binding to its accessory subunit Raptor. In the presence of rapamycin, mTORC1 dissociates from the ULK1-Atg13-FIP200 complex, which allows ULK1 to phosphorylate itself, Atg13 and FIP200, and trigger autophagy (Renna, et al., 2010).
The quality control mechanism that targets depolarized mitochondria for mitophagy involves the proteins Parkin and PINK1 (Figure 7B). In healthy mitochondria, PINK1 is imported to the inner mitochondrial membrane and degraded by the rhomboid protease PARL. However, when mitochondria become sufficiently depolarized, PINK1 import ceases and it accumulates in the outer mitochondrial membrane. Parkin is normally found in the cytosol, but PINK1 accumulation serves as a signal of mitochondrial damage and recruits Parkin to ubiquitinate outer mitochondrial proteins and target that particular mitochondrion for autophagic elimination (Narendra and Youle, 2011; Youle and Narendra, 2011). Polyubiquitination of mitochondrial outer membrane proteins is recognized by phagophore proteins as a signal for mitochondrial degradation (Narendra and Youle, 2011; Youle and Narendra, 2011). The known Parkin substrates are VDAC1, Mfn1/2, and MIRO. Therefore, PINK1 and Parkin add selectivity to mitophagy and ensures that only dysfunctional mitochondria are eliminated by autophagosomes (Kubli and Gustafsson, 2012; Youle and van der Bliek, 2012). By ubiquitinating Mfn1/2, Parkin also ensures that mitochondria targeted for degradation will not undergo mitochondrial fusion and effectively disappear into the mitochondrial network. It is interesting to note that both Parkin and PINK1 have been implicated as genes causing some hereditary forms of Parkinson’s disease (Chen and Chan, 2009). While knockdown mouse models of these genes fail to present with Parkinson’s symptoms, they do have some mild defects and sensitivity towards oxidative stress (Chen and Chan, 2009). Human dopaminergic neurons lacking PINK1 have reduced viability and abnormal mitochondrial morphology (Chen and Chan, 2009).

In order to initiate autophagy, an activation cascade of autophagy proteins (Atg proteins) generates a membrane vesicle known as a phagophore (Gump and Thorburn, 2011). After its initial formation, elongation of the membrane is mediated by microtubule-associated protein 1 light chain 3 (LC3), which becomes lipidated by Atg proteins and accumulates in the phagophore. The phagophore elongates until it is able to fuse around the degradation target to form a double-membrane structure called an autophagosome (Gump and Thorburn, 2011). The autophagosome fuses with a lysosome so that its contents can be degraded by lysosomal enzymes (Kubli and Gustafsson, 2012). The initial step in which the phagophore engulfs its target organelle is mediated by p62/SQSTM1 (p62). p62 is an autophagy adaptor protein that binds both to ubiquitinated proteins in the targeted mitochondria, and to LC3 in the phagophore,
thus bringing these two structures together (Kubli and Gustafsson, 2012). Another important protein in the early initiation of mitophagy is AMBRA1, which interacts with Parkin following membrane depolarization and helps initiate the formation of the phagophore at the mitochondria (Kubli and Gustafsson, 2012).

In certain cases mitochondria may be so severely damaged that allowing it to fuse with healthy mitochondria would contaminate the mitochondrial network. To prevent fusion of severely damaged mitochondria, the protease OMA1 becomes activated by severely low membrane potential and low ATP levels and deactivates OPA1, preventing fusion of the inner mitochondrial membrane (Youle and van der Bliek, 2012). Fusion is also prevented by the recruitment of PINK1 and Parkin to the outer membrane of depolarized mitochondria. Parkin ubiquitinates the mitofusins Mfn1 and Mfn2, which causes them to be extracted from the outer membrane, and degraded by proteasomes. Through these mechanisms, severely damaged mitochondria lose both their inner and outer membrane fusion machinery, and are prevented from joining the healthy mitochondrial network before being eliminated by mitophagy (Youle and van der Bliek, 2012). Another way in which the cell separates dysfunctional mitochondria intended for autophagy from the rest of the mitochondrial network is by inhibiting its motility. MIRO, one of the substrates for Parkin ubiquitination, plays an important role in motility by connecting mitochondria to cellular microtubule tracks. Since fusion occurs as mitochondria come in contact with each other while moving along these microtubule tracks (Chan, 2006b), disruption of mobility will further prevent these dysfunctional mitochondria from interacting with the rest of the mitochondrial pool (Kubli and Gustafsson, 2012).

The main autophagy-regulating pathway in the cell is the mTOR (mammalian or mechanistic target of rapamycin) pathway (Figure 7C). Activity of mTOR inhibits autophagy. mTORC1 suppresses autophagy by interacting with the ULK1-Atg13-FIP200 complex and phosphorylating ULK1 and Atg13, which inhibits the ULK1-Atg13-FIP200 complex and prevents autophagy (Renna et al., 2010). Rapamycin is an inhibitor of mTOR, and can enhance autophagy (Pan et al., 2009). Rapamycin binds to the immunophilin FKBP12, and the rapamycin-FKBP12 complex inhibits mTOR kinase activity by preventing mTORC1 from binding to its accessory subunit regulatory associated protein of mTOR (Raptor) (Oshiro et al., 2004). In the presence of rapamycin, mTORC1 dissociates from the ULK1-Atg13-FIP200 complex, which allows ULK1 to phosphorylate itself, Atg13 and FIP200, and trigger autophagy.
Cytochrome c release from the mitochondria to the cytosol can be initiated by a decrease in mitochondrial membrane potential, and activates caspases that induce apoptosis. By enhancing the degradation of dysfunctional mitochondria, rapamycin allows for these pro-apoptotic organelles to be degraded before cytochrome c release can occur (Pan et al., 2009).

### 1.5.3 Mitochondrial dynamics in neurons

Mitochondrial disease tends to disproportionately affect muscles and the central nervous system (CNS), which indicates that mitochondrial function is especially important for these tissues (Chen and Chan, 2006). The brain represents only 2% of the total body weight, but accounts for 20% of the total oxygen consumption (de Castro et al., 2010). Neurons are very metabolically active, with high energy demands far away from the cell body (Chen and Chan, 2009). Neurons also require a high concentration of mitochondria at the nerve terminal in order for synapses to function properly (Chen and Chan, 2006). There appears to be a high degree of organization of mitochondrial localization within neurons, and for this reason, neurons are especially susceptible to defects in the dynamic properties of mitochondria, including fusion, fission, and mobility (Chen and Chan, 2009). Neuronal mitochondria must be highly mobile, able to move at speeds of 2-3 µm per second. These organelles are often generated at cellular locations different from their ultimate site of action, and some human neurons may be over one meter in length. Therefore, mitochondria may need to travel considerable distances and require functional mitochondrial dynamics machinery (Dykens and Will, 2008). As an example, Charcot-Marie-Tooth disease type 2A is a hereditary peripheral neuropathy caused mainly by Mfn2 mutations, resulting in a primary neuronal defect that affects sensory and motor neurons and leads to motor impairment and muscle atrophy (Chen and Chan, 2006). Dominant optic atrophy is another neurological disease caused by mutations in an important mitochondrial dynamics protein. This disease is often caused by mutations in OPA1, and leads to loss of retinal ganglion cells that give rise to the fibers of the optic nerves (Chen and Chan, 2006).

The sub-cellular organization of mitochondria within neurons is not random, but appears to be highly organized. Mitochondria are most abundant in the nerve terminal (Chen and Chan, 2006). Mutations to Drp1 or other impairments of mitochondrial fission results in elongated mitochondria with compromised mobility, that are mostly absent from the nerve terminal (Chen and Chan, 2006). Mitochondria are also abundant in the axon in sites where action potentials...
originate. However, few mitochondria are found in the myelinated portions of the axon, and are present mainly in the non-myelinated areas of the axon such as the nodes of Ranvier (Chen and Chan, 2006). This observation indicates that mitochondrial localization is tightly controlled, that they are located near the main sites of ATP demand within the neuron. This also indicates that the maintenance of neuronal transmissions requires more ATP in non-myelinated areas of the axon (Chen and Chan, 2006).

Some types of neurons appear to be more seriously affected by mitochondrial dysfunction than others. Several characteristics that make neurons unable to tolerate mitochondrial defects seem to include high amounts of dendritic branching and long axon length (Chen et al., 2007). Purkinje cells have extensive dendritic branching, and mitochondrial clumps in dendritic banch junctions can impede passage of other important cellular components (Chen et al., 2007). The motor neurons affected in Charcot-Marie-Tooth disease type 2A are extremely long, and therefore susceptible to defects that impair mitochondrial mobility (Chen et al., 2007). Mitochondrial localization is also important in hippocampus neurons, where growth of dendritic spines requires the recruitment of mitochondria from nearby within the cell (Chen et al., 2007).

Neurodegenerative diseases such as Parkinson’s, Alzheimer’s, and Huntington’s disease appear to have a mitochondrial component, and in some models the modulation of mitochondrial dynamics can rescue disease phenotypes (Chen and Chan, 2009). Parkinson’s disease is the progressive loss of dopaminergic neurons from the substantia nigra, leading to tremor, rigidity, and bradykinesia. Reduced levels of complex I activity are associated with the disease (de Castro et al., 2010). Drugs that inhibit complex I of the ETC such as rotenone and MPTP can cause parkinson’s symptoms in humans and rodent models (Chen and Chan, 2009), showing a very strong relation between toxin-induced mitochondrial stress and Parkinson’s disease (de Castro et al., 2010). In hereditary cases of Parkinson’s, the genes for Parkin and PINK1 have been implicated, and both of these proteins are involved in the process of mitochondrial autophagy (Chen and Chan, 2009). Mutations in the HTRA2 gene, which encodes for a mitochondrial serine protease, is also associated with hereditary cases of Parkinson’s disease (de Castro et al., 2010).

Alzheimer’s disease is the most common of all neurodegenerative disorders and is caused by the accumulation of toxic intracellular neurofibrillary tangles and extracellular protein
aggregates called amyloid plaques that lead to death of neurons in the cerebral cortex and ultimately leads to cognitive dysfunction and memory loss (Chen and Chan, 2009). Abnormal mitochondrial structure and decreased complex IV activity has been observed in the brains of Alzheimer’s patients (de Castro et al., 2010). The amyloid plaques believed to play a major role in this disease can localize to the mitochondria, possibly contributing to their cytotoxicity (Chen and Chan, 2009). These amyloid aggregates can accumulate in the mitochondria prior to their extracellular accumulation. Their presence in the mitochondria has been shown to impair cytochrome c oxidase enzymatic activity, increase ROS generation, lead to lipid peroxidation, and deplete ATP (de Castro et al., 2010). Brain samples from Alzheimer’s patients also contained high levels of S-nitrosylated –Dpr1, which has increased fission activity compared to regular Drp1 (Chen and Chan, 2009).

In contrast to Parkinson’s and Alzheimer’s, the cause of Huntington’s disease is exclusively genetic (de Moura et al., 2010). It is an autosomal dominant disease due to a CAG trinucleotide extension in the Huntingtin gene that causes the progressive loss of striatal and cortical neurons ultimately leading to cognitive and motor impairment (Chen and Chan, 2009). Huntington’s disease is only one of nine human disorders known to be caused by a CAG trinucleotide extension, and the presence of the disease is determined by the number of repeats. No diseases occurs between 10 and 35 repeats, 35 to 39 repeats cause incomplete penetrance of the disease, and 40 or more repeats lead to complete penetrance of Huntington’s disease (de Castro et al., 2010; de Moura et al., 2010). There is some evidence to suggest that the mutant Huntingtin gene is associated with mitochondrial dysfunction. These include: observations of high lactate levels, decreased mitochondrial respiratory function and membrane potential, and decreased mitochondrial motility (Chen and Chan, 2009). HeLa cells expressing a Huntingtin mutant presented with fragmented mitochondria with reduced ATP and increased cell death, and these effects were rescued by expression of either Mfn2 or dominant-negative Drp1 (Chen and Chan, 2009). HTT is suggested to bind to Htt-associated protein 1 (HAP1) to form a complex that regulates movement of vesicle and mitochondrial transport (de Castro et al., 2010). Mutant Htt has abnormal interactions with motor proteins, which leads to impaired cellular trafficking. With impaired trafficking, mitochondria fail to reach the synapse, which causes synapse damage and impaired neuronal transmissions (de Castro et al., 2010). It is interesting to note that neurons lost in Huntington’s disease are medium spiny neurons with long projections, which would be
especially susceptible to any defect in mitochondrial motility that would hinder mitochondrial transport across long distances from the cell body to the synapse (de Castro et al., 2010).

### 1.5.4 Role of mitochondria in calcium homeostasis

Mitochondria can also protect the cell from apoptosis through its role in Ca\(^{2+}\) buffering and regulation of calcium homeostasis. Release of Ca\(^{2+}\) from the endoplasmic reticulum (ER) by inositol 1,4,5-triphosphate receptors can initiate apoptosis. Mitochondria and ER interact closely with each other. To protect the cell, much of the Ca\(^{2+}\) released from the ER by inositol 1,4,5-triphosphate signaling can be rapidly absorbed into the mitochondrial matrix in order to prevent apoptosis (Raffaello et al., 2012). Mitochondrial Ca\(^{2+}\) uptake is also advantageous to the cell because it increases the activity of Ca\(^{2+}\)-sensitive dehydrogenases of the TCA cycle, including pyruvate dehydrogenase, isocitrate dehydrogenase, and \(\alpha\)-ketoglutarate dehydrogenase, which together represent a rate-limiting step of the TCA cycle (Raffaello et al., 2012). Their enhanced activity results in increased NADPH generation, which will feed additional electrons into the ETC, and increase ATP generation (Raffaello et al., 2012). However, excess Ca\(^{2+}\) uptake by the mitochondria can open the mPTP and induce cell death by necrosis (Kubli and Gustafsson, 2012). The anti-apoptotic protein Bcl-2 has also been shown to protect cells by reducing the ER Ca\(^{2+}\) pool, which has an effect of reducing cellular sensitivity to apoptosis (Pinton and Rizzuto, 2006), while the pro-apoptotic protein Bax has the opposite effect, increasing ER Ca\(^{2+}\) levels (Chami et al., 2004). These observations indicate that Ca\(^{2+}\) dysregulation is highly involved in the initiation of apoptosis, and that by buffering intracellular Ca\(^{2+}\) levels, mitochondria may help the cell respond to additional forms of stress.

Calcium signaling can also affect mitochondrial stress responses by indirectly modulating autophagy. Basal levels of Ca\(^{2+}\) release from the ER to the mitochondria are required to maintain sufficient NADH production (Cardenas et al., 2010). The absence of this basal Ca\(^{2+}\) transfer to the mitochondria causes phosphorylation and inhibition of pyruvate dehydrogenase, which decreases oxidative phosphorylation and ATP generation (Cardenas et al., 2010). The subsequent increase in the AMP/ATP ratio activates AMP-activated protein kinase (AMPK) and induces mTOR-independent autophagy mechanisms (Cardenas et al., 2010; Rubinsztein et al., 2012).
1.6 Mitochondria-targeted chemotherapeutic agents

1.6.1 Cell-penetrating peptides

The cellular plasma membrane is a highly organized and selectively permeable barrier that protects the cell from exogenous bioactive molecules and tightly controls the intake of xenobiotics (Fonseca et al., 2009; Stewart et al., 2008). Many pharmacological agents must gain entry into the cell in order to carry out their effect, so the ability of drugs to cross the plasma membrane is crucial for proper activity (Fonseca et al., 2009). Small exogenous molecules or protein-based drugs can gain access to the cell through direct diffusion across the membrane or through cellular transport machinery (Stewart et al., 2008). However, for larger molecules that do not resemble the substrates for any cellular transporters, entry into the cell can be inefficient, slow, difficult, and a rate limiting factor in their pharmacological activity. One way to improve the entry of xenobiotics into the cell is through the use of short peptide sequences that could efficiently cross the plasma membrane. One example is the Tat peptide, derived from the HIV Tat transactivator protein, which can efficiently enter cells (Stewart et al., 2008). Several such peptides have been identified. Their sequences and important molecular properties have helped optimize a novel class of molecular transporters known as cell penetrating peptides (CPP) (Stewart et al., 2008). They include peptides that replicate naturally occurring sequences, and synthetic constructs with sequences based on the important features of natural systems that are designed to provide the highest possible levels of cellular uptake. CPPs represent a powerful tool for transporting wide ranging cargo molecules into the cell. CPPs have been used to transport DNA, RNA, polymers, proteins, antibodies, nanoparticles, radiolabels, and liposomes across plasma membranes (Stewart et al., 2008). Relevant properties that affect the uptake efficiency of CPPs include peptide length, chemical properties, lipophilicity, and size (Stewart et al., 2008). Based on these properties, CPPs can enter the cell either through energy-dependent vesicular mechanisms of endocytosis, or through a direct process of translocation across the lipid bilayer (Stewart et al., 2008).

Endocytosis is a highly regulated process that can include phagocytosis and pinocytosis. Endocytosis encompasses several different mechanisms, such as: clathrin lattice, dynamin, caveloase, or a lipid raft. Ultimately, the desired extracellular molecules become encapsulated into lipid vesicles formed from the plasma membrane. These vesicles become internalized, and
the transport molecules must then escape the vesicles into the cytosol before the vesicles can be recycled back into the plasma membrane (Stewart et al., 2008). The exact mechanism of endocytosis depends not only on the CPP, but also on the attached cargo molecule, as the same CPP may enter the cell through different mechanisms depending on the type of cargo it is transporting (Stewart et al., 2008). Alternatively, CPPs can cross the membrane using an energy-independent, non-endocytic mechanism by perturbing the structural integrity of the membrane. These mechanisms are sensitive to changes in membrane properties, such as fluidity of membrane potential (Stewart et al., 2008). For example, uptake of poly-arginine CPPs and the naturally occurring CPP penetratin, are dependent on membrane potential. Electrostatic interaction with surface molecules and negatively charged lipids serve to initiate transport across the membrane (Stewart et al., 2008). These uptake mechanisms are not necessarily mutually exclusive, as CPPs may simultaneously use multiple uptake mechanisms (Stewart et al., 2008). In recent years CPPs have been shown to improve the efficacy of several pharmaceutical agents by increasing their cellular uptake. Furthermore, CPPs may allow for the use of certain compounds that exhibit desirable pharmacological properties in vitro but lacked the solubility or lipophilicity required for cellular uptake (Stewart et al., 2008).

1.6.2 Mitochondria-penetrating peptides

The ability of CPPs to cross cellular membranes may also be used to specifically target other membrane-bound compartments within the cell. Of particular interest is the targeting of drugs to the nucleus and the mitochondria. The nucleus is a desirable target because it houses the nuclear genome, where chemotherapeutic agents act to interfere with DNA replication in cancer cells. Mitochondria are also a potential target for drug delivery because they play a crucial role in energy production, cancer, neurodegenerative diseases, and the induction of apoptosis (Horton et al., 2008; Stewart et al., 2008).

Alternative approaches for targeting xenobiotics to mitochondria include delocalized lipophilic cations (DLCs), mitochondrial target sequences (MTSs), and lipid vesicles. DLCs are positively charged lipophilic molecules that accumulate in the mitochondria due to their attraction to the negative charge across the mitochondrial membrane potential, and can be used to transport conjugated cargo. Unlike inorganic cations, delocalized cations can stabilize the positive charge through resonance structures. This causes a much more energetically favourable
uptake because the charge can be spread over a larger area and will have a lower entrophy of desolvation (Yousif et al., 2009b). Examples of DLCs are the MitoTracker and JC-1 dyes (Yousif et al., 2009b). Drawbacks of DLCs are that potential cargo will be limited by size and polarity, and these compounds can cause mitochondrial toxicity at higher concentrations (Yousif et al., 2009b). MTSs are naturally occurring peptide sequences of 20 to 40 amino acids that can be recognized by receptors in the outer mitochondrial membrane, and are actively transported into the mitochondrial matrix by the TOM and TIM proteins complexes (Yousif et al., 2009b). MTSs have been used to deliver many types or cargo into the mitochondria, however, these are fairly long peptide sequences that have poor solubility and limited cellular uptake (Yousif et al., 2009b). Finally, lipid vesicles have also been used to transport cargo molecules into the mitochondria, but this approach is limited to negatively charged cargo, and has a very low delivery efficiency (Yousif et al., 2009b).

Targeting peptides to the mitochondria presents additional challenges. Mitochondria have double membranes, so as opposed to a CPP that must only penetrate a single lipid membrane, mitochondria-penetrating peptides (MPPs) must penetrate three very different lipid membranes to reach their target. The plasma membrane has a resting membrane potential that ranges from -30 to -90 mV, while the inner mitochondrial membrane has a resting membrane potential of -180 mV (Yousif et al., 2009b).

Positive charge improves penetration of peptides through lipid membranes carrying a potential. Since the inner mitochondrial membrane carries strong potential, cationic residues were included into initial MPPs. The inner mitochondrial membrane functions as a rigid barrier against passive diffusion (Yousif et al., 2009b). It is highly impervious, densely packed with proteins, and much more hydrophobic than the plasma membrane. To account for this and penetrate the inner mitochondrial membrane, MPPs must also include lipophilic residues (Horton et al., 2008). With these requirements as a framework, oligomers composed of alternating cationic and lipophilic residues were synthesized and assayed for mitochondrial uptake. Lysine (K) and arginine (R) residues were used to provide positive charge, while the unnatural cyclohexylalanine (Fx) residue was used to provide lipophilicity to the peptide (Horton et al., 2008). There was a positive relationship between lipophilicity and mitochondrial localization (Horton et al., 2008). Treatment with endocytosis-inhibitors did not alter the uptake of MPPs, suggesting that the mechanisms of MPP uptake into the cell occurs predominantly by direct,
potential-driven diffusion. Pharmacological increase to the membrane potential greatly enhanced MPP transport across the plasma membrane, suggesting that MPP uptake is driven by membrane potential (Horton et al., 2008). Altering the mitochondrial membrane potential also has an effect on MPP localization. Treatment with the mitochondrial decoupling agent FCCP dissipates mitochondrial membrane potential, and significantly decreased the localization of MPP into the mitochondria (Horton et al., 2008).

The MPP with the sequence \(F_x r F_x r F_x r\) (where \(F_x = \text{cyclohexylalanine}\) and \(r = \text{d-arginine}\)) is considered ideal based on its ability to penetrate both the plasma and mitochondrial membranes, and localize specifically in the mitochondria (Fonseca et al., 2011). The fact that both cyclohexylalanine and d-arginine are artificial amino acids not normally found in nature makes this peptide resistant to intracellular degradation by proteases, which greatly increases its stability and half-life \textit{in vivo} (Fonseca et al., 2011). The \(F_x r 3\) MPP has cellular uptake similar to classic CPPs such as the Tat peptide, while also presenting mitochondrial localization when inside the cell (Fonseca et al., 2011; Stewart et al., 2008). Cargo drugs to be transported into the mitochondria are conjugated to the peptide via a c-terminal lysine residue.

### 1.6.3 Cargo molecules targeted to the mitochondria

Chemotherapeutic agents that have been conjugated to MPP to date and targeted to the mitochondria include chlorambucil, doxorubicin, and a synthetic platinum compound. Chlorambucil (Cbl) is a nitrogen mustard compound and a potent alkylating agent used in the treatment of leukemia. Cbl alkylation leads to DNA crosslinks that induce apoptosis in leukemia cells (Begleiter et al., 1996).

Doxorubicin (Dox) is a potent antineoplastic agent commonly used for the treatment of solid tumors and leukemia, and is associated with a high incidence of cardiotoxicity (Wallace, 2003). Dox is capable of causing toxicity through a variety of mechanisms. Dox has a planar anthracycline ring that is able to intercalate into the DNA double helix to inhibit DNA and RNA polymerases (Wallace, 2003). Dox is also able to inhibit topoisomerase II, an important enzyme responsible for the opening of nuclear DNA for replication (Wallace, 2003). There is some evidence in the literature to suggest that topoisomerase II is also present in mitochondria (Chavalitsheiwinkoon-Petmitr et al., 2001; Shapiro, 1994; Shapiro and Showalter, 1994). If this were true, doxorubicin should technically be able to inhibit mtDNA replication. In addition, Dox
has electrochemical properties that allow it to undergo redox cycling and act as an alternate electron acceptor at complex I, causing electron transport chain (ETC) inhibition and a massive increase in free-radical production (Cohen, 2010; Oliveira et al., 2004; Wallace, 2003).

A synthetic platinum compound targeted to the mitochondria is acyl-acyl-cis-Diammineplatinum (II)-β-Diketonate (Wilson and Lippard, 2012) termed platinum-acylacyl, or simply Pt-AcAc. In contrast to chlorambucil and doxorubicin, Pt-AcAc is not a clinical chemotherapeutic agent, but a newly derived synthetic compound that has a similar mechanism of action as cisplatin (Wilson and Lippard, 2012). In this mechanism, the two chlorides of cisplatin become displaced by water, resulting in an activated cis-diammineplatinum (II) moiety, a highly reactive electrophile that can bind to purine bases of DNA (Wilson and Lippard, 2012). Pt-AcAc acts through the same mechanism, but since it has a much less reactive leaving group, it is approximately 16-fold less toxic than cisplatin (Wilson and Lippard, 2012). Taken together, mitochondria-targeting drugs represent an emerging strategy for probing mitochondrial function.
1.7 Hypothesis

Mitochondrial DNA (mtDNA) is important for mitochondrial function because it encodes for crucial proteins of the electron transport chain (ETC). We sought to further investigate the relationship between mtDNA and mitochondrial health by determining what role mtDNA integrity may play in the ability of mitochondria to respond to toxic xenobiotic stress. There are examples in the literature of gene-environment interactions relating to the mitochondria, where mutations to mitochondrial proteins sensitize individuals to drug-induced mitochondrial toxicity. We sought to determine whether mtDNA replication fidelity also serves as a genetic predisposing factor for drug-induced mitochondrial toxicity. Our hypothesis is that mitochondrial DNA replication fidelity protects against toxicity initiated by drugs that impair mitochondrial function. This hypothesis has been investigated through three specific aims.

AIM 1: To determine whether mtDNA replication fidelity protects against toxicity initiated by drugs that impair the electron transport chain (ETC). We have identified mitochondrial DNA mutation load as a risk factor for toxicity initiated by the ETC complex I inhibitor rotenone and by other ETC complex inhibitors. This is the first study of the combined effects of the drug rotenone with a genotype of decreased mtDNA replication fidelity (Polg<sup>m/m</sup>), and has the potential to indicate that mtDNA mutations may predispose individuals to increased toxicity of such xenobiotics.

AIM 2: To determine the role of mitochondrial stress responses in toxicity initiated by rotenone. The mechanisms chosen for investigation were mitochondrial dynamics (fusion and fission) and mitochondrial autophagy (mitophagy). We have examined how these mechanisms differ in Polg<sup>m/m</sup> cells compared to wild type cells, and whether modulating these stress responses could rescue the Polg<sup>m/m</sup> susceptibility to bioenergetic stress observed in aim 1. While increased mitochondrial fusion did not affect rotenone toxicity, we observed that induction of mitophagy attenuated the rotenone susceptibility of Polg<sup>m/m</sup> MEFs.

AIM 3: To determine whether mtDNA replication fidelity protects against toxicity initiated by mitochondria-targeted chemotherapeutic drugs. In the mtDNA targeted damage model we have identified mitochondria-targeted acyl-acyl-cis-Diammineplatinum (II)-β-Diketonate (mt-Pt), a novel platinum-based crosslinking agent targeted to the mitochondria, as being hypertoxic to Polg<sup>m/m</sup> compared to wild type MEFs. mt-Pt increased apoptosis in Polg<sup>m/m</sup>
but not wild type MEFs. Bioenergetic differences were observed between $Polg^{m/m}$ and wild type MEFs exposed to mitochondria-targeted chlorambucil, but this did not translate into a genotype difference in cell viability. No differences in toxicity were observed in $Polg^{m/m}$ and wild type MEFs exposed to mitochondria-targeted doxorubicin.
2 Materials and Methods

2.1 Polg\textsuperscript{m/m} mouse model

The Polg\textsuperscript{m/m} mouse model has been described previously (Kujoth et al., 2005) and were kindly provided by Dr. T. A. Prolla, University of Wisconsin. Tail samples were utilized as a source of genomic DNA; gDNA was extracted using the REDExtract-N-Amp Kit (Sigma) according to the manufacturer’s instructions. Genotyping primer sequences are: Polg58: 5’-GCCTCGCTTTCTCCGTGACT-3’ and Polg59: 5’-GGATGTGGCCCAGGCTGTAACTA-3’. The thermocycling conditions are 5 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 45 seconds at 64°C, 45 seconds at 72°C, followed by 5 minutes at 72°C. PCR products were run on a 2% agarose gel at 120V for 1h. The wild type allele generates a 296 base pair product while the Polg\textsuperscript{m/m} allele will generate a 468 base pair product. Animals were maintained by heterozygous crosses. All experiments were performed in accordance with guidelines from the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee.

2.2 Mouse Embryonic Fibroblast (MEF) cell culture

Wild type and Polg\textsuperscript{m/m} mouse embryonic fibroblasts (MEFs) were prepared from embryonic day 14.5 embryos derived from matings of Polg\textsuperscript{+/m} heterozygous mice. The head and internal organs were removed from the E14 embryos, and the tails were collected for genotyping purposes. The embryo bodies were transferred to microfuge tubes and cut into small pieces. The tissues were then incubated with 0.05% trypsin-EDTA (Gibco) for 30 minutes in 6 well plates, dissociated by pipetting, and incubated again in Trypsin-EDTA for an additional 20 minutes. The tissue digestion was inactivated with fetal bovine serum and the cells were centrifuged at 524 g for 10 minutes. The top pellet layer containing MEF cells was maintained in DMEM containing 25 mM glucose, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 55μM β-mercaptoethanol, 100 units/mL penicillin, 100 μg/mL streptomycin (all reagents from Life Technologies, Burlington, ON, Canada) at 37°C and 5% CO₂. To permit analyses by clonogenic assays, passage 0 to 3 primary fibroblasts from wild type and Polg\textsuperscript{m/m} littermates were immortalized with Simian virus large T antigen (SV40gp6) in PsG5 (Stratagene, Mississauga, ON, Canada) together with a puromycin resistance plasmid and selected in puromycin-containing media (Tamblyn et al., 2009). Immortalized MEFs were used for experiments from passage 4 to 10. To examine the impact of glucose utilization on cellular physiology, glucose-free media
formulations were employed. Galactose media was made with glucose-free DMEM (Life Technologies, Burlington, ON, Canada) supplemented with 10% FBS and 10 mM D(+) galactose (Bioshop, Burlington, ON, Canada) according to published methods (Marroquin et al., 2007).

2.3 Clonogenic assay

For clonogenic assays, 0.5-1 x 10^3 cells were seeded in 60 mm dishes (BD Falcon, Mississauga, ON, Canada) and exposed to 0.25-5 nM rotenone (Sigma, Oakville, ON, Canada) for seven days. The assay dishes were washed once with PBS, and colonies were fixed and stained with 10.3 mM methylene blue in methanol for 10 minutes with motion. The dishes were washed twice with deionized water to remove excess dye, and allowed to dry overnight. The number of colonies was counted using a dissecting microscope. Colonies containing at least 50 cells were scored. Four to six replicates of each concentration and cell line were counted. Rotenone was dissolved in DMSO and vehicle controls consisted of 0.1% DMSO. For experiments on mitochondrial-targeted compounds, MEFs were exposed to 0-4 µM of Fxr3k-Cbl, 0-8 µM of Chlorambucil, in serum-free OPTI-MEM media (Invitrogen), or 0-10 µM Fxr3 in maintenance media for 24h. After 24h media was replaced by the maintenance media and colonies were allowed to grow for 7 days.

2.4 Mitochondrial DNA mutations

mtDNA mutation levels were measured using the random mutation capture (RMC) assay, essentially as described (Bielas and Loeb, 2005; Ericson et al., 2012; Vermulst et al., 2008). The first step in the protocol is isolation of mitochondria from fresh cell cultures. 20 strokes of a Dounce homogenizer are used to disrupt the cell membrane and lyse the cells. The homogenate is centrifuged at 1000 g for 10 minutes at 4°C to pellet nuclei and whole cells. The supernatant containing mitochondria is centrifuged again at 13000 rpm for 15 minutes at 4°C to pellet the mitochondria. Next, the mtDNA is extracted by proteinase K digestion containing 30 ng/mL RNaseA at 55°C for 30 minutes. Following proteinase K digestion, phenol-chloroform-isoamyl alcohol (25:24:1) is added to the lysis reaction at a 1:1 ratio, mixed by shaking and centrifuged at 13000 rpm for 2 minutes. The aqueous layer is extracted and mixed again with phenol-chloroform-isoamyl alcohol (25:24:1) and the aqueous layer is extracted again as before. The final extraction step is done with chloroform only at a 1:1 ratio to remove phenol. A 1:20 volume of 3 M sodium acetate is added to the final aqueous extract, and the mtDNA is precipitated by...
addition ice-cold 100% ethanol in a ratio of 2.5:1. This solution is mixed by shaking, stored at -80°C for 15 minutes, -20°C for 30 minutes, and then centrifuged at 13000 rpm for 30 minutes at 4°C. The pellet containing mtDNA is rinsed with 70% ethanol and centrifuged again for 5 minutes. Once all the ethanol has been removed from the pellet, the mtDNA is dissolved in 10 mM Tris-HCl. mtDNA from primary MEFs, immortalized MEFs derived from Polg<sup>m/m</sup>, Polg<sup>+/m</sup>, or wild type mice was digested with TaqI restriction enzyme (NEB cat.# RO149M) in 100 µL reaction volumes containing 100 units of TaqI, 100 mM NaCl, 10 mM Tris-HCl, 1x BSA, and 10 mM MgCl<sub>2</sub>. Mutation frequencies were determined using real-time PCR in two reactions: one using primers flanking the TaqI restriction site in the mitochondrial 12s rRNA gene to amplify molecules resistant to TaqI digestion, and one using primers to amplify a region with no TaqI restriction site as an index of total mtDNA. PCR amplification was carried out in 25 µL reaction volumes with 12.5 µL 2x SYBR Green Brilliant Mastermix, 0.2 µL UDG, 2 µL 10 pM/µL primers and 3.3 µL H<sub>2</sub>O. Primer sequences were: mControl forward: TCGCGCTAAAACGTGTCAAC; mControl reverse: CCGCCAAGTCTTTTGAAGTTT; mTaq634 forward: ACTCAAAGGACTTGGCGGTA; mTaq634 reverse: AGCCCATTTCCTTCCCATTTTC (Vermulst et al., 2008). A schematic with the concept and experimental protocol of the RMC assay can be found in Vermulst et al., 2008.

2.5 Mitochondrial mass

MitoTracker deep red 633 dye (Invitrogen, Burlington, ON, Canada) was used to stain mitochondria and quantify mitochondrial mass by flow cytometry analysis. The MitoTracker dye accumulates specifically in the mitochondria and the fluorescence signal will be proportional to the mitochondrial mass in each cell. Since depolarized mitochondrial will not uptake MitoTracer deep red 633, the fluorescence will also be somewhat dependent on membrane potential. Three biological replicates of 5x10<sup>5</sup> primary Polg<sup>m/m</sup> or wild type MEFs cultured in glucose media were incubated with 100 nM MitoTracker dye in DMSO for 20 minutes, then pelleted by centrifugation at 1000 rpm for 5 minutes and resuspended in PBS for flow cytometry analysis using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada) and FLOJO software (Tree Star, Ashland, OR, USA). 10,000 events were recorder per sample, and single cells were gated using forward and side scatter. Mitochondrial mass in each cell line was quantified as the median FL4 fluorescence. The experiment was performed with 3 independent cells lines per genotype.
2.6 Flow Cytometry analysis of Apoptosis in MEFs

Immortalized MEFs were grown in galactose media for 24h then seeded at 3.3x10⁵ / 60mm dish. 24 h later cells were cultured in 10nM rotenone or 0.1% DMSO in galactose media for 24h. The dishes were washed once with PBS and incubated with 0.05% Trypsin-EDTA (Gibco) for 7 minutes at 37°C. Trypsinized cells were collected as a single cell suspension in 3mL of MEF media in a 15 mL conical tube. Cells were centrifuged at 524 g for 5 minutes, the supernatant was removed, and the cells were resuspended in PBS, filtered through a 0.22 µm filter and transferred to 5 mL round-bottom tubes (BD). The cells were centrifuged again as before, the supernatant was removed and cells were resuspended in 150 µL of binding buffer (BD Pharmingen, Mississauga, ON, Canada) containing 5 µL of FITC-Annexin-V following the manufacturer’s instructions (BD Pharmingen, Mississauga, ON, Canada) and 50µg/mL propidium iodide (PI) (Sigma). The cells were incubated at room temperature for 10 min and analysis was performed using a FACS Calibur flow cytometer (BD Biosciences, Mississauga, ON, Canada) and FLOJO software (Tree Star, Ashland, OR, USA). 10,000 events were recorder per sample, and single cells were gated using forward and side scatter. The experiment was performed with immortalized MEFs in galactose media in triplicate.

In experiments assessing the cytoprotective effects of rapamycin, immortalized MEFs were seeded at 5x10⁵ / 10cm dish and treated with 0.1% DMSO vehicle control or 3 µM rapamycin for 48h, then trypsinized and re-seeded at 3.3x10⁵ / 60mm dish with 0.1% DMSO vehicle control or 3 µM rapamycin as before. 24 h later media was aspirated dishes were washed once with galactose media. Cells were treated with either 0.1% DMSO vehicle control or 3 µM rapamycin, in the presence or absence of 10 nM rotenone in galactose media for 24 h. After 24 h all samples were prepared for flow cytometry analysis with Annexin-V and PI as described above. The experiment was performed with 3 independent cells lines per genotype.

2.7 Measurement of Acute Necrosis

Acute necrosis was measured using the Mitochondrial ToxGlo Assay Kit (Promega, Madison, WI, USA) following the manufacturer’s instructions. The presence of protease activity associated with necrosis is measured using a fluorogenic peptide substrate that only crosses the membrane of dead cells. The fluorogenic peptide substrate bis-AAF-R110 cannot cross the intact membrane of life cells, but can only enter cells that have lost membrane integrity. Once inside
the cell this substrate can detect the activity of proteases associated with necrosis. 1x10^4 cells were seeded per well of white opaque walled clear bottom 96 well plates, and exposed to 5, 10, or 50 nM rotenone or DMSO vehicle control for 24h. Following exposure, cytotoxicity reagent containing the fluorogenic peptide substrate was added, the plate was incubated at 37°C for 30 min, and fluorescence was measured at 490nm/510-570nm. Experiments were performed with primary MEFs in galactose media with 3 independent cells lines per genotype in duplicate.

2.8 Flow Cytometry cell cycle analysis

Immortalized MEFs were seeded at 3.3x10^5 / 60mm dish. 24 h later cells were cultured in 10 nM rotenone or 0.1% DMSO vehicle control in glucose or galactose media for 24 h, and pulsed with 10 µg/mL BrdU for 1 h. Cells were washed once with PBS and incubated with 0.05% Trypsin-EDTA (Gibco) for 7 minutes at 37°C. The trypsinized cells were filtered through a 0.22µm filter and collected as a single cell suspension into 15 mL conical tubes. The cells were centrifuged at 524 g for 5 minutes the supernatant was removed. The cells were then fixed and resuspended in cold 70% ethanol with gentle vortexing, allowed 20 minutes for complete fixation to occur, and stored overnight at -20°C. Cells were transferred to 5mL round-bottom FACS tubes, centrifuged at 524 g for 5 minutes and washed with FACS wash buffer (phosphate buffered saline with 0.5% BSA) to remove ethanol. Following another centrifugation step, cells were permeabilized with 2N HCl for 20min, washed with wash buffer, incubated with 0.1M sodium borate pH 8.5 for 2 min to neutralize the HCl, washed with wash buffer, and incubated with FITC-conjugated anti-BrdU (BD Biosciences, Mississauga, ON, Canada) in PBS/0.5% BSA/0.5% Tween20 in the dark for 30 min. After incubation the cells were washed with wash buffer and stained with 50 µg/ml PI for 30 min. Analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada) and FLOJO software (Tree Star, Ashland, OR, USA). 10,000 events were recorder per sample, and single cells were gated using forward and side scatter. BrdU-pulsed and non-pulsed controls were used to identify the boundary between BrdU-positive and BrdU-negative staining along the FITC fluorescence axis, and these gates were used to identify the percentage of cells with BrdU-positive staining in experimental samples. BrdU-positive staining was used as a marker of cells in S-phase. The experiment was performed with 3 independent cells lines per genotype.
2.9 Luminescence measurement of ATP content

The CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA) was used to measure ATP content in a cell population following manufacturer’s instructions. 3x10^3 immortalized MEFs of either wild type or Polg^m/m genotype were seeded per well of a 96 well plate and exposed to 1.25 - 10 nM rotenone or DMSO vehicle control for 48 h. Following exposure, an equal volume of CellTiter-Glo reagent was added to each well and allowed to incubate at room temperature for 10 min. Luminescence was measured in relative luminescence units and ATP luminescence was normalized to DMSO vehicle controls. The number of viable cells was also measured as part of this experiment. Cells were seeded in 24 well plates at equal seeding density and underwent the same treatments and drug exposures. Following the 48h drug exposure, cells were washed once with PBS and incubated with 0.05% Trypsin-EDTA (Gibco) for 7 minutes at 37°C. An equal volume of trypan blue exclusion dye was added to each well, and the number of trypan-excluding cells was counted for each well. Experiments were performed with immortalized MEFs in glucose or galactose media with 3 independent cells lines per genotype.

2.10 Mitochondrial DNA abundance

Total DNA samples were prepared from 3-5x10^5 cells lysed in 100 mM Tris, 5mM EDTA, 20 mM NaCl and 0.2% SDS pH 8.0, and incubated at 55°C overnight with 0.08 mg/mL Proteinase K (Fermentas). Total DNA was isolated by phenol-chloroform-isoamyl alcohol (25:24:1) extraction. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each sample of digested DNA, it was mixed well by inverting, and centrifuged at 20800 g for 5 minutes. The aqueous layers were extracted and transferred to new microfuge tubes. Another equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each sample, all the samples were mixed well by inverting and centrifuged again as before. The aqueous layers containing DNA were once again extracted and placed in new microfuge tubes. DNA was precipitated by the addition of 100% ethanol with mixing. The samples were centrifuged and the DNA pellet was washed with 70% ethanol and centrifuged again. The supernatant was removed and samples were allowed to dry for 10 minutes to remove residual ethanol from the DNA. The dry DNA pellets were resuspended in TE buffer pH 8.0. Total DNA concentration was measured by A_{260} using a Nanodrop spectrophotometer (Thermo Scientific,
Asheville, NC, USA). mtDNA copy number was estimated by amplifying a portion of the cytochrome b (cyt b) gene of mtDNA and comparing it to the amplification of beta-actin of the nuclear genome. Primers sequences were cytochrome B (CYTB) CYTB_F 5’-GCT TTC CAC TTC ATC TTA CCA T-3’ and CYTB_R 5’-TGT TGG GTT GTT TGA TCC TG-3’, beta-actin-F 5’-GGA AAA GAG CCT CAG GGC AT-3’ and beta-actin-R 5’-GAA GAG CTA TGA GCC TGA-3’ (Ylikallio et al., 2010). Reactions used the SYBR green kit (Applied Biosystems, Burlington, ON, Canada) and an ABI 7500 real-time thermocycler (Applied Biosystems, Burlington, ON, Canada). Relative amplification was calculated using the delta delta Ct (ΔΔCt) method (Livak and Schmittgen, 2001). During optimization, plot of Ct vs log (DNA template) displayed a linear relationship for both the CYTB and beta-actin PCR products at a range of 10-320 ng of total DNA template. Immortalized MEFs were exposed to 10 nM rotenone or DMSO vehicle for 24h. Primary MEFs (passage 1-3) were exposed to 400 µM hydrogen peroxide or untreated control for 1h, then placed in complete growth media for an additional 7 h recovery time before harvest. The experiment was performed with 3 independent cells lines per genotype.

2.11 Measurement of mtDNA and nuclear DNA lesions

2x10^6 immortalized MEFs of wild type and Polg^m/m genotype were seeded in 150mm dishes and exposed to 10nM rotenone and 0.1% DMSO vehicle control for 24h. Following exposure, cells were washed once with PBS and incubated with 0.05% Trypsin-EDTA at 37°C for 7 minutes. The trypsinized cells were collected in MEF media and centrifuged at 524 g for 5 minutes. The cell pellets were washed once with PBS and centrifuged again. Cells were resuspended in resuspension solution (Sigma) containing RNase A (Qiagen). Vortexing was avoided in order to prevent sheared DNA. Cells were lysed by Proteinase K incubation with lysis solution and incubation at 70°C for 10 minutes. Total genomic DNA was isolated with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Oakville, ON, Canada) following manufacturer’s instructions. Total DNA was quantified using PicoGreen dye as previously described (Invitrogen, Burlington, ON, Canada)(Santos et al., 2002; Santos et al., 2006a). Quantitative amplification of the 10kB and 117bp mitochondrial target sequences was performed using the GeneAmp XL PCR kit (Applied Biosystems, Burlington, ON, Canada) as previously described (Santos et al., 2002; Santos et al., 2006b). PCR primers for the 10kB fragment were 2372 5’-GCC AGC CTG ACC CAT AGC CAT ATT AT-3’ and 13337 5’-GAG AGA TTT TAT GGG TGT ATT GCG G-3’. Thermocycler conditions are 94°C for 1 minute, 18 cycles of 94°C
for 1 minute and 68°C for 12 minutes, followed by 72°C for 10 minutes. Primers for the 117bp fragment were 13597 5'-CCC AGC TAC TAC CAT CAT TCA AGT-3' and 13688 5'-GAT GGT TTG GGA GAT TGG TTG ATG-3'. Thermocycler conditions were 75°C for 2 minutes, 22 cycles of 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 45 seconds, followed by 72°C for 10 minutes. Amplification of the 10kB mtDNA segment was normalized to the amplification of the 117bp mtDNA PCR product in order to normalize for total mtDNA content. Lesion number relative to wild type vehicle control was calculated using the equation Lesions = - Ln(PCR productsample/PCR productcontrol). Linearity of amplification was confirmed by using 25% input and 50% input of template DNA.

For the experiments on mitochondrial-targeted compounds, 1.5x10⁶ primary MEFs (passage 3-4) / 100mm dishes were seeded and exposed to 6 µM mt-Cbl for 2 hours, or to 3-400 µM chlorambucil and 0.1% DMSO vehicle control for 2h in serum-free OPTI-MEM media. Cells were collected either immediately after the 2h treatment or after 8h. PCR primers for the 8.7kB beta-globin fragment are 21582(F) 5'- TTG AGA CTG TGA TTG GCA ATG CCT-3' and 30345(R) 5'- CCT TTA ATG CCC ATC CCG GAC T-3'. PCR primers for the 6.5kB beta-polymerase fragment are MBF (F) 5'- TAT CTC TCT TCC TCT TCA CTT CTC CCC-3' and MBEX (R) 5' CGT GAT GCC GCC GTT GAG GGT CTC CTG-3'. The thermocycler conditions are 94°C for 1 minute, 36 cycles of 94°C for 1 minute, 55°C for 1 minute, 68°C for 13 minutes, followed by 72°C for 10 minutes.

2.12 Measurement of ROS generation

Total cellular ROS generation was measured using dichlorofluoroscein diacetate (DCFH-DA, Sigma), an oxidation-sensitive fluorescent probe. This assay is based on the ROS-dependent oxidation of DCF-DA to the highly fluorescent compound dichlorofluorescein (DCF). 3x10³ MEFs of wild type or Polg⁻/⁻ genotype were seeded in wells of a 96-well plate. Prior to rotenone exposure, some wells were pre-treated with 500 µM N-acetyl-cysteine (NAC) for 1h. Cells were exposed to 10 nM rotenone for 6 hours. Cells were washed twice with PBS, and then incubated with 50 µM DCFH-DA for 1h at 37°C. Following incubation, cells were washed twice with PBS, and fluorescence was measured at 485/528 nm using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, US).
2.13 Generation and maintenance of primary cortical neurons

Wild type and Polg\textsuperscript{m/m} cortical neuron cultures were prepared from embryonic day 16 embryos derived from matings of Polg\textsuperscript{m/+} heterozygous mice. The cortex was dissected from E16 embryo brains in cold dissecting solution, which contains 137 mM NaCl, 5.4 mM KCl, 0.17 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.22 mM KH\textsubscript{2}PO\textsubscript{4}, 9.9 mM Hepes, 33.3 mM D (+) glucose, and 43.8 mM sucrose in filtered water. The dissected cortices were digested in dissecting solution containing 9 units/mL of Papain (Worthington, Lakewood, NJ, USA), 10 ng/mL 2-amino-5-phosphonopentannoic acid (APV) (Sigma), and 20 mg/mL L-cysteine at 37°C for 30 minutes. The digested tissue was neutralized by transferring it to an enzyme inhibitor solution containing 20 mg/mL bovine albumin (BSA) (Sigma), 20 mg/mL Trypsin inhibitor and 10 ng/mL APV using a Pasteur pipette. The tissue was washed once in cortical neuron media and then dissociated by pipetting until a single cell suspension was achieved. Cortical neuron media was made by adding 2% B-27 serum-free supplement, 0.25% glutaMAX\textsuperscript{TM}, 100 units/mL penicillin and 100 µg/mL streptomycin to Neurobasal medium (all reagents from Life Technologies, Burlington, ON, Canada). Prior to seeding the primary cortical neurons, cultureware was coated with 50 µg/mL poly-L-lysine (Sigma, Oakville, ON, Canada). 1 x 10\textsuperscript{5} cells were seeded in 60 mm dishes for flow cytometry analysis, and 9.4x10\textsuperscript{4} cells were seeded in glass coverslips for microscopy imaging. The neuron cultures were maintained at 37°C and 5% CO\textsubscript{2}. Neurons were cultured for 4 days in vitro (DIV) before being treated with 1 to 50 nM rotenone or DMSO for 24 hours. Experiments were performed with three embryos of each genotype.

2.14 Neuronal Immunohistochemistry

Wild type and Polg\textsuperscript{m/m} primary cortical neurons were seeded in glass coverslips coated with poly-L-lysine for 4 days. At 4 days following neuron isolation media was removed and neurons were treated with 0.1% DMSO vehicle or 1-50 nM rotenone. After 24h of rotenone exposure, the neurons were fixed with 4% paraformaldehyde (PFA) for 10 minutes with motion, washed with PBS, and then permeabilized with 0.4% triton-100 in PBS for 15 minutes with motion. Next the cells were incubated in blocking solution containing 1% goat serum and 0.2% triton-100 in PBS for 30 minutes with motion. Following the blocking step the cells were incubated with the primary antibody mouse anti-tubulin β III isoform (diluted 1:1000, StemCell Technologies, 01409) overnight at 4°C in blocking solution. This primary antibody reacts with
the β III isoform of tubulin, which is specific for immature neurons. Next, the cells were washed three times with PBS and incubated with the secondary antibody goat anti-mouse Alexa-568 (Invitrogen) for one hour, washed again with PBS twice, and mounted onto slides using Vectashield Mounting Media containing the nuclear stain DAPI (Vector). The neurons were imaged using the AxioImager.M2 Epifluorescence Microscope (Zeiss).

2.15 Cell counting kit 8 (cck-8) assay

WST-8 viability dye (Dojindo, Rockville, Maryland), a water soluble tetrazolium reduction-based dye, was used to measure cell viability following manufacturer’s instructions. The amount of tetrazolium dye reduced to a water-soluble formazan is directly proportional to the number of viable cells, and has been shown to correlate well with ³H-thymidine incorporation in measurements of cellular proliferation (Stoddart, 2011). Standard curves with known numbers of cells were routinely performed as controls for cck-8 assays, and linear relationships were observed between cell number and absorbance at 450 nm. 7.5x10² immortalized MEFs of wild type and Polg\textsuperscript{m/m} genotype were seeded per well of 96-well plates in 100 µL volumes in either glucose or galactose media conditions. The following day, cells were exposed to: rotenone, clofibrate, ketoconazole, antimycin-A, potassium cyanide, flutamide (all Sigma, Oakville, ON, Canada), or vehicle controls in 100 µL volumes for 48h. All drugs were made into 500x stocks in DMSO or 200x stocks in ethanol vehicle and diluted in media. Following exposure, 10 µL of CCK-8 dye was added to each well and allowed to incubate at 37°C for 3.5h. Absorbance was measured at 450 nm using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, US). Background absorbance was measured in wells containing media, and this background absorbance was subtracted from the samples.

In experiments with mitochondrial-targeted chemotherapeutic drugs, 1x10³ immortalized MEFs of wild type and Polg\textsuperscript{m/m} genotype were seeded per well of 96-well plates in 100 µL volumes in MEF media. The following day, cells were exposed to up to 60 µM of mitochondrial-targeted doxorubicin, mitochondrial-targeted cis-Diammineplatinum (II)-β-Diketonate (Wilson and Lippard, 2012) or vehicle controls in 100 µL volumes for 72h. Following exposure, media was removed and replaced with serum-free media containing 10% CCK-8 dye, and plates were allowed to incubate at 37°C for 30 minutes. Absorbance was measured at 450 nm using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, US).
2.16 Mitochondrial-Targeted drugs

Chlorambucil, doxorubicin, and the synthetic compound acyl-acyl-cis-Diammineplatinum (II)-β-Diketonate (Wilson and Lippard, 2012) were conjugated to the mitochondria-penetrating peptide of the sequence F₅rF₅rF₅r (Fx = cyclohexylalanine) as previously described (Fonseca et al., 2011; Horton et al., 2008). This was performed by collaborators from the Shana Kelley lab.

2.17 Cellular bioenergetic profiling

Cellular energy metabolism in intact cells was profiled by the measurement of the two main bioenergetic pathways: oxidative phosphorylation (OXPHOS) and glycolysis by established methods (Qian and Van Houten, 2010). Metabolic profiles over time were measured in a Seahorse XF24 Extracellular Flux analyzer (Seahorse Biosciences, Billerica, MA). Oxidative phosphorylation was measured by the oxygen consumption rate (pmol/min) and glycolysis was measured by the extracellular acidification rate (ECAR). During the pharmacological profiling assay of OXPHOS and glycolysis the following pharmacological probes were added sequentially: (1 µM oligomycin, an F₁F₀-ATPase inhibitor; 300 nM carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), an electron transport chain uncoupler; 100 mM 2-deoxy-D-glucose (2-DG), an inhibitor of glycolysis followed by 1 µM rotenone, an inhibitor of complex I of the electron transport chain). These experiments were performed by the laboratory of Bennet VanHouten in the University of Pittsburgh.

2.18 Measurement of mitochondrial length

This experiment was performed in primary MEFs of wild type and Polg⁻/⁻ genotype by a collaborator Dr. Robert Screaton from the Children’s Hospital of Eastern Ontario, University of Ottawa. 450 cells were seeded per well of a 384-well plate in 40 µL of media. Virus infection to overexpress mouse Drp1(K38A) was performed at 48h after seeding. At 72h after seeding cells were fixed with 3.7% formaldehyde, and stained with TOMM20 antibody (1:1000, Santa Cruz Biotechnologies), which targets the translocase of outer mitochondrial membrane 20 homolog, a protein found throughout the mitochondrial outer membrane. Automated high-throughput immunofluorescence imaging was carried out with a Cellomics microscope, and used to calculate aspect ratio, a quantitative measure of individual mitochondrial length.
2.19 Colourimetric MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) -reducing activity was measured by a colourimetric assay. MTT is a yellow tetrazole that becomes reduced to a purple formazan by cellular metabolic activity (van Meerloo et al., 2011). 1.8x10^4 immortalized MEFs of wild type and Polg^{m/m} genotype were seeded per well of 24well plates and pre-treated with 1 µM MDIVI-1 for 24h, then exposed to 5,10 nM rotenone and 0.1% DMSO vehicle control for 48h. Following exposure, media was removed, cells were washed once with PBS, and incubated in OPTI-MEM with 0.5mg/mL MTT (Sigma, Oakville, ON, Canada) for 1h at 37°C. Blue crystals that formed were dissolved in DMSO. Samples were transferred to 96 well plates and optical density was measured using a spectrophotometric plate reader at 560nm and 620nm (Quincozes-Santos et al., 2010).

2.20 Transfection of MEFs with pEGFP-LC3

Wild type immortalized MEFs were seeded at a density of 3.3x10^5 cells per 60mm dish and allowed cells to settle and attach overnight. The cells were transfected with pEGFP-LC3 (Addgene plasmid 21073) by adding 3 µg of plasmid and 12 µL of Lipofectamine2000 (Life Technologies) in 1 mL OPTI-MEM to the cells. Once the transfection plate became confluent, cells were seeded into glass coverslips at a density of 1.6x10^5 cells per coverslip, and cells were treated with 1 µM rapamycin and 10 mM LiCl for 24 hours. Autophagy was visualized by GFP-LC3 fluorescence using the AxioImager.M2 Epifluorescence Microscope (Zeiss). Autophagy was identified as the presence of GFP-punca.

2.21 Over-expression and western blot analysis of Mfn1/2

Immortalized MEFs of wild type and Polg^{m/m} genotype were seeded at a density of 3.3x10^5 cells per 60mm dish and allowed cells to settle and attach overnight. The cells were transfected with addgene plasmids 23212 and 23213 (Chen et al., 2003) to overexpress Mfn1-Myc and Mfn2-Myc, respectively. Transfection was performed by adding 3 µg of plasmid and 12 µL of Lipofectamine2000 (Life Technologies) in 1 mL OPTI-MEM to the cells. GFP transfection was also carried out as a control of positive transfection efficiency. Overexpression of Mfn1/2 was confirmed by western blot analysis. Transfected wild type cells were collected using non-enzymatic cell dissociation solution (Sigma). Collected cells were washed once with PBS and
centrifuged at 524 g for 5 minutes. The supernatant was removed and cells were lysed by incubation with 1x RIPA buffer supplemented with EDTA-free proteinase inhibitors (Roche 11-836-1170-001) and 1 mM PMSF on ice for 5 minutes. The cell lysates were centrifuged at 14000 g for 10 minutes at 4°C, and the supernatant containing proteins was collected. The protein concentration in the protein lysates was quantified using a BCA protein assay kit (Thermo Scientific) following manufacturer’s instructions. Protein lysates were boiled with 6x SDS buffer at 95°C for 5 minutes and loaded into wells of a 10% SDS-PAGE gel and the gel was run at 100 V for 2 hours. The protein bands were transferred from the SDS-PAGE gel to a PVDF membrane using a 48 mM Tris, 39 mM glycine transfer buffer and running the transfer at 15 V for 20 minutes in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Non-specific sites were blocked with 1 hour incubation in 5% milk in PBS. After blocking, the membrane was washed three times in 0.05% Tween20 in PBS for 5 minutes and incubated with anti-C-Myc primary antibody (1:2000 C3956 Sigma) for 2 hours. After incubation with primary antibody, the membrane was washed three times in 0.05% Tween20 in PBS and incubated with secondary antibody (1:10000 anti-mouse ECL) in wash buffer for 1 hour. The membrane was washed three times in wash buffer and immunoreactivity was visualized using the Western Lightning Plus-ECL substrate (PerkinElmer, Waltham, MA, USA) following manufacturer’s instructions. Clonogenic assay on transfected cell lines was performed as described above (Section 2.3).

2.22 LC3 western blot analysis

Immortalized MEFs of wild type and \( Polg^{m/m} \) genotype were pre-treated with 1µM rapamycin or 0.1% DMSO vehicle control for 48h. In the last 4 h of incubation, media was replaced with fresh rapamycin or vehicle media containing 100 nM bafilomycin A. Cells were harvested non-enzymatically in 1 mM EDTA and lysed in 1x RIPA buffer containing protease inhibitors (cOmpleteMini, EDTA-free, Roche) and 1 mM PMSF. The cell lysates were cleared by centrifugation at 14000g for 10min at 4°C, and protein concentration in the supernatants was quantified using the BCA protein assay kit (Thermo scientific). Equal amounts of lysate protein were loaded and separated on 15% SDS-PAGE gel run at 100 V for 1:45 h. The proteins were transferred to a PVDF membrane and nonspecific sites were blocked with 5% nonfat dry milk in PBS with 0.3% tween20 for 1h. The membrane was incubated with LC3 antibody in blocking solution (1:500) (clone 5F10, nanotools) at 4 °C overnight. The membrane was washed 3 times
with 0.1% tween20 in PBS and incubated in secondary antibody (1:10000 anti-mouse ECL) in wash buffer for 45 min. Immunoreactivity was visualized using the Western Lightning Plus-ECL substrate (PerkinElmer, Waltham, MA, USA). The expected molecular weight is 14-16 kDa, with the pro-autophagy lipidated LC3-II form migrating faster than the non-lipidated cytosolic LC3-I form. The membrane was stripped using a mild stripping buffer with 200 mM glycine, 3.47 mM SDS, and 1% tween20 (Abcam) for 20 min, followed by two washes with PBS. Following membrane stripping, the protocol was repeated on the same membrane from the blocking step using anti-α-Tubulin (1:1000 Millipore) primary antibody as a loading control.

2.23 Statistical analyses

Statistical analysis was performed using two-tailed t tests, non-linear regression for estimation of IC$_{50}$, one-way ANOVA with bonferroni post-tests, or two-way ANOVA with bonferroni post-tests, as appropriate, using Graphpad Prism software. p values < 0.05 were considered statistically significant ( *p < 0.05, **p < 0.01, ***p < 0.001).
3 Results

3.1 mtDNA replication fidelity protects against drugs that impair the electron transport chain

Data presented within this section addresses the first aim of the hypothesis, which is whether mtDNA replication fidelity conferred by Polg is protective against toxicity initiated by drugs that impair the ETC.

In the measurement of mtDNA mutation load experiments (Figure 8A, 16B), the random mutation capture assay was carried out by Dr. Nolan Ericson and Dr. Jason Bielas at the Fred Hutchinson Cancer Research Center. Fernando Bralha provided the MEF and neuronal cell lines and received raw data for data analysis.

In the flow cytometry experiments for Annexin-V (Figure 9 C,D, 16B) and BrdU incorporation (Figure 10A,B), sample preparation and data analysis were carried out by Fernando Bralha, and acquisition of flowgrams was performed by Nishani Rajakulendran.

In the flow cytometry experiments for the measurement of mitochondrial mass (Figure 8C, D), sample preparation and data analysis were carried out by Fernando Bralha, and acquisition of flowgrams was performed by Laura Tamblyn. All other experiments presented in this section were performed by Fernando Bralha.
3.1.1 Measurement of mtDNA mutation load

The primary molecular phenotype of the Polg

exonuclease activity resulting in increased de novo mtDNA point mutations (Kujoth et al., 2005; Trifunovic et al., 2004). In order to confirm this phenotype on Polg

MEFs generated in our laboratory, the mtDNA mutation load was measured in primary and immortalized MEFs of wild type, Polg

and Polg

genotype. This experiment was performed in order to determine the effects of impaired mtDNA replication fidelity on mtDNA mutation load. The method of mtDNA mutation load measurement chosen was the random mutation capture (RMC) assay. The RMC assay is highly sensitive, able to detect one mutation in $10^9$ bases, and relies on single molecule amplification to detect rare mutations even among an overwhelming number of wild type copies (Vermulst et al., 2008). Spontaneous mutations are rare events that occur randomly throughout the mitochondrial genome. Since mutations need to be present in 10-25% of the population of molecules in order to be detected by sequencing, most de novo mtDNA mutations cannot be readily detected by sequencing (Vermulst et al., 2008). Several sequencing methods account for this by amplifying individual DNA molecules before sequencing, but this amplification may introduce additional mutations, and this creates a detection limit for spontaneous mutations (Vermulst et al., 2008). The RMC assay is a very sensitive method for the measurement of mtDNA mutation load that is designed to avoid these technical limitations. In this assay, the restriction enzyme TaqI is used to digest wild type genome copies, but preserve mutated copies harboring a point mutation within the restriction site (Vermulst et al., 2008). These mutated mtDNA molecules can then be quantified by real-time PCR using primers flanking the TaqI restriction site. The number of total mtDNA copies assayed can be quantified using another set of real-time primers that are not affected by TaqI digestion (Vermulst et al., 2008). This experiment was performed by collaborators in the genomics lab in the Fred Hutchinson Cancer Research Center.

As predicted, the level of mtDNA mutations was significantly higher in Polg

MEFs than in wild type (Figure 8A). This was observed both in primary cells and those immortalized with SV40 large-T antigen. In primary MEFs, mtDNA mutation load was approximately 50-fold higher in Polg

compared to wild type (p<0.05) (Figure 8A), while in immortalized MEFs the mutation load was approximately 300-fold higher in Polg

compared to wild type (p<0.001). Polg

heterozygous MEFs were also analyzed, and exhibited an intermediate level of mtDNA
mutations, 25-fold higher than wild type in primary MEFs and approximately 100-fold higher than wild type in the immortalized MEFs. The mtDNA mutation load in immortalized MEFs was 3-fold higher in Polg<sup>m/m</sup> compared to Polg<sup>+/m</sup> MEFs (p<0.01). The immortalization process did not significantly alter mtDNA mutation load in any of the three genotypes. No differences were observed between primary and immortalized MEFs. The mutation load in Polg<sup>n/m</sup> MEFs trended towards an increase in immortalized compared to primary MEFs, but this trend did not reach statistical significance.
Figure 8. Characterization of Polg^{mim} MEFs. (A) Increased random mitochondrial DNA mutations in primary and immortalized Polg^{mim} MEFs. Mutation frequency (+ S.D.) was measured at Taq I restriction sites within 12s rRNA gene within mitochondrial DNA isolated from wild type (open bars), Polg^{mim} (grey bars) or Polg^{mim} (black bars) MEFs (n=3 independent cell lines per group) (*p<0.05, **p<0.01, ***p<0.001). (B) Proliferation curves (mean cumulative cell number +/- S.E.M., n=3 technical replicates) of SV40-immortalized Polg^{mim} (solid circles and squares, dotted lines) and wild type (open circles and squares, solid lines) MEFs grown in glucose-containing (squares) or galactose-containing (circles) media (*p<0.001). (C) Mitochondrial mass assessed by MitoTracker Deep Red fluorescence (mean +/- S.E.M., n=3 independent cell lines per group) in Polg^{mim} (black bars) and wild type (open bars) MEFs. (D) Representative flow cytometry graphs of mitochondrial mass assessed by MitoTracker Deep Red in Polg^{mim} (red) and wild type (blue) MEFs.
3.1.2 Cellular growth curve under glucose and glucose-free media conditions

A cell proliferation curve was carried out in order to identify any basal defects in the cellular proliferation of the Polg<sup>m/m</sup> model. This was done in glucose and glucose-free media conditions. The two major metabolic pathways for generating ATP are oxidative phosphorylation (OXPHOS) and glycolysis (Marroquin et al., 2007). Standard cell culture media typically contains glucose concentrations much higher than physiological levels (Marroquin et al., 2007). This allows cells to generate most of their ATP via glycolysis and not mitochondrial respiration (Rodriguez-Enriquez et al., 2001). For this reason, cells cultured under routine glucose media conditions may gain increased resistance to drugs and xenobiotics that impair mitochondrial function (Marroquin et al., 2007). However, if galactose instead of glucose is the only carbon source, no net ATP-equivalents will be generated by glycolysis. This forces cells to once again rely on mitochondrial respiration for ATP energy production, and as a consequence restores sensitivity to xenobiotics that impair mitochondrial function (Marroquin et al., 2007).

There was a significant difference in cell number between Polg<sup>m/m</sup> and wild type MEFs after 12 days in both media conditions (p<0.001) (Figure 8B). While the switch from glucose to galactose media caused an approximate 5-fold decrease in wild type cumulative cell number over 12 days, it caused an approximate 30-fold decrease in Polg<sup>m/m</sup> cumulative cell number.

3.1.3 Mitochondrial mass

The mitochondrial mass was measured to further characterize the mitochondrial health of Polg<sup>m/m</sup> MEFs. Abnormal mitochondrial mass is a common sign of mitochondrial dysfunction, as it serves as a compensatory measure in response to reduced mitochondrial function (Apostolova et al., 2010; Lee et al., 2002; Nugent et al., 2007). Abnormally large mitochondria have been observed in the cardiomyocytes of Polg<sup>m/m</sup> mice (Trifunovic et al., 2004). In order to measure the overall mitochondrial mass in Polg<sup>m/m</sup> MEFs, we performed flow cytometry analysis of primary MEFs stained with the mitochondrial-specific dye MitoTracker, which accumulates in the mitochondria in a manner relatively dependent on mitochondrial membrane potential. There was no significant difference in the intensity of MitoTracker fluorescent signal between Polg<sup>m/m</sup> and wild type MEFs (Figure 8C). Representative flow cytometry graphs of one of the three wild type
and $Polg^{m/m}$ MEFs pairs analyzed shows that the distribution of MitoTracker FL4 fluorescence is very similar for both genotypes (Figure 8D).

### 3.1.4 Decreased colony forming ability in $Polg^{m/m}$ MEFs exposed to rotenone

In order to examine the role of mtDNA replication fidelity in the cellular sensitivity to rotenone, a clonogenic survival assay was performed using immortalized $Polg^{m/m}$, $Polg^{+/m}$, and wild type MEFs. The clonogenic assay provides a very sensitive measure of cellular toxicity because it requires cells to survive drug exposure and proliferate into colonies. Rotenone caused a concentration-dependent decrease in the colony-forming ability of $Polg^{m/m}$ MEFs which was significantly different from wild type at concentrations of 1, 2, and 5 nM rotenone ($p<0.001$) (Figure 9A). The IC$_{50}$ of rotenone for clonogenic lethality was 2.5 nM for $Polg^{m/m}$ and $>5$ nM for wild type and $Polg^{+/m}$ MEFs. $Polg^{+/m}$ did not differ from wild type in their survival following rotenone exposure.
Figure 9. Rotenone reduces survival and increases apoptosis of Polg<sup>mim</sup> MEFs. (A) Clonogenic survival curve of Polg<sup>mim</sup> (solid circles and dotted line), Polg<sup>im</sup> (solid triangles and dashed line) and wild type (solid squares and solid line) MEFs cultured in 0.25 to 5 nM rotenone for 1 week; % colony forming units normalized to DMSO vehicle control, mean +/- S.E.M. (n=4-6 experimental replicates) (**p<0.001, different from wild type). (B) Acute necrosis assessed by plasma membrane integrity 24 h after 5 – 50 nM rotenone in Polg<sup>mim</sup> (dashed line) and wild type (solid line) MEFs; data normalized to DMSO vehicle control, mean +/- S.E.M., (n=3 independent cell lines per group). (C) Cell survival assessed by flow cytometric analysis of Annexin V negative, PI negative population of Polg<sup>mim</sup> (black bars) and wild type (open bars) MEFs exposed to 10 nM rotenone for 24 h, mean +/- S.E.M., (n=3 experimental replicates) data normalized to DMSO vehicle control (**p<0.001). (D) Apoptosis assessed by flow cytometric analysis of Annexin V positive population of Polg<sup>mim</sup> (black bars) and wild type (open bars) MEFs exposed to 10 nM rotenone for 24 h, mean +/- S.E.M. (n=3 experimental replicates), data normalized to DMSO vehicle control (**p<0.001).
3.1.5 Rotenone reduces survival of Polg\textsuperscript{m/m} MEFs through apoptosis and not necrosis

Following the observation of a strong sensitivity of Polg\textsuperscript{m/m} MEFs to rotenone, we performed a series of experiment to elucidate the mechanism of cell death. We assessed apoptosis and necrosis in wild type and Polg\textsuperscript{m/m} MEFs exposed to rotenone. Acute necrotic protease activity as a percentage of DMSO vehicle control did not significantly increase from baseline in either genotype (Figure 9B) even when cells were exposed to rotenone concentrations as high as 50 nM. Flow cytometry analysis for apoptosis was also performed in cells exposed to DMSO vehicle control or 10 nM rotenone for 24h. In the absence of rotenone, both genotypes had a baseline cell viability of approximately 85-90\%. Polg\textsuperscript{m/m} MEFs trended towards slightly lower viability relative to wild type, but this was not significantly different. Following the 24h exposure to 10 nM rotenone, wild type viability was not significantly altered, while the viability of Polg\textsuperscript{m/m} MEFs dropped to approximately 65-70\%. Under this condition, the viability of Polg\textsuperscript{m/m} MEFs was significantly lower than wild type (p<0.001) (Figure 9C). In the absence of rotenone, the Annexin-V positive cell population was approximately 10-15\% for both genotypes. There was a trend of higher baseline apoptosis in Polg\textsuperscript{m/m} MEFs compared to wild type, but this was not significant. Following exposure to rotenone, the Annexin-V positive population of wild type cells was not changed, while it increased to approximately 28\% for Polg\textsuperscript{m/m} MEFs. This resulted in an almost 3-fold increase in Annexin-V positive cells in the Polg\textsuperscript{m/m} MEFs relative to wild type (p<0.001) (Figure 9D).

3.1.6 Rotenone decreases cellular proliferation equally in Polg\textsuperscript{m/m} and wild type MEFs

As an alternate explanation for the decreased colony forming ability of Polg\textsuperscript{m/m} MEFs exposed to rotenone, we sought to determine whether rotenone was affecting cellular proliferation in addition to causing cell death preferentially in Polg\textsuperscript{m/m} MEFs. In order to determine the effect of rotenone on cellular proliferation, BrdU was used to label cells in S-phase during a flow cytometry experiment on cells treated with rotenone. Since the cell growth curves for Polg\textsuperscript{m/m} immortalized MEFs differed in glucose and galactose media (Fig. 8B), the effect of rotenone on cell proliferation was assessed in both media conditions. In glucose media, the percentage of cells in S-phase remained constant at approximately 35-45\%, and was not altered in either genotype by the presence of rotenone (Figure 10A). In galactose media, the percentage
of cells in S-phase remained in the 35-45% range for both genotypes in DMSO vehicle control samples. Cells grown in galactose media exposed to rotenone had an approximate 50% reduction in the proportion of cells in S-phase compared to DMSO vehicle (p<0.01) (Figure 10B). This rotenone effect was observed equally in both genotypes. In representative flow cytometry graphs the data can be plotted with cell counts on the y-axis and BrdU incorporation (FL1-FITC fluorescence) in the x-axis. The peak closest to the y-axis represents cells considered negative for BrdU incorporation which are not in S-phase. The peak further away from the y-axis represents cells positive for BrdU incorporation which are considered to be in S-phase. The exact boundary between the two peaks was determined through the appropriate control samples. The percent of cells in S-phase for each sample is the area under the BrdU-positive peak, as a percentage of the area under both peaks (Figure 10C).
Figure 10. Effects of Rotenone on cellular proliferation of Polg<sup>mm</sup> and wild type MEFs. Effect of rotenone on the proportion of MEFs in S-phase in cells grown in glucose media (A) or galactose media (B). Polg<sup>mm</sup> (black bars) and wild type (white bars) immortalized MEFs were treated with 10 nM rotenone for 24 hours and BrdU for one hour to mark DNA replication. The proportion of S-phase cells was determined by flow cytometry (mean +/- S.E.M., n=3 independent cell lines per group) (**p<0.01). (C) Representative flow cytometry graphs of BrdU incorporation in Polg<sup>mm</sup> and wild type MEFs. Each flow cytometry graph shows plots of 3 BrdU-negative populations (brown, red and orange peaks) and 3 BrdU-positive populations (blue, purple and green peaks) representative of 3 independent cell lines per group.
3.1.7 No detectable change in ROS generation caused by rotenone in \( Polg^{m/m} \) and wild type MEFs

Since one of the main mechanisms of toxicity of rotenone is reactive oxygen species (ROS) generation, we sought to determine whether this plays a role in the observed \( Polg^{m/m} \) sensitivity to rotenone. A DCF fluorescence assay was used to measure relative ROS generation in MEFs in response to rotenone exposure. This experiment was used to investigate whether the susceptibility arises due to increased generation of ROS in \( Polg^{m/m} \) compared to wild type MEFs.

The DCF fluorescence of \( Polg^{m/m} \) DMSO control samples was approximately 10% higher than that of wild type MEFs, but this was not statistically significant \( (t = 0.6952, p > 0.05) \) (Figure 11). In rotenone-treated cells, DCF-fluorescence trended towards an increase in fluorescence for both genotypes to approximately 120% of wild type DMSO control, but this increase was not statistically significant. The antioxidant N-acetyl-cysteine (NAC) was also tested as a positive control of ROS modulation. MEFs treated with NAC trended towards a decrease in DCF fluorescence to approximately 70% for wild type and 80% for \( Polg^{m/m} \) MEFs. This decrease in DCF fluorescence was statistically significant for \( Polg^{m/m} \) \( (p < 0.05) \) but not wild type MEFs. The difference between genotypes was also not significant in these samples. When cells were co-treated with both rotenone and NAC, the apparent trends caused by the two treatments seemed to cancel each other off, as the fluorescence remained very close to base line, with approximately 110% fluorescence for wild type and 105% fluorescence for \( Polg^{m/m} \) MEFs, relative to wild type DMSO controls.
Figure 11. No detectable change in ROS generation caused by rotenone in Polg<sup>mtm</sup> and wild type MEFs. Effects of rotenone and N acetyl-cysteine on ROS generation in Polg<sup>mtm</sup> (black bars) and wild type (white bars) MEFs treated with DMSO vehicle control, 10 nM rotenone, or 500 µM N acetyl-cysteine (n=3 technical replicates, +/- S.E.M.) (*p<0.05 different from DMSO).
3.1.8 Rotenone decreases ATP levels in Polg\textsuperscript{m/m} MEFs

Since rotenone inhibits complex I of the mitochondrial electron transport chain, we assessed whether rotenone preferentially decreased ATP levels in Polg\textsuperscript{m/m} MEFs. Furthermore, ATP is the final product of mitochondrial oxidative phosphorylation and the major source of energy in the cell. It is an important endpoint of cellular toxicity and correlates well with cell viability in most cell lines. In glucose media, the number of viable cells determined by trypan exclusion was not significantly altered in either Polg\textsuperscript{m/m} or wild type MEFs (Figure 12A). In glucose media conditions, rotenone caused a concentration-dependent decrease in ATP in Polg\textsuperscript{m/m} but not wild type MEFs (Figure 12C). In galactose media, the number of viable cells determined by trypan exclusion was lower in Polg\textsuperscript{m/m} than wild type MEFs at rotenone concentrations of 0 – 5 nM (p < 0.01) (Fig. 12B). In galactose media, rotenone caused a concentration-dependent decrease in ATP in both genotypes and the ATP levels in Polg\textsuperscript{m/m} MEFs were significantly less than those in wild type MEFs at rotenone concentrations of 0, 1, 2.5 and 5 nM (p < 0.01)(Figure 12D).
Figure 12. Effect of rotenone on ATP content of Polg<sup>m/m</sup> and wild type MEFs. (A,B) Number of cells at 48h resulting from 1 X 10<sup>5</sup> seeded. (A) Glucose-grown or (B) galactose-grown Polg<sup>m/m</sup> (dashed lines) and wild type (solid lines) MEFs were treated with 1 to 10 nM rotenone for 48 h and counted. Asterisks indicate different from Polg<sup>m/m</sup>, **p< 0.01, ***p<0.001 (mean +/- S.E.M., n=3 independent cell lines per group). C,D) ATP levels indicated by relative luminescence units in MEFs at 48 hours. (C) Glucose-grown or (D) galactose-grown Polg<sup>m/m</sup> (dashed lines) or wild type (solid lines) MEFs were treated with 1 to 10 nM rotenone for 48 h and ATP levels were measured. Asterisks indicate different from Polg<sup>m/m</sup>, *p<0.05, **p< 0.01, ***p<0.001 (mean +/- S.E.M., n=3 independent cell lines per group).
3.1.9 Increased mtDNA damage in $Polg^{m/m}$ MEFs compared to wild type

In this experiment we sought to characterize what effect the $Polg^{m/m}$ proofreading mutation has on endogenous levels of mtDNA damage, and what effect rotenone exposure will have on the levels of mtDNA lesions on each genotype. In this quantitative PCR assay $Polg^{m/m}$ MEFs had decreased mtDNA long fragment amplification compared to wild type ($p<0.01$) (Figure 13A). The relative amplification of long PCR was normalized to wild type DMSO values. In vehicle treated $Polg^{m/m}$ MEFs, the relative amplification was approximately half that of wild type. Rotenone treatment did not significantly alter the mtDNA lesion levels for either wild type or $Polg^{m/m}$ MEFs. The increase in polymerase-blocking lesions in the mtDNA of DMSO treated $Polg^{m/m}$ MEFs compared to wild type is approximately 0.6 lesions per 10kB. Since the mitochondrial genome is 16.5kB, this translates to approximately 1 additional polymerase-blocking lesion per $Polg^{m/m}$ mtDNA molecule compared to wild type.
Figure 13. Increased polymerase-blocking mitochondrial DNA damage in Polg<sup>m/m</sup> MEFs.
Long PCR analysis of a 10 kbp mitochondrial DNA fragment was performed on total DNA isolated from Polg<sup>m/m</sup> (black bars) or wild type (white bars) MEFs. (A) Relative levels of amplification of the 10 kbp mtDNA amplicon normalized to mtDNA copy number using the 117 bp mtDNA amplicon (n = 3 independent cell lines per group, long PCR analyses performed in duplicate) in MEFs exposed to 10 nM rotenone or DMSO vehicle control for 24 h. Asterisk indicates different from wild type, *p<0.05, **p< 0.01 (mean +/- S.E.M.). (B) Mitochondrial DNA copy number in Polg<sup>m/m</sup> and wild type MEFs (n = 3 independent cell lines per group, QPCR analyses performed in duplicate) exposed to 400 μM H<sub>2</sub>O<sub>2</sub> for one hour and then harvested at 8 hours, or exposed to rotenone or DMSO vehicle for 6 or 24 h, normalized to wild type vehicle control. Asterisk indicates significantly different from wild type, *p<0.05, **p< 0.01, ***p<0.001 (mean +/- S.E.M.).
3.1.10 Increased mtDNA copy number in \textit{Polg}^{m/m} MEFs

Alterations in mtDNA abundance are another common sign of mitochondrial disease (Cohen and Naviaux, 2010; Lebedeva et al., 2009). Real-time PCR was used to determine whether mtDNA abundance was altered in immortalized \textit{Polg}^{m/m} MEFs relative to wild type MEFs. In cells treated with 0.1\% DMSO vehicle control, mtDNA abundance relative to nuclear genome was significantly higher in \textit{Polg}^{m/m} MEFs compared to wild type (p<0.05). mtDNA copy number was approximately 25\% higher in \textit{Polg}^{m/m} than in wild type MEFs (Figure 13B).

3.1.11 Rotenone does not alter mtDNA copy number

Since excessive oxidative stress can result in mitochondrial DNA depletion (Clay Montier et al., 2009; Gilkerson, 2009), we assessed whether rotenone exposure caused mtDNA depletion in either genotype, to determine if this was a possible mechanism for the rotenone toxicity observed. \textit{Polg}^{m/m} MEFs and wild type MEFs were exposed to 10 nM rotenone or 0.1\% DMSO vehicle control for either 6 or 24h. Following drug exposure the total DNA was isolated and real-time PCR was used to quantify mtDNA abundance relative to genomic DNA. In vehicle control samples for both time points, \textit{Polg}^{m/m} MEFs had increase mtDNA abundance compared to wild type (p< 0.05) (Figure 13B). Rotenone treatment did not alter mtDNA copy number of either genotype at 6 or 24h after exposure (Figure 13B). In the 6h rotenone exposure samples, the increased mtDNA abundance in \textit{Polg}^{m/m} MEFs relative to wild type was also observed (p<0.01). At 24h of rotenone exposure, mtDNA abundance was no longer significantly different between \textit{Polg}^{m/m} and wild type by post hot tests.

3.1.12 Hydrogen peroxide causes mtDNA depletion in wild type but not \textit{Polg}^{m/m} MEFs

In a separate experiment, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was used as a positive control for mtDNA depletion. High concentrations of hydrogen peroxide cause excessive oxidative stress, leading to degradation of damaged mtDNA molecules and severe mtDNA depletion (Shokolenko et al., 2009; Van Houten et al., 2006). As expected, 400 \textmu M H\textsubscript{2}O\textsubscript{2} acutely reduced mtDNA copy number 4-fold in wild type MEFs compared to untreated controls (p<0.001)(Figure 13B). However, in \textit{Polg}^{m/m} MEFs H\textsubscript{2}O\textsubscript{2} did not alter mtDNA copy number relative to untreated controls. There was a significant difference between mtDNA copy number of H\textsubscript{2}O\textsubscript{2} treated \textit{Polg}^{m/m} and wild type MEFs (p< 0.001).
3.1.13 Polg<sup>m/m</sup> MEFs have cellular sensitivity to inhibition of other ETC complexes

Following our observations of increased cellular toxicity in Polg<sup>m/m</sup> MEFs exposed to rotenone, we sought to determine whether this was an effect unique to complex I, or if similar observations could also be made with other ETC complexes. In order to assess whether mitochondrial DNA replication fidelity protects against the toxicity of other ETC complex inhibitors, we tested antimycin A, an inhibitor of complex III (van Keulen and Berden, 1985) and cyanide, an inhibitor of complex IV (Way, 1984). Rotenone was included again in this set of experiments as a positive control. A soluble and non-toxic tetrazolium salt reduction assay similar to MTT was used to assess cell viability. This experiment was carried out in glucose and galactose media conditions. As previously observed, rotenone was more toxic to Polg<sup>m/m</sup> MEFs than wild type (Fig. 14 A, C). In glucose media viability was significantly lower in Polg<sup>m/m</sup> than wild type at 2.5, 5 and 10 nM rotenone (Fig. 14A). In galactose media, viability was significantly lower in Polg<sup>m/m</sup> than wild type at 2.5, 5, 10 and 20 nM rotenone (Fig. 14D). For antimycin A, viability was significantly lower in Polg<sup>m/m</sup> than wild type at 10 nM antimycin A in glucose media (Fig. 14B) while in galactose media, antimycin A reduced viability to a similar extent in both Polg<sup>m/m</sup> and wild type (Fig. 14E). For cyanide, viability was significantly lower in Polg<sup>m/m</sup> than wild type at 1, 5, 10 and 25 nM potassium cyanide in glucose media (Fig. 14C), while no genotype differences were observed in galactose media (Fig. 14F).
Figure 14 Polg\textsuperscript{mim} MEFs have cellular sensitivity to other ETC complex inhibitors. The effect of mitochondrial electron transport chain inhibitors on the viability of wild type (squares and solid lines) and Polg\textsuperscript{mim} (circles and dashed lines) MEFs in glucose (A, B, C) or galactose (D, E, F) media conditions 48 h after the addition of drugs/chemicals. Viability was assessed by tetrazolium salt reduction. (A, D) Viability after rotenone. (B, E) Viability after antimycin-A. (C, F) Viability after potassium cyanide. Asterisks indicate different from Polg\textsuperscript{mim}, n = 3 technical replicates, mean +/- S.E.M., *p<0.05, **p<0.01, ***p<0.001.
3.1.14 Polg<sup>m/m</sup> MEFs have cellular sensitivity to clinical drugs with off-target ETC complex I inhibition

Following our observations of increased cellular toxicity in Polg<sup>m/m</sup> MEFs exposed to rotenone, we sought to determine whether this was an effect unique to rotenone, or if similar observations could also be made with other complex I inhibitors. We tested three drugs with off-target inhibition of complex I: the lipid-lowering drug clofibrate (Brunmair et al., 2004a), the antifungal drug ketoconazole (Rodriguez and Acosta, 1996), and the anti-androgen drug flutamide (Fau et al., 1994; Hynes et al., 2006). A soluble and non-toxic tetrazolium salt reduction assay similar to MTT was used to assess cell viability. This experiment was carried out in glucose and galactose media conditions. Clofibrate at 1 mM was found to be more toxic to Polg<sup>m/m</sup> compared to wild type in both media conditions (Figure. 15A,C). Ketoconazole was more toxic to Polg<sup>m/m</sup> than wild type MEFs at 5 and 10 µM in glucose media (Fig. 15B). No genotype differences were observed for ketoconazole toxicity in galactose media (Fig. 15E). For flutamide in either glucose media or galactose media, viability was similarly reduced in Polg<sup>m/m</sup> and wild type MEFs (Fig. 15C,F).
Figure 15 Polg<sup>−/−</sup> MEFs have cellular sensitivity to clinical drugs with off-target Complex I inhibition. The effect of mitochondrial electron transport chain inhibitors and drugs with off-target inhibition of complex I on the viability of wild type (squares and solid lines) and Polg<sup>−/−</sup> (circles and dashed lines) MEFs in glucose (A, B, C) or galactose (D, E, F) media conditions 48 h after the addition of drugs/chemicals. Viability was assessed by tetrazolium salt reduction. (A, D) Viability after Clofibrate. (B, E) Viability after ketoconazole. (C, F) Viability after flutamide. Asterisks indicate different from Polg<sup>−/−</sup>, n = 3 technical replicates, mean +/- S.E.M., **p<0.01, ***p<0.001.
3.1.15 Apoptosis in Polg^{m/m} or wild type cortical neurons is not increased by rotenone

After observing strong sensitivity in Polg^{m/m} MEFs to rotenone, we next sought to repeat this experiment in a secondary cell type that would be clinically relevant to mitochondrial dysfunction. Cortical neurons were chosen due to their additional biological relevance, as neurons are highly metabolically active, and neurological defects are clinically associated with POLG mutations (Hudson and Chinnery, 2006). A qualitative assessment of neuronal health following rotenone treatment was made by immunohistochemical analysis using β-tubullin-III as a marker for immature neurons (Figure 16A). At rotenone concentrations of 5 nM, neuronal viability appeared qualitatively to be reduced in both genotypes. This was observed through the presence of broken neuronal extensions, shrunken cell bodies, and an apparent decrease in number of neurons still attached to the coverslip and present during imaging (Figure 16A). In order to quantify these observations, flow cytometry analysis for apoptosis using Annexin-V and the vital dye PI was performed on wild type and Polg^{m/m} primary cortical neurons treated with rotenone for 24h. There was a slight trend towards increasing Annexin-V staining with higher rotenone concentrations in both genotypes, but this was not statistically significant. We observed no significant dose-dependent increase in apoptosis caused by rotenone. There was no significant difference in apoptosis between Polg^{m/m} and wild type neurons (Figure 16B).

3.1.16 Polg^{m/m} cortical neurons have increased mtDNA mutation load

In order to address the lack of observed sensitivity of Polg^{m/m} cortical neurons to rotenone, our collaborators measured mtDNA mutation load in wild type and Polg^{m/m} cortical neurons. This mtDNA mutation load measurement was performed by our collaborators using the random mutation capture assay similarly to the experiment on primary and immortalized MEFs. The level of mtDNA mutations in Polg^{m/m} cortical neurons was 25-fold higher than in wild type (p<0.01) (Figure 16C). However, this mutation load was 3 to 6-fold lower than that observed in Polg^{m/m} MEFs.
Figure 16. Polg<sup>mm</sup> and wild type cortical neurons are equally sensitive to rotenone. (A) Representative immunohistochemical imaging of wild type primary cortical neurons stained for the neuronal-specific marker beta-tubulin III (green) and the nuclear dye DAPI (blue), treated with DMSO vehicle control, 1 nM and 5 nM rotenone for 24h. Scalebar in DMSO image applies to all three images. (B) Apoptosis (n=3 independent cell lines per group) assessed by flow cytometric analysis of the Annexin-V positive, PI negative population of wild type (open bars) and Polg<sup>mm</sup> (solid bars) cortical neurons exposed to 1-50 nM rotenone for 24 h. Data normalized to DMSO vehicle. (C) Mitochondrial DNA mutation frequency in cortical neurons from wild type (open bars) and Polg<sup>mm</sup> (solid bars) mice, mean +/- S.E.M. (n=3 independent cell lines per group), *p < 0.05.
3.2 Role of mitochondrial stress responses in toxicity initiated by rotenone

Data presented within this section addresses the second aim of the hypothesis, which is to determine the role of mitochondrial stress responses in toxicity initiated by rotenone. Towards this aim, we assessed the function of mitochondrial dynamics and mitochondrial autophagy endogenously in $Polg^{m/m}$ MEFs relative to wild type. We also examined how their modulation affects the rotenone hypersensitivity observed in $Polg^{m/m}$ MEFs.

The measurement of mitochondrial length by high throughput microscopy (Figure 17B) was performed by Dr. Robert Screaton at the University of Ottawa. Fernando Bralha provided the MEF cell lines, received raw data, performed data analysis and figure preparation. All other experiments presented in this section were performed by Fernando Bralha.
3.2.1 Decreased mitochondrial length in $Polg^{m/m}$ MEFs

In order to determine whether mitochondrial dynamics are altered by impaired mtDNA replication fidelity, fluorescent microscopy using the mitochondrial dye MitoTracker deep red was used as a qualitative measurement of mitochondrial length and morphology in $Polg^{m/m}$ and wild type MEFs. Fluorescent microscopy images of $Polg^{m/m}$ and wild type MEFs stained with MitoTracker deep red suggest that mitochondria in $Polg^{m/m}$ MEFs exist as a population of more punctuate isolated organelles, while in wild type cells the mitochondria population form a more interconnected network (Figure 17A). In order to quantitatively confirm these observations, measurements of mitochondrial length were performed by our collaborator Dr. Robert Screaton in the University of Ottawa. Aspect ratio is a quantitative measure of individual mitochondrial length by high throughput immunofluorescence with a mitochondrial antibody TOMM20, immunoreactive against the mitochondrial outer membrane protein TOMM. Mitochondrial aspect ratio was lower in $Polg^{m/m}$ compared to wild type MEFs ($p<0.001$). As a technical positive control of increased mitochondrial length, MEFs overexpressing a dominant negative Drp1 mutant protein (K38A) were also analyzed. The dominant negative Drp1 protein inhibits mitochondrial fission and therefore is predicted to increase the aspect ratio for both genotypes. Wild type and $Polg^{m/m}$ MEFs expressing the dominant negative Drp1 protein both had an increase in aspect ratio compared to each genotype’s untransfected control. Even in the Drp1 dominant negative samples, mitochondrial length in $Polg^{m/m}$ MEFs remained significantly lower than wild type ($p<0.001$) (Figure 17B).
Figure 17. Decreased mitochondrial length in Polg<sup>m/m</sup> MEFs. (A) Fluorescent microscopy images of Polg<sup>m/m</sup> and wild type MEFs stained with 100 nM MitoTracker deep red and the nuclear dye DAPI. Insert shows 5x increased magnification. 63x magnification, scale bar represents 5 μm. (B) Measurement of mitochondrial morphology of Polg<sup>m/m</sup> (red boxes) and wild type (blue boxes) MEFs either untreated (solid boxes) or overexpressing a dominant negative Drp1 mutant protein (hatched boxes) by high throughput immunofluorescence with TOMM20 antibody. Aspect ratio is a measure of length of individual mitochondria. *P< 0.001 (N=2 independent cell lines per genotype, 30 mitochondria scored in untreated samples, 6 mitochondrial scored in Drp1(K83A) samples).
3.2.2 Pharmacological inhibition of mitochondrial fission does not attenuate rotenone toxicity

In order to determine whether this apparent decrease of mitochondrial length in Polg\textsuperscript{m/m} MEFs is mechanistically relevant to the rotenone sensitivity of this genotype, we attempted to decrease mitochondrial fission and assess if this has a protective role against rotenone susceptibility in Polg\textsuperscript{m/m} MEFs. The inhibition of fission results in reduced cellular sensitivity to apoptosis (Frank, 2006). Mitochondrial fission was inhibited pharmacologically using MDIVI-1 (mitochondrial division inhibitor) in Polg\textsuperscript{m/m} and wild type MEFs. These cells were subsequently challenged with rotenone and viability was measured using the MTT reduction assay. A MDIVI-1 cell toxicity curve was generated to determine the optimal concentration of MDIVI-1 to use as a pre-treatment. MDIVI-1 was highly toxic with an EC\textsubscript{50} of approximately 8 µM for both genotypes. The concentration chosen for subsequent pre-treatments was 1 µM, as this MDIVI-1 concentration was not toxic to either genotype. Contrary to the predicted rescue effect, 1 µM MDIVI-1 caused a decrease in viability for both genotypes. MDIVI-1 co-treatment decreased cell viability of Polg\textsuperscript{m/m} MEFs at rotenone concentrations of 0 and 5 nM (p<0.001). MDIVI-1 co-treatment decreased cell viability of wild type MEFs at rotenone concentrations of 5 and 10 nM (p<0.001) (Figure 18).
Figure 18. MDIVI-1 treatment does not attenuate rotenone toxicity. MTT-reduction levels at 48h normalized to DMSO vehicle. Polg<sup>nm</sup> (red lines) and wild type (blue lines) MEFs were treated with 5 and 10 nM rotenone for 48h in the presence (dotted lines) or absence (solid lines) of the mitochondrial fission inhibitor MDIVI-1 (**p<0.001) (Mean +/- S.E.M., n=3 technical replicates).
3.2.3 Overexpression of mitofusins does not alter rotenone toxicity

As an alternate way to determine whether increasing mitochondrial length can rescue the observed susceptibility to mitochondrial toxicity caused by impaired mtDNA replication fidelity in the Polg<sup>m/m</sup> MEFs, mitochondrial fusion was genetically enhanced by over-expression of mitofusins 1 and 2. It has been shown that mitofusin over-expression can reduce apoptosis (Chan, 2006b). Since the Polg<sup>m/m</sup> MEFs exhibited decreased mitochondrial length compared to wild type, increasing fusion may bring Polg<sup>m/m</sup> mitochondrial dynamics to comparable levels with wild type, and protect Polg<sup>m/m</sup> MEFs from the increased rotenone toxicity we have observed. A colony-forming assay was performed with Polg<sup>m/m</sup> and wild type MEFs overexpressing Mfn1/2 in the presence of rotenone or DMSO vehicle control.

Over-expression of Mfn 1/2 was confirmed by western blot analysis, where the Myc-tagged Mfn1 and Mfn2 proteins both have an apparent band size of 130kDa (Figure 19B). This over-expression did not significantly change the toxicity profile of rotenone. Polg<sup>m/m</sup> MEFs showed increased susceptibility to rotenone either in the absence or presence of Mfn 1, Mfn2, or Mfn1/2 over-expression (Figure 19A). Wild type MEFs exposed to 5 nM rotenone had 75-90% colony forming units relative to DMSO. Wild type MEF colony forming ability was not affected by transfection with mitofusins. Polg<sup>m/m</sup> MEFs exposed to 5 nM rotenone had 35-40% colony forming units relative to DMSO. Polg<sup>m/m</sup> MEF colony forming ability was also not altered by transfection with mitofusins.
Figure 19. Overexpression of mitofusins does not alter rotenone toxicity. (A) Clonogenic survival curve (n=3 technical replicates) of Polg<sup>nm</sup> (red bars) and wild type (blue bars) MEFs cultured in 5 nM rotenone for 1 week; data normalized to DMSO vehicle control. Prior to clonogenic assay, MEFs were transfected with vectors containing mitofusin1,2 or both (NT represents non-transfected control). (B) Overexpression of mitofusins was confirmed by western blot analysis of Myc epitope tag.
3.2.4 Rapamycin induces autophagy in MEFs

The second mechanism of mitochondrial stress response investigated was mitochondrial autophagy. Technical experiments were performed to determine whether autophagy could be induced pharmacologically in MEFs using 1 µM rapamycin. In order to monitor the induction of autophagy in our cells, we used western blot analysis to detect the conversion of LC3-I to LC3-II, which is a well recognized measure of cellular autophagy (Klionsky et al., 2008). Once the autophagosome merges with a lysosome, LC3 is also degraded. In order to prevent this degradation and preserve the LC3 signal we also incubated the cells with bafilomycin A1 (Mizushima et al., 2010). Addition of bafilomycin A1 to the cells greatly improved the LC3 band intensities by preventing mature autophagosomes from being degraded by lysosomes. The LC3-I and LC3-II bands have an apparent band size of approximately 15kDa (Gusdon et al., 2012; Kabeya et al., 2000), with the lipidated LC3-II form running slightly faster than the non-lipidated LC3-I (Kabeya et al., 2000). Subcellular fractionation studies have confirmed the differential distribution of the LC3-I and LC3-II proteins. Following ultracentrifugation (100,000 g) the LC3-I band has been shown to be recovered in the supernatant, consistent with a cytosolic protein. LC3-II was recovered only in the pellet fraction, and immunoblot analysis found LC3-II enriched in the autophagic vacuole fraction (Kabeya et al., 2000). Several non-specific bands are found above 35 kDa but did not interfere with quantification of the LC3-specific bands at approximately 15 kDa. In the bafilomycin A1-treated samples, autophagy, as measured by the ratio of LC3-II to LC3-I was increased almost 2-fold by 1 µM rapamycin-treated MEFs relative to untreated samples (Figure 20A).

3.2.5 Decreased mitophagy in Polg<sup>m/m</sup> MEFs

In order to determine whether mitochondrial autophagy is altered by decreased mtDNA replication fidelity in the Polg<sup>m/m</sup> model, we examined autophagy levels in Polg<sup>m/m</sup> and wild type MEFs. The LC3 western blot analysis described above was used to measure autophagy in MEFs either in vehicle control, or pre-treated with1 µM rapamycin. LC3-II band intensity was normalized to α-tubulin as a loading control. Autophagy was measured by the ratio of the lipidated LC3-II band intensity to the α-tubulin loading control. In DMSO vehicle control samples, Polg<sup>m/m</sup> MEFs showed a 2-fold decrease in autophagy relative to wild type (Figure 20B). Rapamycin increased autophagy by approximately 50% in both genotypes.
Figure 20. Decreased autophagy in Polg<sup>tm</sup> MEFs. (A) Western blot analysis of LC3-II : LC3-I. 100 nM rapamycin induces autophagy in MEFs relative to untreated (UT) control. Addition of bafilomycin A1 2h prior to protein harvest preserves LC3 signal. (B) Western blot analysis of autophagy measured as a ratio of LC3-II : alpha tubulin loading control. Polg<sup>tm</sup> have decreased autophagy compared to wild type. 1μM Rapamycin for 48h induces autophagy in both genotypes.
3.2.6 Autophagy inducer rapamycin attenuates rotenone toxicity in Polg\(^{m/m}\) MEFs

In order to determine whether mitochondrial autophagy can rescue the observed phenotype of rotenone susceptibility in Polg\(^{m/m}\) MEFs caused by impaired mtDNA replication fidelity, mitophagy was induced pharmacologically using rapamycin in Polg\(^{m/m}\) and wild type MEFs. Since the Polg\(^{m/m}\) MEFs exhibited less autophagy than wild type, increasing mitophagy may protect Polg\(^{m/m}\) MEFs from the increased rotenone toxicity we have observed. A 48h treatment with 1 \(\mu\)M rapamycin was able to increase autophagy in both genotypes. Polg\(^{m/m}\) and wild type MEFs were pre-treated with 1 \(\mu\)M rapamycin for 48h and challenged with rotenone. Cell viability was measured using the ATP levels. ATP levels as measured through luminescence by the luciferin-luciferase reaction are directly proportional to the number of live cells (Crouch et al., 1993). ATP standard curves and cell number standard curves are routinely performed and confirm the linear relationship between cell number and ATP luminescence. In the vehicle control samples, Polg\(^{m/m}\) MEFs once again exhibited increased sensitivity to rotenone compared to wild type. Pre-treatment with rapamycin did not significantly alter rotenone toxicity in wild type MEFs. However, pre-treatment with 1 \(\mu\)M rapamycin prior to rotenone exposure significantly attenuated the rotenone toxicity observed in Polg\(^{m/m}\) MEFs (Figure 21A,B). Rapamycin-treated Polg\(^{m/m}\) MEFs had significantly higher viability than untreated Polg\(^{m/m}\) MEFs at 5, 10, and 20 nM rotenone (p<0.001).
Figure 21. Mitophagy Inducer Rapamycin Attenuates Rotenone Toxicity in Polg<sup>mm</sup> MEFs.

ATP levels at 48 h following rotenone treatment, normalized to respective drug control. Wild type (A, blue lines) and Polg<sup>mm</sup> (B, red lines) MEFs were treated with DMSO vehicle control, 5, 10, or 20 nM rotenone for 48 h in the presence (short dotted lines, open symbols) or absence (solid lines, solid symbols) of 1 µM rapamycin. Asterisks indicate different from vehicle control. *p < 0.001, mean +/- S.E.M., n=3 technical replicates.
3.2.7 Autophagy inducer rapamycin is protective against rotenone-induced apoptosis

Flow cytometry analysis was also performed to confirm the observation of attenuated rotenone toxicity in Polg\textsuperscript{m/m} MEFs by rapamycin pre-treatment. Flow cytometry analysis with annexin-V and PI staining is a more sensitive measurement of cell viability than ATP luminescence. MEFs of both wild type and Polg\textsuperscript{m/m} genotype were pre-treated with DMSO vehicle control or 3 µM rapamycin for 72 h. 3 µM rapamycin is the highest concentration that has been shown to induce autophagy while also causing minimal decrease to cell viability (Pan et al., 2009). Following pre-treatment, cells were exposed to DMSO vehicle control, 10 nM rotenone, 3 µM rapamycin, or a combination of 10 nM rotenone and 3 µM rapamycin for 24 h in galactose media. One-way ANOVA with bonferroni post-tests were used to compare different treatments of the same genotype, and two-way ANOVA with bonferroni post-tests were used to compare wild type and Polg\textsuperscript{m/m} MEFs at the same treatment condition.

In DMSO vehicle control samples, both genotypes had a baseline cell viability of approximately 90-97% (Figure 22A). Polg\textsuperscript{m/m} MEFs trended towards slightly lower viability relative to wild type, but this was not significantly different. In rapamycin-treated samples, both genotypes had a cell viability of 95-97%. No genotype difference was observed between wild type and Polg\textsuperscript{m/m} MEFs treated with rapamycin, and although rapamycin-treated Polg\textsuperscript{m/m} MEFs trended towards higher viability than DMSO-treated Polg\textsuperscript{m/m} MEFs, this was not significantly different. Rotenone treatment caused the viability of wild type MEFs to drop to approximately 90%, which was significantly different from DMSO (p<0.01). Rotenone treatment also caused a Polg\textsuperscript{m/m} MEFs viability to drop significant to approximately 28% (p<0.001). Rotenone exposure also caused Polg\textsuperscript{m/m} MEFs viability to become significantly different from rotenone-treated wild type viability (p<0.001). Co-treatment with rotenone and rapamycin caused viability of approximately 96% in wild type MEFs, which indicates a complete rescue of rotenone toxicity. In Polg\textsuperscript{m/m} MEFs, rotenone and rapamycin co-treatment resulted in viability of approximately 81%, which is not statistically different from DMSO or rapamycin-treated Polg\textsuperscript{m/m} MEFs. The Annexin-V positive cell population was approximately 2-3% for wild type MEFs treated with DMSO, rapamycin, or rapamycin and rotenone (Figure 22B). Rotenone-alone treatment caused the annexin-V population to increase to approximately 9%, which was statistically significant compared to DMSO (p<0.01). In Polg\textsuperscript{m/m} MEFs, the baseline Annexin-V positive population was
approximately 8%. It trended towards a decrease in rapamycin-treated cells to 4%, but did was not statistically significant. The Annexin-V positive population increased significantly to approximately 60% in rotenone-treated Polg\textsuperscript{in/m} MEFs (p<0.001), and decreased to approximately 17% in Polg\textsuperscript{in/m} MEFs treated with rapamycin and rotenone, which was no longer statistically different from DMSO.
Figure 22. Rapamycin is protective against rotenone-induced apoptosis. (A) Cell viability assessed by flow cytometric analysis of Annexin-V negative, PI negative population of Polg<sup>−/−</sup> (red bars) and wild type (blue bars) MEFs, mean ± S.E.M., (n=3 independent cell lines per genotype) asterisks indicate difference from DMSO vehicle control of own genotype (**p<0.01, ***p<0.001). Cells were pretreated with DMSO vehicle control or 3 μM rapamycin for 72 h, then exposed to DMSO vehicle control, 3 μM rapamycin, 10 nM rotenone, or 3 μM rapamycin and 10 nM rotenone for 24 h. (B) Apoptosis assessed by flow cytometric analysis of Annexin-V positive population of Polg<sup>−/−</sup> (red bars) and wild type (blue bars) MEFs, mean ± S.E.M., (n=3 independent cell lines per genotype) asterisks indicate difference from DMSO vehicle control of own genotype (**p<0.01, ***p<0.001). Cells were pretreated with DMSO vehicle control or 3 μM rapamycin for 72 h, then exposed to DMSO vehicle control, 3 μM rapamycin, 10 nM rotenone, or 3 μM rapamycin and 10 nM rotenone for 24 h.
3.3 Role of mtDNA replication fidelity in toxicity initiated by mitochondria-targeted chemotherapeutic drugs

Data presented within this section addresses the third aim of the hypothesis, which is whether mtDNA replication fidelity conferred by Polg is protective against toxicity initiated by mitochondria-targeted chemotherapeutic drugs.

In the measurement of mitochondrial bioenergetics through oxygen consumption rate experiments (Figure 23), the extracellular flux oxygen consumption rate assay was carried out by Dr. Bennet Van Houten at the University of Pittsburgh. Fernando Bralha provided the MEF cell lines and received raw data to perform data analysis, statistical analysis, and preparation of figures.

In the flow cytometry experiments for Annexin-V (Figure 27), sample preparation and acquisition of flowgrams was performed by Simon Wisnovsky. Fernando Bralha provided the MEF cell lines, received raw, performed data analysis and figure preparation. All other experiments presented in this section were performed by Fernando Bralha.
3.3.1 Effects of mt-Cbl and Cbl on mitochondrial bioenergetics

The nitrogen mustard chlorambucil (Cbl) was selected as a potential cargo for mitochondrial localization via the MPP, in the form of mitochondrial-targeted chlorambucil (mt-Cbl). Cbl is a commonly used chemotherapy agent for the treatment of leukemia that acts through the alkylation of nuclear DNA (Fonseca et al., 2011).

In order to monitor potential alterations in cellular energy metabolism by mt-Cbl, Cbl, and MPP, the metabolic profile (oxygen consumption rate, OCR) of live cells was measured in response to mt-Cbl or Cbl using an extracellular flux analyzer. We hypothesized that mtDNA replication fidelity mediated by DNA Polg would protect against toxicity initiated by drugs targeted to mitochondria and predicted that the strongest effect would be a decrease in OCR in Polg\(^{m/m}\) MEFs treated with mt-Cbl.

Bioenergetic analysis in untreated MEFs confirmed the low oxidative phosphorylation of Polg\(^{m/m}\) MEFs compared to wild type (Figure 23A) that has been previously reported (Kujoth et al., 2005; Trifunovic et al., 2005; Trifunovic et al., 2004). We observed that both the maximal respiration (the oxygen consumption rate following ETC uncoupling by FCCP) and the total reserve capacity (the oxygen consumption rate following inhibition of glycolysis by 2-DG) were lower in Polg\(^{m/m}\) MEFs compared to wild type (p<0.001). In response to mt-Cbl (Figure 23B), wild type MEFs responded with an increase in OCR (p<0.01 in the total reserve capacity time points), while Polg\(^{m/m}\) MEFs trended towards a decrease in OCR, but this was not significant. In response to the targeting peptide alone (Figure 23C), OCR was not significantly altered in either genotype, although it trended towards an increase in wild type and a decrease in Polg\(^{m/m}\) MEFs. For Cbl (Figure 23D), wild type cells showed no change in OCR over time relative to vehicle controls. In Polg\(^{m/m}\) MEFs, Cbl caused a decrease in maximal respiration (p<0.05 and p<0.01) and total reserve capacity (p < 0.05).
Figure 23. Pharmacological profiles of oxygen consumption. Oxygen consumption rate was determined with a Seahorse XF24 Extracellular Flux analyzer. Metabolic inhibitors injected sequentially were F1F0-ATPase inhibitor oligomycin, electron transport chain uncoupler FCCP, glycolysis inhibitor 2-DG, and complex I inhibitor rotenone. Mouse embryonic fibroblasts from Polg<sup>mm</sup> or wild type mice were cultured for 2 hours prior to and during bioenergetic measures. (A) Untreated Polg<sup>mm</sup> (open circles) and wild type (open squares) MEFs. (B) Polg<sup>mm</sup> (circles) and wild type (squares) MEFs cultured with 3 µM mt-Cbl (black symbols) or vehicle controls (open symbols). Asterisks represent difference from wild type vehicle in 2-way-repeated measures ANOVA with bonferroni posttests, **<p<0.01. (C) Polg<sup>mm</sup> (circles) and wild type (squares) MEFs cultured with 3 µM of the mitochondria-penetrating peptide Fxr3 (black symbols) or vehicle control (open symbols). (D) Polg<sup>mm</sup> (circles) and wild type (squares) MEFs cultured with 3 µM Cbl (black symbols) or vehicle control (open symbols). Asterisks represent difference from Polg<sup>mm</sup> vehicle in 2-way-repeated measures ANOVA with bonferroni posttests, *<p<0.05, **p<0.01.
3.3.2 DNA lesion levels following mt-Cbl or Cbl exposure in wild type and Polg\(^{m/m}\) MEFs

Since the mechanism of cell death caused by Cbl involves bulky DNA damage in the form of Cbl-DNA adducts and inter-strand cross-links (Begleiter et al., 1996), we sought to measure this DNA damage using quantitative long PCR amplification of the mitochondrial and nuclear DNA (Santos et al., 2006a) in cells treated with mt-Cbl and Cbl. DNA lesions cannot physically be bypassed by Taq DNA polymerases. Thus, DNA templates with higher levels of polymerase-blocking lesions will be amplified to a lower extent in a PCR reaction. (Santos et al., 2006a).

Previous studies of mt-Cbl and Cbl have demonstrated that mt-Cbl damages mitochondrial DNA but not nuclear DNA, while Cbl damages nuclear DNA but not mitochondrial DNA (Fonseca et al., 2011). Wild type and Polg\(^{m/m}\) MEFs were treated with either mt-Cbl or Cbl, and DNA was isolated for the assay either immediately after exposure or following a 6 hour recovery period (Figure 24A).

We observed no significant difference in PCR amplification of the mitochondrial genome between the vehicle and mt-Cbl groups in either genotype in the pair of MEFs examined (Figure 24D). Untargeted Cbl also did not generate any detectable lesions in the mtDNA at either time point (Figure 22B,C). Within the nuclear genome, the lesion level within a transcribed gene (DNA polymerase beta, pol\(\beta\)) and a non-transcribed gene (beta-globin) was measured. The level of lesions in the transcribed gene pol\(\beta\) at 2 hours after Cbl treatment increased in a concentration-dependent manner (p < 0.001) (Fig. 24E). The contribution of genotype to DNA lesion level was statistically significant (p < 0.05), with lesion levels being higher in the wild type (Fig. 24E).

When an additional 6 hours were allowed for repair, the level of DNA lesions after Cbl treatment still varied with Cbl concentration (p < 0.001) but there was no longer a statistically significant contribution by genotype (Fig. 24F). For the untranscribed gene \(\beta\)-globin, at 2 hours after Cbl treatment, the lesion level increased in a concentration-dependent manner (p < 0.001) (Fig. 24G). The contribution of genotype to DNA lesion level was statistically significant (p < 0.05), with lesion levels being higher in the wild type (Fig. 24G). When an additional 6 hours were allowed for repair, the level of DNA lesions after Cbl treatment still varied with Cbl concentration (p < 0.001) but there was no longer a statistically significant contribution by genotype (Fig. 24H).
Figure 24. DNA damage levels in mitochondrial or nuclear DNA after Cbl or mt-Cbl. Long PCR analysis of a 10 kbp nuclear or mitochondrial DNA fragment was performed on total DNA isolated from wild type and Polg<sup>mm</sup> MEFs. A) schematic of timecourse of drug treatments and cell harvests. DNA lesions were measured at 2 hours of drug exposure (B, D, E, G) or 8 hours (C, F, H) after the start of drug exposure. (B-D) DNA damage levels in mitochondrial DNA in wild type (squares and solid lines) or Polg<sup>mm</sup> (circles and dashed lines) treated with Cbl, or wild type (grey bars) or Polg<sup>mm</sup> (black bars) MEFs treated with mt-Cbl (D). DNA damage levels after (B) 2 h of Cbl, (C) 2 h of Cbl followed by 6 h of recovery, or (D) after 2 h of mt-Cbl. (E-F) DNA damage levels in the transcribed gene DNA polymerase β in wild type or Polg<sup>mm</sup> MEFs. DNA damage levels after (E) 2 h of Cbl, or (F) 2 h of Cbl followed by 6 h of recovery. (G-H) DNA damage levels in the transcriptionally silent β-globin gene DNA in wild type or Polg<sup>mm</sup> MEFs. DNA damage levels after (G) 2 h of Cbl, or (H) 2 h of Cbl followed by 6 h of recovery. Asterisks represent difference between Polg<sup>mm</sup> line and wild type line, *P<0.05. Error bars represent S.E.M. of three experiments.
3.3.3 Clonogenic survival after Cbl or mt-Cbl is similar in \(Polg^{m/m}\) and wild type

In order to determine whether mtDNA replication fidelity is protective against direct mitochondrial damage, we assessed the effects of mt-Cbl or Cbl on cellular viability. Clonogenic survival assays were performed in wild type and \(Polg^{m/m}\) MEFs. ATP levels were also measured to determine the acute effects of these drugs on cellular toxicity. mt-Cbl induced a concentration-dependent decrease in clonogenic survival (\(p < 0.0001\)). The effect was not different between wild type and \(Polg^{m/m}\) MEFs (Fig. 25A). The MPP targeting peptide on its own was not appreciably toxic in either genotype, at concentrations up to 10 \(\mu M\), the highest concentration tested (Fig. 25C). Cbl induced a concentration-dependent decrease in clonogenic survival (\(p < 0.0001\)) and again, no differences were observed between wild type and \(Polg^{m/m}\) MEFs (Fig. 25B).

A similar pattern was observed in the ATP endpoint (Figure 25D,E,F). mt-Cbl induced a concentration-dependent decrease in ATP levels from 3 to 12 \(\mu M\) mt-Cbl (\(p < 0.0001\)). The effect was not different between wild type and \(Polg^{m/m}\) MEFs (Fig. 25D). Untargeted Cbl did not cause a concentration-dependent decrease in ATP levels even at concentrations as high as 200 \(\mu M\) (\(p < 0.0001\)) and no differences were observed between wild type and \(Polg^{m/m}\) MEFs (Fig. 25E). The MPP targeting peptide on its own did not alter ATP levels in either genotype at concentrations of 3 and 6 \(\mu M\) (Fig. 25F).

Acute necrosis was also measured as an endpoint of cellular toxicity in MEFs exposed to mt-Cbl, untargeted Cbl, and the MPP targeting peptide. Mt-Cbl caused a concentration-dependent increase in acute necrotic protease activity in both genotypes. mt-Cbl concentration of 12 \(\mu M\) caused a 3-4-fold increase in necrotic activity relative to DMSO vehicle controls (Figure 25G). Untargeted Cbl did not increase necrosis of either genotype, even in concentrations as high as 200 \(\mu M\) (Figure 25H). The MPP targeting peptide was assessed at concentration of 3 and 6 \(\mu M\), and no increase in necrosis was observed in either genotype (Figure 25I).
Figure 25. Cellular survival following Cbl or mt-Cbl are similar in Polg<sup>min</sup> and wild type. (A-C) Clonogenic survival assays in wild type (squares and solid lines) or Polg<sup>min</sup> (circles and dashed lines). Clonogenic survival after (A) mt-Cbl, (B) Cbl or (C) MPP. Error bars represent S.E.M. of three to six experiments. (D-F) ATP levels in wild type (squares and solid lines) or Polg<sup>min</sup> (circles and dashed lines) MEFs 2 hours after drug treatment. ATP levels after treatment with (D) mt-Cbl, (E) Cbl or (F) MPP. (G-I) Necrotic protease activity in wild type and Polg<sup>min</sup> MEFs. Error bars represent SEM of three to six experiments.
3.3.4  \textit{Polg}^{m/m} MEFs are not susceptible to mitochondria- targeted doxorubicin.

We sought to determine if \textit{Polg}^{m/m} MEFs would show increased sensitivity towards other mitochondrial targeted chemotherapeutic agents other than Cbl. Another chemotherapy agent conjugated to MPP and targeted to the mitochondria is doxorubicin (Dox). A soluble and non-toxic tetrazolium salt reduction assay similar to MTT was used to assess cell viability in wild type and \textit{Polg}^{m/m} MEFs treated with mitochondrial-targeted doxorubicin (mt-Dox) for 72 hours. mt-Dox caused a concentration-dependent decreased in cell viability (p<0.0001) at a concentration range of 0.4 to 60 µM. the EC50 was 4.2 µM for wild type MEFs, and 4.0 µM for \textit{Polg}^{m/m} MEFs. No genotype difference was observed in viability between wild type and \textit{Polg}^{m/m} MEFs treated with mt-Dox (Figure 26).
Figure 26. Polg<sup>m/m</sup> MEFs are not susceptible to mitochondria-targeted doxorubicin. cck8 viability assay of wild type (blue) and Polg<sup>m/m</sup> (red) MEFs exposed to mt-Dox for 72 h. (N=3 technical replicates, mean +/- S.E.M.).
3.3.5 \textit{Polg}^{m/m} MEFs are susceptible to mitochondria- targeted platinum-AcylAcyl agent.

The final mitochondrial-targeted chemotherapeutic agent assessed for increased \textit{Polg}^{m/m} MEFs sensitivity was the synthetic platinum compound acyl-acyl-cis-Diammineplatinum (II)-\(\beta\)-Diketonate (Wilson and Lippard, 2012) (Platinum-acylacyl, or simply Pt-AcAc). A soluble and non-toxic tetrazolium salt reduction assay similar to MTT was used in a colourimetric assay to measure cell viability in wild type and \textit{Polg}^{m/m} MEFs treated with mitochondrial-targeted Pt-AcAc (mt-Pt) for 72 hours. mt-Pt caused a concentration-dependent decrease in cell viability in both genotypes (Figure 27A). mt-Pt appeared to cause a greater decrease in tetrazolium salt absorbance at 450 nm in \textit{Polg}^{m/m} than in wild type MEFs. However, the two genotypes had differing baseline absorbances in this experiment. This variation in baseline absorbance in these data makes it difficult to compare the two genotypes and prevents any conclusions from being made.

An additional experiment was performed to determine whether mt-Pt was more toxic to \textit{Polg}^{m/m} than wild type MEFs. The toxicity of mitochondrial-targeted Pt-AcAc (mt-Pt) was measured by Annexin-V and PI flow cytometry analysis of wild type and \textit{Polg}^{m/m} MEFs exposed to Pt-AcAc for 24 hours. The baseline cell viability of both genotypes was approximately 90%. With increasing concentration of mt-Pt, there was a slight drop in wild type MEF viability, but a much more pronounced drop in \textit{Polg}^{m/m} MEF viability. At 30 µM mt-Pt, the highest concentration assessed, the viability of wild type MEFs was 83.8%, while the viability of \textit{Polg}^{m/m} MEFs at the same concentration dropped to 59.3% (Figure 27B). At a mt-Pt concentration of 30 µM, the percentage of apoptotic cells was 15.5% for wild type, and 40.5% for \textit{Polg}^{m/m} MEFs (Figure 27C). The cellular toxicity of the untargeted parent compound Pt-AcAc was also assessed as a control. Concentrations of Pt-AcAc as high as 60 µM, twice the maximum mt-Pt concentration tested, only decreased cell viability to 83.6% for wild type and 89.3% for \textit{Polg}^{m/m} MEFs (Figure 27D). The percentage of apoptotic cells exposed to 60 µM Pt-AcAc was 16.2% for wild type and 10.5% for \textit{Polg}^{m/m} MEFs (Figure 27E).
Figure 27. Polg<sup>m/m</sup> MEFs are susceptible to mitochondria-targeted mt-Pt. (A) cck8 viability assay of wild type (blue) and Polg<sup>m/m</sup> (red) MEFs exposed to mt-Pt for 72 h. (N=3 technical replicates, mean +/- SEM). (B-E) Flow cytometry analysis of wild type (blue) and Polg<sup>m/m</sup> (red) MEFs exposed to mt-Pt (B, D) or the untargeted control compound Pt-AcAc (C, E).
4 Discussion

4.1 Characterization of $Polg^{m/m}$ MEFs

The results of the random mutation capture assay quantified the mtDNA mutation load in all three genotypes (wild type, $Polg^{+/m}$, and $Polg^{m/m}$) and identified the number of mtDNA mutations as increasing with increasing number of knock-in $Polg$ alleles. This observation is in agreement with other published results that characterized increased mtDNA mutations as the primary phenotype of the $Polg^{m/m}$ mouse model (Kujoth et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2007). While publications from several groups indicate that the $Polg^{m/m}$ mouse model has an increased rate of mtDNA mutations, the extent of mtDNA mutation rate has been a matter of some disagreement (Edgar and Trifunovic, 2009). The reason for discrepancies in quantifying the actual number of mtDNA point mutations is that different techniques have been used to measure point mutations in the mtDNA of these animals. While the level of mtDNA mutations in $Polg^{m/m}$ is always higher than wild type, the magnitude of mtDNA mutation load varies considerably with different techniques. The random mutation capture (RMC) method detects 500-fold less mtDNA mutations in wild type animals than the more common cloning-sequencing method (Edgar and Trifunovic, 2009). In cloning-sequencing methods, PCR amplification of mtDNA is followed by cloning and sequencing of the amplified region to determine mtDNA point mutations. The initial PCR amplification step may introduce artificial mutations, which will then be detected as false positives during the sequencing reaction. As a result, the cloning-sequencing method has a lower sensitivity and detects 10-1000 times more mutations than the RMC method (Edgar and Trifunovic, 2009). This will mean that in wild type, the mtDNA mutation load may actually fall below the detection limit of the cloning-sequencing method (Edgar and Trifunovic, 2009). Since the mutation load of the $Polg^{m/m}$ genotype is routinely reported as a fold-increase relative to wild type controls, the detection limit in wild type controls can influence the perceived extent of $Polg^{m/m}$ mutation load.

An important difference between the primary and immortalized MEFs in this study arises from the immortalization process. MEFs were immortalized by transfection with the Simian virus large T antigen, which effectively deactivates cellular p53. In addition to its well known roles in cell cycle arrest, nuclear DNA repair, senescence and apoptosis (de Moura et al., 2010), p53 can also be recruited to the mitochondria where it has been proposed to play a similar role in
maintaining mtDNA integrity by suppressing mtDNA mutagenesis (Holt, 2010; Lebedeva et al., 2009). Therefore, inhibiting p53, as has been done during the MEFs immortalization process of this study, may further compromise mtDNA integrity in these cells and lead to additional mtDNA mutations. While mtDNA mutation load in immortalized MEFs trended towards an increase for all three genotypes, this trend did not reach statistical significance. This suggests that inhibition of p53 did not have a significant role in mtDNA mutation load, and that the leading factor driving mtDNA mutation load is these MEFs is the Polg\textsuperscript{m/m} allele.

The comparison between glucose and galactose media conditions is intended to offer clues regarding which metabolic pathway (oxidative phosphorylation or glycolysis) the cells rely most heavily upon for their energy production. Cells grown on glucose media are able to utilize both glycolysis and oxidative phosphorylation. Under physiological conditions, cells generate 95% of their cellular ATP through mitochondrial oxidative phosphorylation (Dykens and Will, 2008). However, cells may exhibit an increased reliance on aerobic glycolysis for ATP generation due to the high glucose concentration in normal media (known as the Crabtree effect). Glucose-free media conditions with galactose as the carbon source for glycolysis eliminates the Crabtree effect because glycolytic metabolism of galactose is much less efficient, and does not generate any net ATP molecule. As a result, cells increase their reliance on mitochondrial respiration for energy production. One notable exception is leukemia stem cells, which rely strongly on mitochondrial respiration for their energy production, even when grown in high glucose concentrations (Lagadinou et al., 2013). Glycolysis is an important metabolic process even in cells utilizing mitochondrial respiration because it generates electron donor molecules such as NADH, and generates pyruvate to feed the TCA cycle.

A 12 day cellular proliferation curve revealed that Polg\textsuperscript{m/m} MEFs had decreased cell number compared to wild type MEFs in both glucose and galactose media conditions. This suggests that Polg\textsuperscript{m/m} MEFs may have a basal deficit in mitochondrial respiration that becomes significant after 12 days. Although both genotypes exhibited a decrease in cell number when grown in glucose-free media relative to glucose media, this decrease was more pronounced in Polg\textsuperscript{m/m} MEFs. This suggests that Polg\textsuperscript{m/m} MEFs have higher reliance on aerobic glycolysis than wild type, possibly as a compensatory measure for impaired mitochondrial activity. This observation is corroborated by earlier reports that Polg\textsuperscript{m/m} MEFs grown to 70-100% confluency acidified the media after 1-3 days, while media pH from wild type MEFs remained neutral even
after 1 week. Media acidification due to lactic acid production suggests an increased reliance on glycolysis (Trifunovic et al., 2005). Polg$^{m/m}$ MEFs have also been shown to have increased aerobic glycolysis compared to wild type MEFs (Kukat et al., 2011). One important limitation in interpreting a cellular proliferation curve is that the endpoint measured is simply the number of cells present after 7 days in culture. When a significantly lower number of cells is present, as is the case with Polg$^{m/m}$ MEFs, this assay offers no information of the fate of the missing cells. Two possible mechanisms for decreased cell number are decreased proliferation and increased cell death. Therefore, additional follow-up experiments were carried out to determine whether Polg$^{m/m}$ MEFs have decreased proliferation or increased cell death. Since potential differences in proliferation and cell death were observed when the cells were grown in glucose versus galactose media, several subsequent experiments were performed under both media conditions.

In order to further characterize the Polg$^{m/m}$ MEFs, MitoTracker fluorescent dye was used as a surrogate to indirectly measure mitochondrial mass in Polg$^{m/m}$ and wild type MEFs. We found that mitochondrial mass is not altered in Polg$^{m/m}$ MEFs. This observation is in agreement with published results that demonstrated that Polg$^{m/m}$ MEFs have mitochondrial mass and membrane potential similar to those in wild type (Kukat et al., 2011). Increased mitochondrial mass is a commonly observed compensatory measure in response to mitochondrial dysfunction. For example, oxidative stress causes an increase in mitochondrial mass in human lung fibroblast cells (Lee et al., 2000), and a mouse model of mitochondrial myopathy in muscle fibers also associated with increased mitochondrial mass as a response to respiratory chain deficiency (Wredenberg et al., 2002). Similarly, the Polg$^{m/m}$ mouse model was found to have cardiomyocytes with enlarged and abnormally shaped mitochondria (Trifunovic et al., 2004). This illustrates that the cellular response to mitochondrial dysfunction may be tissue specific. Even within the same genetic model, such as the Polg$^{m/m}$ mouse model, increased mitochondrial mass as a compensatory measure may be tissue and cell type specific, being observed in cardiomyocytes but not in cultured fibroblasts.

4.2 Rotenone reduces the survival of Polg$^{m/m}$ MEFs

The observation of increased rotenone-initiated lethality in Polg$^{m/m}$ MEFs relative to wild type supports the hypothesis that mitochondrial DNA replication fidelity protects against toxicity initiated by xenobiotics that impair the ETC. We also observed that Polg$^{+/m}$ heterozygous MEFs
were not hypersensitive to rotenone despite a mtDNA mutation load 100-fold higher than wild type. This lack of Polg\(^{+/m}\) sensitivity to rotenone suggests that an elevated mtDNA mutation load alone does not predispose to toxicity. Our data suggest that a certain threshold for rotenone sensitivity has been reached by Polg\(^{m/m}\) but not by Polg\(^{+/m}\) MEFs, and based on our measurement of mtDNA mutation load this threshold must be between 1000 and 3000 mtDNA point mutations per million base pairs, which is equivalent to 16.5 to 49.5 point mutations per mtDNA molecule. The lack of rotenone hypersensitivity in the Polg\(^{+/m}\) MEFs relative to wild type is in agreement with a lack of premature aging in the heterozygous mice despite their elevated mtDNA mutation frequency in vivo (Kujoth et al., 2005; Trifunovic et al., 2004). Mutation threshold is a common requirement for mitochondrial disease and toxicity to occur. The exact threshold depends on the severity of the mutation phenotype, and can range between 50-95% (Smith and Lightowlers, 2011).

Inhibition of complex I by rotenone leading to toxicity and cell death is well documented at a wide concentration range. Rotenone concentrations of 25-30nM are able to inhibit complex I activity enough to cause a lethal decrease in mitochondrial membrane potential (Xiong et al., 2012). The extent of rotenone toxicity on different cultured cell lines varies depending on the cell type, media conditions, and passage number of the cells (Xiong et al., 2012). For example, 200 nM rotenone can cause complete death of SH-SY5Y neuroblastoma cells (Sawmiller et al., 2012), 10 \(\mu\)M rotenone exposure caused 25% cell death in primary MEFs (Ungvari et al., 2011), and MEFs had an EC\(_{50}\) of <10 \(\mu\)M rotenone (Pereira et al., 2012). The EC\(_{50}\) of rotenone in 3 different cancer cell lines (SAS, Cal27, and Tw2.6) was 0.95, 0.11, and 1.24 \(\mu\)M rotenone, respectively (Lee et al., 2008a). Rotenone exposure to SH-SY5Y cells caused 50% cell death at approximately 5 nM (Giordano et al., 2012). The colony forming assay that we have used to evaluate toxicity is highly sensitive because it requires cells to not only survive the drug stress, but also to proliferate into colonies of >50 cells. This dual requirement may underlie our observation of rotenone toxicity in the nanomolar concentration range. In our flow cytometry experiments, 10 nM rotenone was used to investigate rotenone-induced apoptosis following a 24 h exposure. The observation that rotenone increased apoptosis in Polg\(^{m/m}\) but not wild type MEFs also supports the hypothesis that mitochondrial DNA replication fidelity protects against toxicity initiated by xenobiotics that impair the ETC.
Rotenone-initiated apoptosis has been reported to result from caspase cascades (Gao et al., 2012; Lee et al., 2008a). Apoptosis induction by rotenone can occur due to three mechanisms: oxidative stress (Deng et al., 2010; Gao et al., 2012; Li et al., 2003; Sanchez-Reus et al., 2005), decreased mitochondrial membrane potential (Hu et al., 2009; Isenberg and Klaunig, 2000; Menke et al., 2003), and ATP energy depletion (Giordano et al., 2012; Wang et al., 1999), all of which can lead to the release of pro-apoptotic mitochondrial factors. In the current study apoptosis was assessed via the marker Annexin-V that monitors the loss of membrane phospholipid asymmetry (Koopman et al., 1994), which leads to the exposure of phosphatidylserine at the surface of the cell. The presence of phosphatidylserine at the surface of the cell membrane coincides with chromatin condensation, and therefore occurs very early in the process of apoptosis at which point dying cells are still intact. Annexin V is a protein that binds strongly to phosphatidylserine, and FITC-bound Annexin-V detects cells in the early phase of apoptosis (Koopman et al., 1994). While this method is very sensitive in its detection of apoptosis, it does not provide information on whether rotenone exposure leads to the induction of apoptosis through ROS stress, decreased membrane potential, or bioenergetic insufficiency. Rotenone can also cause cell death through caspase-independent, non-apoptotic mechanisms (Newhouse et al., 2004; Shimizu et al., 1996). We tested for protease activity associated with necrosis in either genotype in response to acute rotenone exposure, but observed no evidence suggesting that rotenone kills Polg<sup>m/m</sup> MEFs by necrosis.

In addition to increased cell death, another possible explanation for the decreased colony-forming ability of rotenone-treated Polg<sup>m/m</sup> MEFs is decreased proliferation (Goncalves et al., 2011). Rotenone inhibits mammalian cell proliferation with an EC<sub>50</sub> concentration of 200-400 nM by disrupting assembly of spindle microtubules crucial for cell division (Barham and Brinkley, 1976; Marshall and Himes, 1978; Srivastava and Panda, 2007). Rotenone can inhibit the assembly of mitotic spindle microtubules by direct binding to tubulin (Goncalves et al., 2011). Cell cycle arrest through microtubule inhibition occurs at concentrations 10 to 50-fold higher than those required for complex I inhibition (Armstrong et al., 2001).

BrdU-incorporation flow cytometry analysis was used to measure the percentage of cells in S-phase of the cell cycle in order to investigate the effect of rotenone exposure on Polg<sup>m/m</sup> MEF proliferation. Since potential differences in proliferation were observed in the growth curve when the cells were grown in glucose versus galactose media, this experiment was performed under
both media conditions. No genotype difference was observed between wild type and \( \text{Polg}^{m/m} \) MEFs at any of the conditions tested, which suggests that the \( \text{Polg}^{m/m} \) sensitivity to rotenone observed in the clonogenic assays was not due to a differential effect of rotenone on proliferation. Rotenone was found to reduce proliferation of both genotypes equally, but only in glucose-free conditions where cells are predicted to have an increased reliance on mitochondrial respiration for ATP production. The low nanomolar concentrations of rotenone used in this present study are likely not sufficient to cause inhibition of microtubule assembly, and so decreased cellular proliferation we observed is not likely to occur by this mechanism. The decreased percentage of cells at S-phase that occurred as a result of rotenone exposure in glucose-free media conditions may likely be due to insufficient cellular ATP levels. However, this decrease was observed equally in both genotypes, indicating that it is not the mechanism of \( \text{Polg}^{m/m} \) MEF hypersensitivity to rotenone. These results, along with the Annexin-V flow cytometry experiment, indicate that in the \( \text{Polg}^{m/m} \) model, rotenone treatment leads to a decreased cell number due to cell death by apoptosis rather than to decreased cell proliferation.

ROS generation is considered an important mechanism of rotenone toxicity leading to apoptosis and cell death (Deng et al., 2010; Gao et al., 2012; Li et al., 2003; Sanchez-Reus et al., 2005). ROS damage to macromolecules can be observed at the level of mtDNA. 300 nM rotenone exposure increases the amount of 8-oxo-dGuanine lesions in the mtDNA by almost 3-fold (Achanta et al., 2005). In order to determine whether rotenone exposure caused higher ROS generation in \( \text{Polg}^{m/m} \) MEFs compared to wild type, we performed a ROS assay using a fluorescent ROS-sensitive probe. While the lack of statistical significance does not allow for conclusions to be made based on this set of experiments, our data suggests that \( \text{Polg}^{m/m} \) MEFs do not increase ROS to a greater degree than wild type following rotenone exposure. Rotenone induces apoptosis at concentrations of 1 µM in cancer cell lines, and inhibition of ROS by the antioxidants NAC, GSH, or Tiron significantly inhibits rotenone-induced apoptosis (Lee et al., 2008a). In our experiments, nanomolar concentrations of rotenone were not sufficient to induce observable increases in ROS generation. Since \( \text{Polg}^{m/m} \) MEFs exhibit rotenone hypersensitivity at nanomolar concentration, the mode of toxicity is likely bioenergetic and not ROS-mediated.

Another potential mechanism of rotenone-induced toxicity is ATP insufficiency due to inhibition of complex I of the ETC. Rotenone exposure impairs mitochondrial function by inhibiting complex I of the electron transport chain and impairing ATP production (Ravanel et
In galactose media, where the bioenergetics gain of glycolysis is neutral, the number of viable cells and ATP levels were lower in $Polg^{m/m}$ compared to wild type MEFs, even in the absence of rotenone. This suggests that the shift towards reliance on oxidative phosphorylation that occurs in galactose media is especially stressful to $Polg^{m/m}$ MEFs. Alternatively, $Polg^{m/m}$ MEFs may be unable to shift from glycolysis to mitochondrial respiration. Since $Polg^{m/m}$ MEFs have pre-existing respiratory chain deficiency and reduced complex I activity (Edgar et al., 2009; Trifunovic et al., 2005) this genotype is more severely impacted by the effects of rotenone.

The structural integrity of mtDNA in $Polg^{m/m}$ MEFs is considered to be relatively poor compared to wild type. In addition to mtDNA point mutations, $Polg^{m/m}$ MEFs also have an increased number of linear deletions, replication pausing and chromosomal breaks in mtDNA (Bailey et al., 2009; Hiona et al., 2010). A semi-quantitative PCR assay that detects polymerase-blocking structural abnormalities through decreased amplification of mitochondrial genome was used to determine the effect of rotenone on mtDNA integrity of $Polg^{m/m}$ MEFs relative to wild type. We detected reduced amplification of $Polg^{m/m}$ MEF mtDNA compared to wild type. This was observed even in the absence of rotenone. The reduced amplification observed in $Polg^{m/m}$ MEFs can be estimated to be on average approximately one additional polymerase-blocking structure per mitochondrial genome relative to wild type. Possible interpretations of the decreased amplification are increased lesions, an increase in linear mtDNA molecules and increased double stranded breaks. Micromolar concentrations of rotenone can cause lipid peroxidation and radical species capable of generating polymerase-blocking oxidative stress lesions in mtDNA (Kasiviswanathan et al., 2013; Zhang et al., 2001). In our present study, 10 nM rotenone did not significantly alter the mtDNA lesion levels in either wild type or $Polg^{m/m}$ MEFs. This suggests that the observed $Polg^{m/m}$ MEF hypersensitivity to rotenone does not occur through a mechanism of increased polymerase-blocking mtDNA structural abnormalities.

mtDNA depletion is a common mechanism of mitochondrial dysfunction that leads to impaired mitochondrial respiration due to the lack of sufficient mtDNA-encoded proteins (Cohen and Naviaux, 2010). mtDNA depletion often occurs due to mutations in either the polymerase or the exonuclease domain of human DNA polymerase gamma (Ashley et al., 2008; Dimmock et al., 2010). Conversely, increased mtDNA copy number is observed in aging (Barrientos et al., 1997; Gadaleta et al., 1992), and has also been suggested as an adaptive response to mtDNA damage or mutations (Bai and Wong, 2005). Following genotoxic stress, mtDNA synthesis is
increased as part of a feedback response to impaired ATP generation (Graziewicz et al., 2006). With respect to rotenone, 100 nM rotenone can increase mtDNA content up to 2-fold (Miyako et al., 1997). In order to assess the effect of rotenone on mtDNA abundance in our model, real-time PCR of the cytochrome b gene (on mtDNA) relative to the beta-actin gene (nuclear DNA) was employed. We observed that \( \text{Polg}^{m/m} \) MEFs have endogenously increased mtDNA abundance relative to wild type. Increased mtDNA abundance in \( \text{Polg}^{m/m} \) MEFs likely occurs as a compensatory mechanism to correct for mitochondrial protein insufficiency due to mtDNA mutations (Bai and Wong, 2005). Increased mtDNA copy number is also a molecular marker of normal aging (Gadaleta et al., 1992). The knock-in mutation present in the \( \text{Polg}^{m/m} \) model does not affect the polymerase activity of DNA Polymerase Gamma, but only inhibits its exonuclease proofreading activity (Kujoth et al., 2005; Trifunovic et al., 2004). Therefore while the exonuclease-deficient variant of DNA Polymerase Gamma is predicted to generate mutated mtDNA copies, it is not expected to have any deficiency in the synthesis of new mtDNA. With respect to mtDNA abundance, hydrogen peroxide strongly depletes mtDNA acutely (Shokolenko et al., 2009; Van Houten et al., 2006). We observed substantial hydrogen peroxide-induced mtDNA depletion in wild type MEFs, but no depletion in \( \text{Polg}^{m/m} \) MEFs. This observation suggests that the proper stress-handling response to oxidative stress is impaired in \( \text{Polg}^{m/m} \) MEFs. One possible interpretation is that due to its impaired mitochondrial respiration (Trifunovic et al., 2005) \( \text{Polg}^{m/m} \) MEFs lack sufficient ATP energy to initiate the active degradation of so many damaged mtDNA molecules.

In testing the hypothesis that mtDNA replication fidelity protects against xenobiotics that impair ETC activity, we chose to focus on rotenone, a classic inhibitor of complex I. Additional experiments were performed to determine whether the observations with rotenone also hold true for the inhibition of other ETC complexes. The proofreading defect during mtDNA replication in the \( \text{Polg}^{m/m} \) model leads to decreased stability of ETC complexes I, III and IV (Edgar et al., 2009), likely because mtDNA-encoded subunits are crucial for the proper function of these three complexes (Lamantea et al., 2002; Saraste, 1990; Ugalde et al., 2003). Since these three ETC complexes have base-line impairment in the \( \text{Polg}^{m/m} \) model, we assessed how their inhibition with pharmacological agents would affect viability of \( \text{Polg}^{m/m} \) MEFs compared to wild type. Cell viability was assessed indirectly by colourmetric measurement of non-mitochondrial dehydrogenase activity. Decreased viability in \( \text{Polg}^{m/m} \) MEFs relative to wild type was observed
for all three inhibitors: complex I (rotenone), complex III (antimycin A) and complex IV (potassium cyanide). These finding suggest that increased susceptibility in Polg<sup>m/m</sup> MEFs to ETC inhibitors is not limited to inhibitors of complex I, but also occurs in response to inhibition of other ETC complexes.

Over 60 different classes of clinically used drugs can cause undesired side effects and toxicity through off-target inhibition of complex I of the ETC (Dykens and Will, 2008). These include fibrates, thiazolidinediones such as metformin, and antidepressants such as nefazodone (Brunmair et al., 2004a; Brunmair et al., 2004b; Pereira et al., 2012). Mechanisms by which drugs can impair complex I include: direct inhibition (amytal), increased ROS production (capsaicin), electron cycling (doxorubicin and haloperidol), or co-enzyme Q analog (risperidone, metformin, halothane, and fibrates) (Cohen, 2010; Hynes et al., 2006). Drugs with off-target complex I inhibition typically cause toxicity in tissues that have a strong reliance on mitochondrial respiration with high energy demand such as kidneys, muscles and heart, or tissues that are directly exposed to the drug as high concentrations such as the liver (Dykens and Will, 2007). It is interesting to note that often there is no correlation between toxicity and drug dose, and genetics are an important factor in determining whether toxicity will occur (Dykens and Will, 2007). For example, individuals with mutations in the DNA Polymerase gamma gene (POLG) are at an increased risk of rare but potentially life-threatening hepatotoxicity following valproic acid exposure (Saneto et al., 2010).

As examples of clinical drugs with known off-target complex I inhibition, we assessed the relative toxicity of clofibrate, ketoconazole, and flutamide on wild type and Polg<sup>m/m</sup> MEFs. All three drugs carry black box warnings from the Food and Drug Administration (FDA), which calls attention to serious or life-threatening risks (Dykens and Will, 2007). Clofibrate is an antihyperlipidemic drug prescribed to prevent heart disease. However, like most fibrates clofibrate inhibits complex I by acting as a CoQ analog and can cause liver and muscle toxicity (Cohen, 2010; Dykens and Will, 2008). Ketoconazole is an anti-fungal agent that can cause hepatotoxicity and can act as a potent inhibitor of complex I (Rodriguez and Acosta, 1996). Flutamide is a nitroaromatic agent that acts as an anti-androgen drug for the treatment of prostate cancer. Flutamide is associated with hepatotoxicity and is able to inhibit complex I and cause ROS generation through redox cycling (Dykens and Will, 2008). Polg<sup>m/m</sup> hypersensitivity was only observed for clofibrate and ketoconazole, but not flutamide. This discrepancy is likely
because while flutamide can inhibit complex I, its toxicity occurs largely as a result of cytochrome P450 (3A and 1A) mediated metabolism, which creates electrophilic metabolites that aggravate the effects of complex I inhibition (Fau et al., 1994). These finding suggest that increased susceptibility in Polg<sup>m/m</sup> MEFs is not unique to rotenone toxicity, but also occurs in response to many other clinically relevant drugs with off-target complex I inhibition.

Neurons are highly susceptible to mitochondrial dysfunction due to their high ATP energy demand (Chen and Chan, 2006, 2009). For this reason we predicted that Polg<sup>m/m</sup> primary cortical neurons would also exhibit increased sensitivity to rotenone toxicity. However, we observed no rotenone-induced apoptosis in cortical neuron cultures and no genotype difference in rotenone toxicity by Annexin-V flow cytometry analysis. This lack of rotenone toxicity is in contrast to published observations, where primary cortical neurons treated with 10 nM rotenone for 24 hours decreased neuronal viability by 50 % as measured by MTT formazan reduction (Gieseler et al., 2009). An important variable that modulates the extent of neuronal rotenone toxicity is the presence of other cell types in the culture such as astrocytes. Neuron-only cultures are very sensitive to rotenone, with an LD<sub>50</sub> of approximately 2 nM. However, if the primary cortical neurons were co-cultured with astrocytes, the LC<sub>50</sub> shifted to approximately 12 nM rotenone (Mullett and Hinkle, 2009). While our neuronal cultures are predominantly composed of primary cortical neurons, a certain population of astrocytes and other non-neuronal glial cells is also present, and this may increase the resistance to rotenone exposure.

When considering other potential explanations for a lack of rotenone-induced apoptosis in neurons, while a range of rotenone concentrations were tested, the time course for flow cytometry analysis was kept constant at 24h after rotenone exposure. It is possible that an earlier time point may be more appropriate. Other confounding challenges of working with cortical neurons are that, as primary cells, cortical neurons are more difficult to culture than MEFs, with high variability and relatively high baseline cell death. Another important difference between these cell types is proliferative ability. We have observed decreased proliferation in Polg<sup>m/m</sup> MEFs exposed to rotenone, and highly proliferative tissues are the most affected in the Polg<sup>m/m</sup> mice premature aging phenotype (Kujoth et al., 2005). Together this may suggest that proliferative ability may be necessary for Polg<sup>m/m</sup> tissues to present with increased sensitivity to rotenone. This cell type difference in rotenone toxicity may also be due to a certain threshold of mtDNA mutation load that Polg<sup>m/m</sup> MEFs have reached but Polg<sup>m/m</sup> cortical neurons have not.
The mtDNA mutation load was approximately 25-fold higher in Polg\textsuperscript{m/m} than in wild type neurons, as is expected due to the Polg\textsuperscript{m/m} genotype. However, mtDNA mutation load was 3-6-fold lower in Polg\textsuperscript{m/m} neurons than that observed in Polg\textsuperscript{m/m} MEFs.

4.3 Role of mitochondrial stress responses in toxicity initiated by rotenone

4.3.1 Mitochondrial fission/fusion

The cause for decreased mitochondrial length in Polg\textsuperscript{m/m} MEFs compared to wild type can be attributed to either decreased mitochondrial fusion, or increased mitochondrial fission. The overall morphology of a mitochondria population depends on the balance between the opposing actions of fusion and fission. Increased fission will lead to overall fragmentation, while increased fusion will lead to elongation. Both these extremes have negative effects on mitochondrial respiration. Mitochondrial fragmentation due to lack of fusion compromises mitochondrial respiration because mitochondria become autonomous organelles unable to interact with each other and unable to correct defects by complementation with healthy mitochondria (Chan, 2006b). Conversely, excessive elongation of mitochondrial networks may impair their movement, and this can be especially detrimental in cells such as neurons where mitochondria must be transported long distances to synaptic sites (Chen and Chan, 2009).

Since we observed an endogenous decrease of mitochondrial length in Polg\textsuperscript{m/m} MEFs, we hypothesized that increasing mitochondrial elongation, either by inhibiting mitochondrial fission or by inducing mitochondrial fusion, would reduce rotenone toxicity in Polg\textsuperscript{m/m} MEFs. To this end we attempted to modulate mitochondrial elongation both pharmacologically and genetically. Pharmacological inhibition of fission was attempted using the mitochondrial division inhibitor-1 (MDIVI-1), a Drp1 inhibitor. Genetic induction of fusion was attempted by overexpression of the mitofusin proteins Mfn1/2. Overexpression of Myc-tagged Mfn1/2 has been shown to increase fusion (Chen et al., 2003). Neither attempt to increase mitochondrial length rescued the phenotype of rotenone sensitivity in Polg\textsuperscript{m/m} MEFs. In the contrary, MDIVI-1 appeared to cause a slight but significant decrease in cell viability on both genotypes.

One possibility why Mfn1/2 overexpression did not protect against rotenone toxicity is that while mitofusins initiate fusion of the outer mitochondrial membrane, fusion of the inner
membrane is more highly regulated and requires membrane potential to be intact. Under normal conditions, the initiator and rate-limiting step of mitochondrial fusion is outer membrane fusion mediated by Mfn1/2 (Chan, 2012). However, this alone does not necessarily guarantee completion of the process because proper mitochondrial fusion also requires fusion of the inner mitochondrial membrane (Chan, 2012). Fusion of the inner mitochondrial membrane is more tightly controlled, and mediated by optic atrophy 1 (OPA1) (Tolkovsky, 2009). Hydrogen/potassium ionophores that decrease the mitochondrial membrane potential can disrupt mitochondrial fusion by preventing fusion of the inner membrane. This suggests that following outer membrane fusion, progression to fusion of the inner membrane requires the electrical gradient across that membrane to remain intact (Chan, 2006b). It has been previously shown that Polg<sup>m/m</sup> MEFs have an average mitochondrial membrane potential comparable to that of wild type (Kukat et al., 2011). Impaired mitochondrial function is a hallmark of this model, so one might hypothesize that a subset of dysfunctional Polg<sup>m/m</sup> mitochondria have decreased mitochondrial membrane potential, this would prevent inner membrane fusion of this subset of dysfunctional organelles. In the presence of rotenone, ETC inhibition will further impair membrane potential (Hu et al., 2009; Isenberg and Klaunig, 2000; Menke et al., 2003), which will further prevent inner membrane fusion, and therefore overall fusion even in the presence of mitofusin overexpression.

The lack of reduction in rotenone toxicity following Mfn1/2 overexpression and MDIVI-1 treatment goes contrary to our hypothesis, and suggests that perhaps it is mitochondrial fission rather than fusion that is most protective against rotenone toxicity in Polg<sup>m/m</sup> MEFs. Mitochondrial fission is generally considered pro-apoptotic. Mitochondrial fission and fragmentation often occurs during apoptotic cell death (Chan, 2006b). During apoptosis induced by mitochondrial outer membrane permeabilization, self-assembly and mitochondrial recruitment of the mitochondrial fission protein Drp1 is increased, causing a high amount of mitochondrial division and fragmentation (Cassidy-Stone et al., 2008). The inhibition of fission results in reduced cellular sensitivity to apoptosis (Frank, 2006). In the mitochondria, assembled Drp1 co-localizes with the pro-apoptotic activated Bax protein, providing a mechanism coupling mitochondrial fission and apoptosis (Cassidy-Stone et al., 2008). However, mitochondrial fission is not always pro-apoptotic, but can also have important protective roles. Fission can serve to quarantine severely damaged organelles to prevent them from contaminating the rest of the
mitochondrial population (Bess et al., 2012; Bess et al., 2013). Then, working in coordination with mitophagy, fission allows for degradation of severely damaged mitochondria before these activate apoptosis pathways and induce cell death (Bess et al., 2012; Bess et al., 2013). Thus, an alternate explanation for the decreased mitochondrial length observed in Polg\textsuperscript{m/m} mitochondria is that this represents an effort to quarantine and isolate dysfunctional mitochondria that are prevalent in the Polg\textsuperscript{m/m} model. Following rotenone exposure, mitochondrial fission may have a protective effect by quarantining mitochondria damaged by rotenone and targeting them for degradation before they can initiate apoptosis. If this is true, one would predict that induction of fission would protect Polg\textsuperscript{m/m} MEFs from rotenone toxicity. Both genotypes were found to be responsive to mitochondrial length modulation by the dominant negative Drp1 (K38A) protein. The K38A mutation prevents GTP from binding to the mitochondrial fission protein Drp1, inhibits the GTPase activity of Drp1, and therefore prevents mitochondrial fission (Lee et al., 2004). Once Drp1 was inhibited by Drp1 (K38A), fission was impaired and fusion was able to increase the overall length of the mitochondrial population of Polg\textsuperscript{m/m} and wild type MEFs. This indicates that modulation of mitochondrial dynamics is feasible in Polg\textsuperscript{m/m} MEFs, and suggests that mitochondrial fusion mechanisms are not absent, but rather decreased in Polg\textsuperscript{m/m} MEFs as part of an adaptive response.

4.3.2 Mitochondrial autophagy

Mitochondrial autophagy is another important mitochondrial stress response because it maintains a healthy mitochondrial population by selectively eliminating damaged mitochondria. Microtubule-associated light chain 3 (LC3) is a cytosolic protein (LC3-I) that becomes lipitated (LC3-II) and recruited to the autophagosome during autophagy (Kabeya et al., 2000). This makes the pro-autophagy form LC3-II a reliable marker of mature autophagosomes, and a good way to measure autophagy (Klionsky et al., 2008). We were able to detect autophagy in our cell lines through the use of GFP-tagged LC3 followed by fluorescence microscopy. However, the transient nature of the GFP-LC3 transfection, along low transfection efficiency made this method not optimal for quantification of autophagy. Another method we used to detect autophagy is LC3 western blot analysis. This method had several advantages over the fluorescent microscopy. The LC3 western blot allows detection of endogenous LC3, and the difference in migration between the cytosolic form (LC3-I) and pro-autophagy lipitated form (LC3-II) allows for detection of both forms. The LC3 western blot analysis was able to quantitatively detect autophagy in all cells.
and allowed us to measure autophagy modulation. We have observed decreased endogenous autophagy in $Polg^{m/m}$ MEFs compared to wild type. This was an unexpected observation. Considering $Polg^{m/m}$ MEFs have dysfunctional and damaged mitochondria relative to wild type, we anticipated this would result in higher baseline autophagy in order to remove damaged and dysfunctional organelles. One interpretation could be that $Polg^{m/m}$ MEFs have lost the ability to detect dysfunctional mitochondria. Both genotypes seem equally responsive to autophagy induction by rapamycin, as both had an approximate 50% autophagy induction. This suggests that modulation of autophagy is feasible in $Polg^{m/m}$ MEFs, and that mitophagy mechanisms are not impaired, but merely decreased in these cells.

In order to determine whether mitophagy plays a role in the rotenone susceptibility in $Polg^{m/m}$ MEFs, cells of both genotypes were pre-treated with rapamycin and exposed to rotenone. As measured through flow cytometry analysis, rapamycin pre-treatment was able to rescue the rotenone toxicity in both genotypes. There was a complete rescue of rotenone toxicity in wild type MEFs, and since the cell viability of $Polg^{m/m}$ MEFs treated with rapamycin and rotenone was not statistically different from vehicle control, the attenuation of $Polg^{m/m}$ MEF toxicity can also be considered a complete rescue effect. Our results are in agreement with previous reports that autophagy induction with rapamycin can have a protective effect. Pan et al. reported that rapamycin pre-treatment protected cells from rotenone-induced mitochondrial dysfunction, reduced the release of cytochrome c from the mitochondrial into the cytosol, and attenuated rotenone-induced apoptosis (Pan et al., 2009). The authors concluded that enhanced autophagy attenuated rotenone toxicity by clearing the cell of dysfunctional mitochondria that would have a pro-apoptotic effect by undergoing mitochondrial membrane depolarization and cytochrome c release (Pan et al., 2009). We propose that a very similar mechanism of toxicity attenuation is occurring in our present results.

In addition to its role in autophagy, the mTOR pathway is also involved in numerous cellular processes, including cellular growth, cancer, diabetes and aging (Zoncu et al., 2011). The mTOR pathway is responsible for maintaining energy balance in the cell. It is able to sense nutrient abundance, stress signals and growth factors, then regulates cellular growth and proliferation accordingly (Zoncu et al., 2011). mTOR activation drives cellular growth, energy storage and consumption. Conversely, mTOR suppression encourages energy conservation and proliferation arrest (Zoncu et al., 2011). mTOR activity can therefore serve as a switch between conditions of
growth and starvation (Zoncu et al., 2011). mTOR is often implicated in diseases where growth becomes dysregulated, such as cancer, metabolic diseases and aging (Zoncu et al., 2011). Inhibition of mTOR has been shown to increase the translation of several genes that encode for components of the ETC (Zid et al., 2009), which is expected to improve mitochondrial respiration and decrease ROS generation, reducing overall cellular damage from mitochondrial sources (Zoncu et al., 2011). Therefore, it is possible that treatment with the mTOR inhibitor rapamycin may cause attenuation of rotenone toxicity through mechanisms distinct from autophagy induction.

Polg<sup>m/m</sup> MEFs have a phenotype of endogenous mitochondrial dysfunction, and may be susceptible to undergo apoptosis and cell death when exposed to mitochondrial-damaging xenobiotics. Enhanced mitophagy caused by rapamycin pre-treatment clears the cell of many of its dysfunctional mitochondria before these organelles can initiate apoptotic cascades. Overall, our results suggest that enhanced mitochondrial autophagy, but not increased mitochondrial fusion or decreased mitochondrial fission, protects against rotenone susceptibility in cells with impaired mtDNA replication fidelity.

### 4.4 Role of mtDNA replication in toxicity initiated by mitochondria-targeted chemotherapeutic drugs

Mitochondria are a potential target for drug delivery because these organelles play a crucial role in energy production, cancer, and the induction of apoptosis (Horton et al., 2008; Stewart et al., 2008). Delivery of xenobiotics to the mitochondria is challenging because mitochondria have two membranes, with the inner mitochondrial membrane being charged and near impermeable. Mitochondria-penetrating peptides are a potential tool for mitochondrial drug delivery because these peptides have good cellular uptake, and localize to the mitochondria based on its unique charge and lipophilicity (Fonseca et al., 2011; Stewart et al., 2008).

For the purpose of causing alkylation damage to the mtDNA and not the nuclear genome, Cbl has been covalently conjugated to MPP and therefore re-routed to the mitochondria (Fonseca et al., 2011). The ability of MPP and its conjugated cargo to penetrate both the cellular and mitochondrial membranes, and accumulate specifically in the mitochondria depends on the mitochondrial membrane potential ($\Delta\psi_m$) (Fonseca et al., 2011; Horton et al., 2008; Yousif et al., 2009a; Yousif et al., 2009b). Since the mitochondrial membrane potential
(Δψₘ) is similar in Polgₘ/ₘ and wild type MEFs (Kukat et al., 2011), mitochondrial delivery of mt-Cbl is predicted to be similar in Polgₘ/ₘ and wild type. Thus, any differences in biochemical and cellular responses to mt-Cbl between Polgₘ/ₘ and wild type are not due to differences in mitochondrial delivery of this chemical.

Changes in cellular bioenergetics, measured indirectly through changes in oxygen consumption rate (OCR) can be an excellent readout of mitochondrial stress (Qian and Van Houten, 2010). We assessed potential changes in OCR using extracellular flux analysis in intact cells. This approach has the advantage of avoiding artifacts generated during mitochondrial isolation (Qian and Van Houten, 2010). The bioenergetic profile of untreated MEFs suggests that mitochondrial oxidative phosphorylation is endogenously lower in Polgₘ/ₘ compared to wild type MEFs. This observation is in agreement with other published reports of respiratory deficiency in the Polgₘ/ₘ model, assessed by polarographic measurement of ETC complex function and enzymatic colorimetric reactions (Hiona et al., 2010; Kuloth et al., 2005; Kukat et al., 2011; Trifunovic et al., 2005; Trifunovic et al., 2004). We observed that mt-Cbl increased the OCR in wild type but not in Polgₘ/ₘ MEFs. This increase in OCR in wild type cells following drug exposure was interpreted as an increased cytoprotective stress response. We also observed that untargeted Cbl decreased OCR in Polgₘ/ₘ but not in wild type MEFs. Taken together, these results suggest that mitochondrial bioenergetics in wild type MEFs have stress response not found in Polgₘ/ₘ MEFs. In response to mitochondrial damage caused by mt-Cbl, wild type cells were able to increase their OCR as a compensatory measure, while Polgₘ/ₘ MEFs were unable to do this. Similarly, the overall cellular stress of untargeted Cbl caused Polgₘ/ₘ MEFs to decrease their oxygen consumption while wild type cells were able to maintain respiration at baseline levels.

Following observations of a genotype difference in bioenergetic response to mitochondrial-targeted (mt-Cbl), or untargeted (Cbl) alkylating agents, we assessed the extent of DNA damage by these two compounds in wild type and Polgₘ/ₘ MEFs. mt-Cbl has been reported to damage mitochondrial DNA in HeLa cells at levels of 1 DNA lesion per 10 kB at 2 hours after 3 µM mt-Cbl (Fonseca et al., 2011). In contrast, we observed no detectable mtDNA damage caused by 6 µM mt-Cbl in either genotype. The lack of decreased PCR amplification, interpreted as a lack of DNA damage caused by mt-Cbl exposure was unexpected, and could be due to mitochondrial membrane potential in MEFs not being sufficiently high to allow MPP to accumulate inside
mitochondria. Alternatively, mt-Cbl may be predominantly alkylating other macromolecules within the mitochondria other than mtDNA, and this obscures any effects on mtDNA. The quantitative long PCR assay used in these experiments is able to detect DNA lesions in the order of 1 per 10 kb (Santos et al., 2006a). It is possible that mt-Cbl damaged the mtDNA at a frequency below the level of detection of this technique. Proofreading-defective Polg has been reported to bypass irreparable bulky mtDNA damage at a higher frequency that wild type Polg (Kasiviswanathan et al., 2012). Thus, if mt-Cbl does cause mitochondrial DNA alkylation in Polg<sup>m/m</sup> MEFs, such damage may be more mutagenic than in wild type MEFs.

Turning our attention to untargeted Cbl, the observations of lack of untargeted Cbl lesions in the mtDNA are consistent with a previous report that Cbl does not enter mitochondria and does not damage mitochondrial DNA (Fonseca et al., 2011). Damage to the nuclear genome by Cbl is well established (Begleiter et al., 1996). The lesion levels we observed in the current study are of a similar magnitude to those reported in the nuclear genome of HeLa cells after 2 hours of Cbl treatment (Fonseca et al., 2011). A dose-response relationship between Cbl concentration and DNA lesion level was observed in both a transcribed gene and a non-transcribed gene. Lesion levels at 8 hours were generally lower than at 2 hours, consistent with DNA repair of these lesions. At 400 µM Cbl, a concentration in the reported range of the LD<sub>50</sub> of Cbl in other mammalian cell lines (Fonseca et al., 2011), DNA lesion levels were consistently two-fold higher in wild type relative to Polg<sup>m/m</sup> MEFs, both in the transcribed and non-transcribed gene, and at both time points. The most straightforward interpretation of this result is that Polg<sup>m/m</sup> MEFs have enhanced repair of nuclear DNA. Cbl causes monofunctional adducts and interstrand cross-links in nuclear DNA (Begleiter et al., 1996). Theoretically, an increase in nuclear DNA repair in Polg<sup>m/m</sup> MEFs is potentially an example of the broader concept of DNA retrograde communication from the mitochondrial genome to the nuclear genome (Jazwinski, 2012; Spinazzola and Zeviani, 2005). There are reports in literature stating that continuous low doses of oxidative stress to mtDNA leads to mtDNA damage, but in some cases also causes an increase in the integrity of nuclear DNA (Sawyer and Van Houten, 1999). The authors interpreted their data as an example of cross talk between the mitochondria and nucleus that causes up-regulation of DNA repair enzymes for both genomes. If a similar upregulation is the case here, one possible explanation for the apparent lower DNA damage in Polg<sup>m/m</sup> MEFs
compared to wild type could be that endogenous mtDNA damage in the \(Polg^{m/m}\) model causes a baseline increase in integrity of the nuclear genome in these cells.

Although genotype differences were observed in the bioenergetic and long PCR experiments, cell survival did not differ between wild type and \(Polg^{m/m}\) MEFs exposed to mt-Cbl, Cbl, or MPP. mt-Cbl was more potent at causing cell death than untargeted Cbl. The observation that MPP alone was not overly toxic even at concentrations of 10 µM while 3 µM mt-Cbl caused cell viability to fall below 10% suggests that the observed toxicity was likely due to the alkylating activity of mt-Cbl and not the peptide toxicity. Similar observations regarding toxicity were noted when an acute necrosis assay was performed. The increased toxicity of mt-Cbl is in agreement with published results that indicate mt-Cbl has approximately 100-fold increased cytotoxicity in HeLa cells compared to untargeted Cbl and the MPP alone (Fonseca et al., 2011). The \(LC_{50}\) for mt-Cbl and untargeted Cbl in HeLa cells was 3 µM and >100 µM, respectively. In K562 leukemia cells, the \(LC_{50}\) for mt-Cbl and untargeted Cbl was 8.7 µM and 306 µM, respectively (Fonseca et al., 2011). It is possible that due to its high potency and off-target alkylation of mitochondrial macromolecules other than mtDNA, mt-Cbl may be inducing cell death through a variety of different mechanisms (Mourtada et al., 2013). If that is the case, and multiple death pathways are activated simultaneously by mt-Cbl (Mourtada et al., 2013), then it is likely that this compound is not specific enough with respect to mtDNA damage for \(Polg^{m/m}\) sensitivity to be observed. Nevertheless, these results support the null hypothesis that mtDNA replication fidelity does not protect against toxicity of mitochondria-targeted chemotherapeutic drugs.

Targeting the chemotherapeutic agent doxorubicin to the mitochondria was equally toxic to both genotypes. Doxorubicin is able to cause mitochondrial toxicity through many different mechanisms, including mtDNA intercalation, polymerase inhibition, topoisomerase II inhibition, complex I inhibition, and redox cycling leading to ROS generation (Cohen, 2010; Oliveira et al., 2004; Wallace, 2003). Doxorubicin is able to cause mitochondrial damage through direct mtDNA damage, but also through indirect bioenergetic stress. The synergy of all these mechanisms can make mt-Dox extremely toxic to mitochondria, but its toxicity becomes very non-specific. This non-specificity renders doxorubicin a weak tool in this particular study because it may not be possible to distinguish between its genotoxic and bioenergetic mitochondrial stress effects, and its mechanism of toxicity will be too broad. Our observations
suggest that Polg\textsuperscript{m/m} MEFs are hypersensitive to several different bioenergetic stresses. However, the multiple mechanisms of mt-Dox toxicity working together may serve to heighten the toxicity of wild type MEFs as well, therefore negating any genotype sensitivity that might have been observed through a single mechanism. This may be the reason why no genotype difference was observed between wild type and Polg\textsuperscript{m/m} MEFs exposed to mt-Dox.

In contrast to mt-Cbl and mt-Dox, targeting the synthetic platinum compound (Pt-AcAc) to the mitochondria (mt-Pt) resulted in increased apoptosis preferentially in Polg\textsuperscript{m/m} MEFs. Pt-AcAc has a mechanism of toxicity similar to cisplatin, causing DNA crosslinks (Wilson and Lippard, 2012). Targeting this compound to the mitochondria did not appear to significantly alter its toxicity in wild type MEFs, but caused an apparent 4-fold increase in apoptosis in Polg\textsuperscript{m/m} MEFs. This suggests that both genotypes respond very differently to mtDNA crosslinking damage. Mitochondria have limited DNA repair machinery, and are not able to repair cisplatin mtDNA crosslinking damage (Bohr, 2002; Bohr et al., 2002; Cullinane and Bohr, 1998; Liu and Demple, 2010; Singh and Maniccia-Bozzo, 1990). One outcome of persistent mtDNA damage that cannot be repaired is mitochondrial autophagy 24 to 72 hours following exposure to ultraviolet C radiation (UVC) (Bess et al., 2013). Interestingly, this damage removal pathway for persistent irreparable mtDNA lesions caused by UVC requires the coordination of mitochondrial fusion, fission, and autophagy (Bess et al., 2012). Mitochondrial fission and autophagy promote removal of these mtDNA lesions while mitochondrial fusion is required to preserve mitochondrial function by providing complementation of healthy mtDNA and mitochondrial proteins (Bess et al., 2012). Since our data suggest that both the processes of mitochondrial dynamics and mitochondrial autophagy are altered in Polg\textsuperscript{m/m} MEFs, the Polg\textsuperscript{m/m} MEFs may be sensitive to mt-Pt as a result of an inability to remove persistent irreparable mtDNA crosslinks. These data support the hypothesis that mtDNA replication fidelity protects against toxicity initiated by at least one mitochondria-targeted chemotherapeutic drug.

4.5 Conclusions and significance

The phenotype of increased mtDNA mutation load in the Polg\textsuperscript{m/m} MEFs represents an extreme example of mtDNA mutation load not likely to be found within the human population. Nevertheless Polg\textsuperscript{m/m} MEFs serve as a model to examine the potential consequences of mtDNA mutations on toxicity initiated by mitochondrial damage resulting from xenobiotics. Bioenergetic
stress caused by ETC inhibition can be initiated by other xenobiotics; the implication of these findings is that mtDNA replication fidelity may also be protective against toxicity initiated by many other xenobiotics.

Mitochondrial toxicity is recognized as a serious problem by the pharmaceutical industry (Dykens and Will, 2007, 2008). Off-target mitochondrial toxicity often appears idiosyncratic, unrelated to drug dose, and very difficult to model or predict (Pereira et al., 2012). It has been predicted that mitochondrial genetics are a factor in predisposing individuals to drug-induced mitochondrial toxicity. Within the human population, it is estimated that approximately 1 in 200 individuals have potentially pathogenic mtDNA mutations (Elliott et al., 2008). It is hypothesized that in the majority of individuals with mtDNA mutations, the burden of pathogenic mtDNA mutations is below the threshold for disease to occur. However, our data suggest that increased levels of mtDNA mutations may predispose individuals to xenobiotic induced mitochondrial toxicity. We have observed that concentrations of rotenone that cause no detectable cell death in wild type MEFs induced a 3-fold increase in apoptosis in Polg$^{m/m}$ MEFs. This may suggest that clinical drugs and environmental chemicals with off-target ETC inhibition may be harmful to individuals with mtDNA mutations at xenobiotic concentrations that are lower than those considered safe for the general population. Mutations in the nuclear gene POLG occur in approximately 1 in 200 individuals within the general population (Hudson and Chinnery, 2006). These mutations are found throughout the Polg protein, including the exonuclease-proofreading domain (Hudson and Chinnery, 2006). While these variants do not produce a disease phenotype, they may sensitize individuals to drug-induced mitochondrial toxicity. Thus, my work has potential clinical relevance for an appreciable proportion of the population.

With respect to tissue specificity, drug induced mitochondrial toxicity frequently occurs in aerobically-poised tissues that have a high metabolic demand and a special reliance on mitochondrial oxidative phosphorylation, such as the cardiovascular and central nervous systems (Silver and Erecinska, 1998). Other organ systems such as the liver and kidneys are also among the most common targets for drug induced mitochondrial toxicity. Their toxicity can be modulated by genetic predisposition and exacerbated by repeated drug exposures (Dykens and Will, 2008). Mitochondria are especially important for the proper function of the central nervous system because mitochondria in the brain appear to be more active than in other body tissues as
the brain utilizes a disproportionately higher amount of oxygen relative to its overall mass (Silver and Erecinska, 1998). Neurons are potentially highly susceptible to ETC impairment because neurons have unique energy demands compared to other tissues. Over half of the ATP generated in the brain is used to maintain ionic gradients crucial for proper signal transduction (Erecinska and Silver, 1994). Another energetic challenge faced specifically by neurons is that mitochondria must often be transported long distances across the cell, and mitochondrial trafficking requires energy. Drug toxicity that impairs ATP generation is therefore expected to disrupt mitochondrial transport and further disrupt neuronal function (Chang and Reynolds, 2006). Some neuronal populations also appear to be selectively vulnerable to xenobiotic-induced mitochondrial toxicity. As an example, low doses of complex I inhibitors such as rotenone cause preferential toxicity to dopaminergic neurons and can produce Parkinson-like syndromes (Betarbet et al., 2000).

Cardiovascular toxicity is another common effect of drug-induced mitochondrial dysfunction. Over 50% of drugs receiving FDA black box warnings for cardiovascular toxicity have mitochondrial liabilities (Dykens and Will, 2008). The cardiovascular system has a very high metabolic demand and is extremely reliant on aerobic metabolism and mitochondrial oxidative phosphorylation. This makes the heart very sensitive to xenobiotics that inhibit mitochondrial function and ATP generation (Dykens and Will, 2008). Common mechanisms of drug-induced cardiovascular toxicity include inhibition of ETC complex activity, redox cycling, and inhibition of mitochondrial genomic replication and expression (Dykens and Will, 2008). Another tissue with a high prevalence of mitochondrial toxicity is skeletal muscle. Muscle has a high metabolic demand and strong reliance on aerobic respiration. Many different classes of drugs and xenobiotics can cause muscle toxicity through inhibition of mitochondrial function (Dykens and Will, 2008).

Kidney injury can also result from toxicity induced by hundreds of drugs, and many nephrotoxic drugs are known to target mitochondrial function (Dykens and Will, 2008). There are mechanisms of mitochondrial toxicity that are unique to the kidneys because in addition to general bioenergetic functions, nephron mitochondria also play specific roles crucial for proper kidney function (Dykens and Will, 2008). For example, mitochondria of the nephron are involved in generating the active metabolite of vitamin D, and release ammonia into the distal
segments of the nephron in order for proton secretion into the urine to occur (Dykens and Will, 2008).

Mitochondrial toxicity is one of the most frequent mechanisms of drug-induced liver injury, which can be caused by the parent compound or a reactive metabolite (Pessayre, 1995). Drug-induced liver injury is a frequent reason why drugs fail during drug development, and often leads to withdrawal from the market. This makes mitochondrial liver toxicity a major problem for the pharmaceutical industry (Dykens and Will, 2008; Labbe et al., 2008). Preclinical studies during drug development may be able to identify the formation of reactive metabolites or mitochondrial toxicity at clinically relevant concentrations, but such tests are not required by all regulatory agencies and therefore are not always performed (Dykens and Will, 2008). Clinical trials often fail to identify drugs with potential liver toxicity because such events are relatively rare and more likely to occur in susceptible individuals that are not included in standard clinical trials due to exclusion criteria (Dykens and Will, 2008). As a result, the mitochondrial liability of many drugs does not become apparent until after the drug has been approved and entered the market. The most common type of drug-induced liver damage is acute hepatitis, which causes inflammation and destruction of hepatocytes, potentially leading to jaundice, hepatic encephalopathy, and death (Pessayre et al., 1999). A frequent mechanism of drug-induced liver injury is the production of reactive metabolites that cause direct toxicity or immune reactions. Other mechanisms of drug-induced liver toxicity include: anionic uncoupling that diminishes the mitochondrial membrane potential and impairs ATP generation, opening of the mitochondrial permeability transition pore, impairment of mitochondrial β-oxidation and the accumulation of fat in the liver, sequestration of mitochondrial factors such as coenzyme A, and many other mechanisms (Dykens and Will, 2008).

The sensitivity of in vitro detection of mitochondrial toxicity can be increased by simply growing cells in galactose instead of glucose as the carbon source. Under typical culture conditions, cells are grown in glucose concentrations five times higher than physiological levels, which cause most cells to rely on glycolysis instead of OXPHOS for energy production (Marroquin et al., 2007). The addition of galactose instead of glucose as the carbon source forces cells to once again rely on mitochondrial function for ATP generation. This simple change in media formulation can greatly enhance the susceptibility of cells to drug induced mitochondrial toxicity, and can serve to improve the sensitivity of toxicity endpoints (Marroquin et al., 2007;
Pereira et al., 2012). We have observed that $Polg^{m/m}$ MEFs have susceptibility towards several drugs and chemicals with known mitochondrial liabilities. We propose that the $Polg^{m/m}$ MEF model may have potential utility in drug screening as a complementary model to test for mitochondrial toxicity. The potential advantage of drug screening for mitochondrial toxicity using $Polg^{m/m}$ MEFs is that one may be able to identify mitochondrial liabilities that would otherwise go undetected in wild type cells.

In conclusion, the data presented in this thesis indicate that mtDNA replication fidelity is protective against drug-induced mitochondrial toxicity, with the implication being that individuals with mtDNA mutations or defective DNA Polymerase Gamma may be at an increased risk of drug-induced mitochondrial toxicity. This type of drug toxicity may appear idiosyncratic, and would most likely be caused by drugs with known mitochondrial liabilities, such as off-target inhibition of the electron transport chain, inhibition of enzymes required for mtDNA maintenance or direct damage to mtDNA. This mitochondrial toxicity would be expected to occur in susceptible tissues with a strong reliance on mitochondrial respiration, such as the central nervous system, cardiovascular system, muscles, kidneys and liver. Susceptible populations can experience toxicity at much lower concentrations than wild type controls, but traditional methods used by the pharmaceutical industry to screen for mitochondrial toxicity may not be sensitive enough to detect this increased risk. Detection of drug-induced mitochondrial toxicity in susceptible populations may only be feasible using highly sensitive techniques such as the extracellular flux analyzer, coupled with conditions designed to heighten mitochondrial sensitivity, such as media formulations containing galactose instead of glucose. Use of cell lines with a hypersensitivity phenotype, such as the $Polg^{m/m}$ MEFs may also improve the detection of drugs with an increased risk of toxicity in susceptible populations.

4.6 Future directions

**Measurement of mitochondrial membrane potential.** Data presented in this thesis indicates that $Polg^{m/m}$ MEFs have increased apoptosis following rotenone exposure. Since depolatization of the mitochondrial membrane potential ($\Delta \Psi_m$) is a trigger for apoptosis, rotenone-induced depolarization of the mitochondrial membrane preferentially in the $Polg^{m/m}$ MEFs could be a mechanism by which rotenone sensitivity occurs. The proposed experiment would measure $\Delta \Psi_m$ of wild type and $Polg^{m/m}$ MEFs by flow cytometry analysis of cells stained
with the JC-1 dye (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide). JC-1 monomers exhibit green fluorescence, but in response to \( \Delta \Psi_m \), JC-1 molecules will enter the mitochondria and form aggregates that exhibit red fluorescence. Therefore, the ratio of JC-1 red:green fluorescence is a measure of \( \Delta \Psi_m \) (Reers et al., 1991; Smiley et al., 1991). JC-1 measurement of \( \Delta \Psi_m \) would be carried out both endogenously to confirm wild type and Polg\(^{m/m}\) MEFs have similar \( \Delta \Psi_m \), but also following exposure to rotenone, to determine if increased rotenone-induced apoptosis in Polg\(^{m/m}\) MEFs occurs through a mechanism of mitochondrial membrane depolarization. Since mitochondrial targeting of the mitochondria-penetration peptide used to address the third aim of the hypothesis relies on \( \Delta \Psi_m \), an observation of similar endogenous \( \Delta \Psi_m \) between both genotypes would also confirm that wild type and Polg\(^{m/m}\) MEFs are able to sequester equal amounts of mitochondria-penetrating peptides and their cargo molecules.

**Measurement of mitochondrial ROS generation.** Since rotenone inhibition of complex I generates ROS, mitochondrial ROS generation could potentially be implicated in the mechanism of rotenone-induced Polg\(^{m/m}\) MEF sensitivity. The assay to measure ROS using dichlorofluoroscein diacetate has considerable limitations because it measures total cellular ROS as opposed to mitochondrial ROS. Additional experiments measuring a ROS dye specific to mitochondrial ROS would provide a more reliable measure of ROS generation due to ETC, and how rotenone exposure at nanomolar concentrations affects the ROS levels in wild type and Polg\(^{m/m}\) MEFs. The mitoSOX red mitochondrial superoxide indicator is a fluorogenic dye that targets specifically to the mitochondria where it becomes oxidized and fluoresces in the presence of superoxide. Since superoxide is the primary ROS generated by the ETC, particularly at complex I and complex III, the mitoSOX dye would allow for the measurement of mitochondrial ROS generation. The proposed experiment would measure mitochondrial ROS generation in both genotypes endogenously and in the presence of rotenone to determine whether increased ROS generation is involved in the mechanism of Polg\(^{m/m}\) MEF sensitivity to rotenone. Trifunovic et al. have reported that despite having a severe decrease in oxidative phosphorylation, Polg\(^{m/m}\) MEFs did not show any increased generation of ROS (Trifunovic et al., 2005). The predicted result would be equal levels of ROS generation in both genotypes, consistent with the previous publications (Trifunovic et al., 2005), and consistent with the hypothesis that mitochondrial dysfunction in the Polg\(^{m/m}\) model leads to an induction of
apoptosis instead of a vicious cycle of increased ROS production (Trifunovic, 2006). Since increased ROS generation has not been implicated in the premature aging phenotype of the Polg\textsuperscript{m/m} model, rotenone would be predicted to generate an equal amount of mitochondrial ROS in both genotypes.

**Assessment of senescence in wild type and Polg\textsuperscript{m/m} MEFs.** Apoptosis and decreased proliferation were assessed as possible mechanisms for the observed sensitivity of Polg\textsuperscript{m/m} MEFs to rotenone. However, an additional cell fate that could occur in response to rotenone exposure is cellular senescence. Senescence is strongly associated with aging, and since the Polg\textsuperscript{m/m} model has a phenotype of premature aging, it may be possible that Polg\textsuperscript{m/m} MEFs exposed to rotenone could experience increased senescence in addition to increased apoptosis. Kujoth et al. found no difference in replicative senescence between wild type and Polg\textsuperscript{m/m} primary MEFs (Kujoth et al., 2005). By contrast, Kukat et al. observed that paradoxically, while conditions of high oxidative stress (20% O\textsubscript{2}) induced senescence in wild type, Polg\textsuperscript{m/m} primary MEFs underwent spontaneous immortalization and escaped senescence (Kukat et al., 2011). Senescence can be quantified by measuring senescence-associated beta-galactosidase activity. The proposed experiment would include measurement of senescence in wild type and Polg\textsuperscript{m/m} primary MEFs exposed to DMSO vehicle control and rotenone to determine whether Polg\textsuperscript{m/m} primary MEFs have an endogenous increase in senescence, and whether rotenone induces senescence in addition to apoptosis preferentially in Polg\textsuperscript{m/m} primary MEFs. Since there are reports of oxygen concentrations altering senescence in Polg\textsuperscript{m/m} primary MEFs, this experiment would also be carried out in 5% O\textsubscript{2} and 20% O\textsubscript{2} culture conditions.

**Observation of mitochondrial length and connectivity following pharmacological and genetic modulation of mitochondrial dynamics.** The attempted modulation of mitochondrial dynamics, either pharmacologically by MDIVI-1 treatment, or genetically by Mfn1/2 overexpression, caused no attenuation of rotenone toxicity in Polg\textsuperscript{m/m} MEFs. This lack of any rescue effect suggests that mitochondrial dynamics are not involved in the Polg\textsuperscript{m/m} MEFs sensitivity towards rotenone. In order to strengthen this interpretation, additional immunofluorescence experiments would be carried out to confirm that MDIVI-1 treatment and Mfn1/2 overexpression truly modulated mitochondrial dynamics by significantly increasing mitochondrial length and connectivity. The observation of no attenuation of rotenone toxicity
despite successful mitochondrial dynamics modulation would support the conclusion that mitochondrial dynamics are not involved in the Polg<sup>m/m</sup> MEFs sensitivity towards rotenone.

**Confirmation of autophagy as the mechanism responsible for rapamycin protection against rotenone-induced apoptosis.** The mTOR pathway is the main inhibitory pathway of autophagy, and by inhibiting mTORC1, rapamycin acts as a potent inducer of autophagy. However, other mechanisms, such as the phosphoinositide cycle, can also inhibit autophagy independently of mTOR. Lithium and valproic acid, which are both mood stabilizers used in the treatment of bipolar disease, can inhibit the phosphoinositide cycle and induce autophagy (Renna et al., 2010). Lithium can inhibit inositol monophosphatase, which breaks the phosphoinositide cycle by depleting cellular inositol (Renna et al., 2010; Sarkar et al., 2005). Valproic acid can also inhibit inositol synthesis and cause the decrease of cellular myo-inositol-1,4,5-triphosphate levels (Renna et al., 2010). Lithium and valproic acid have both been shown to protect human neuroblastoma cells from rotenone-induced cytotoxicity (Lai et al., 2006). The proposed experiment would attempt to replicate the rescue effect of rapamycin on rotenone-induced apoptosis using lithium as the autophagy-inducing rescue agent. The successful rescue of rotenone-induced apoptosis in Polg<sup>m/m</sup> MEFs through another independent autophagy-induction mechanism would confirm that attenuation of rotenone toxicity occurred due to increased autophagy and not any other effect caused by rapamycin. Furthermore, since lithium and rapamycin both induce autophagy through separate mechanisms, it is possible that co-treatment would have an additive effect, and could therefore rescue the observed rotenone-induced apoptosis in Polg<sup>m/m</sup> MEFs to a greater extent than lithium or rapamycin alone.

**In vivo assessment of drug-induced mitochondrial toxicity in Polg<sup>m/m</sup> mice.** Polg<sup>m/m</sup> MEFs have increased sensitivity to ETC inhibitors and clinical drugs with off-target mitochondrial toxicity. It remains to be seen whether these observations could be replicated with Polg<sup>m/m</sup> mice in vivo. There is evidence in the literature that genetic mouse models with impaired mitochondrial function show increased sensitivity to drug-induced mitochondrial toxicity. Superoxide dismutase (SOD) is an enzyme that converts superoxide ion radicals to hydrogen peroxide, and therefore plays an important role in cellular antioxidant pathways. A homozygous SOD-knockout mouse model (Sod2<sup>−/−</sup>) displays increased oxidative damage and impaired mitochondrial function characterized by decreased complex I activity, decreased ΔΨ<sub>m</sub>, and decreased ATP production (Lee et al., 2008b; Williams et al., 1998).
Nimesulide is a clinical drug that inhibits cyclooxygenase and is able to inhibit mitochondrial function by uncoupling oxidative phosphorylation, and causes rare but serious cases of hepatotoxicity in susceptible patients. Nimesulide was administered at clinically relevant doses of 10mg/kg twice a day for 4 weeks, and caused increased apoptosis in hepatocytes of $Sod2^{+/−}$ but not wild type mice (Ong et al., 2006). Troglitazone is another clinical drug that causes hepatotoxicity by ETC complex V inhibition (New et al., 2007). Troglitazone treatment at clinically relevant doses of 30mg/kg for 4 weeks caused hepatic necrosis in $Sod2^{+/−}$ but not wild type mice (Ong et al., 2007). Flutamide doses of 100mg/kg/day of for 4 weeks caused liver toxicity, characterized by increased apoptosis and necrosis, in $Sod2^{+/−}$ mice but not in wild type mice (Kashimshetty et al., 2009). A single acetaminophen dose of 300mg/kg caused hepatic necrosis in $Sod2^{+/−}$ but not in wild type mice (Fujimoto et al., 2009).

In the proposed experiment, wild type and $Polg^{m/m}$ mice would be treated with clinical drugs that are known to cause mitochondrial toxicity. Drugs selected for this proposed experiment would include nimesulid, troglitazone, acetaminophen, and flutamide following published methods as described above. Liver toxicity would be assessed by TUNEL staining to detect apoptosis, and H&E staining to detect liver injury such as hepatic necrosis. The predicted results would be that, similarly to $Polg^{m/m}$ MEFs, $Polg^{m/m}$ mice would also present with increased sensitivity to drug-induced mitochondrial toxicity in the form of hepatotoxicity.
References


