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# Mitochondrial Mutagenesis in Aging and Disease

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Marc Vermulst, Konstantin Khrapko and Jonathan Wanagat

Additional information is available at the end of the chapter

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## 1. Introduction

Shortly after DNA was discovered to be the carrier of hereditary information, a number of peculiar observations were made. It was found that some traits, like the ability of yeast cells to use non-fermentable sources of energy, were inherited through a cytoplasmic, and not a nuclear mechanism[1]. This observation was rather alarming at the time, since DNA was only known to be present in the nucleus. As a result, some researchers started to doubt whether DNA was truly the sole carrier of hereditary information. Could it be that the original hypothesis about DNA was incorrect? Or did the cytoplasm carry DNA molecules that were yet to be discovered? Ultimately, this conflict was resolved when clever mating experiments, biochemical tests and precise electron microscopy culminated in the discovery of a new DNA molecule, present inside mitochondria[2-4].

Now, approximately 50 years later, we can truly appreciate the historic nature of this discovery. Not only do we realize how essential mitochondrial DNA (mtDNA) is to life itself, but mtDNA has also helped us understand our origin as a species. As our ancestors spread across the globe, they acquired mutations in their mitochondrial genome, which genetically marked the humans that colonized a certain geographical location. Expansive sequencing projects have now documented these mutations, and used them to trace the origin of mankind back to central Africa[5, 6]. In addition to their historical significance, mtDNA mutations are also important in a medical context. Most diseases that are currently endemic in western society have an mtDNA component, including cancer, diabetes and Parkinson disease. As a result, mitochondrial mutagenesis is now one of the focal points of modern biomedical research[7].

This renewed interest in mitochondrial genetics has led to an enormous influx of new researchers, ideas and technology. Because of this enthusiasm new mouse models have been generated, improved mutation detection assays have been developed, and new roles for

mitochondria have been discovered in immunity, signal transduction, development, and countless other biological processes that are essential to human health[8]. As a result, we are starting to expose the molecular mechanisms that underpin mitochondrial mutagenesis, and we are learning how intimately mutagenesis is related to mitochondrial genetics. The following sections of this chapter will describe this relationship in greater detail.

Feature	Nuclear DNA	Mitochondrial DNA
Length	3 billion bases	16.5kb
Shape	Linear	Circular
Copies per cell	2 chromosomes/cell	100-10.000 copies/cell
Packaging	Histones	Nucleoid
Inheritance	Paternal/maternal	Maternal
Introns	Many	None
Geneticcode	AGA = R, AGG = R AUA = I, UGA = Ter	AGA = Ter, AGG = Ter AUA = M, UGA = W
Replication	Symmetrical	Strand-displacement*
Transcription	Gene specific	Multi-cistronic

**Table 1.** Structural and genetic differences between the nuclear and mitochondrial genome.

## 2. The structure of mtDNA

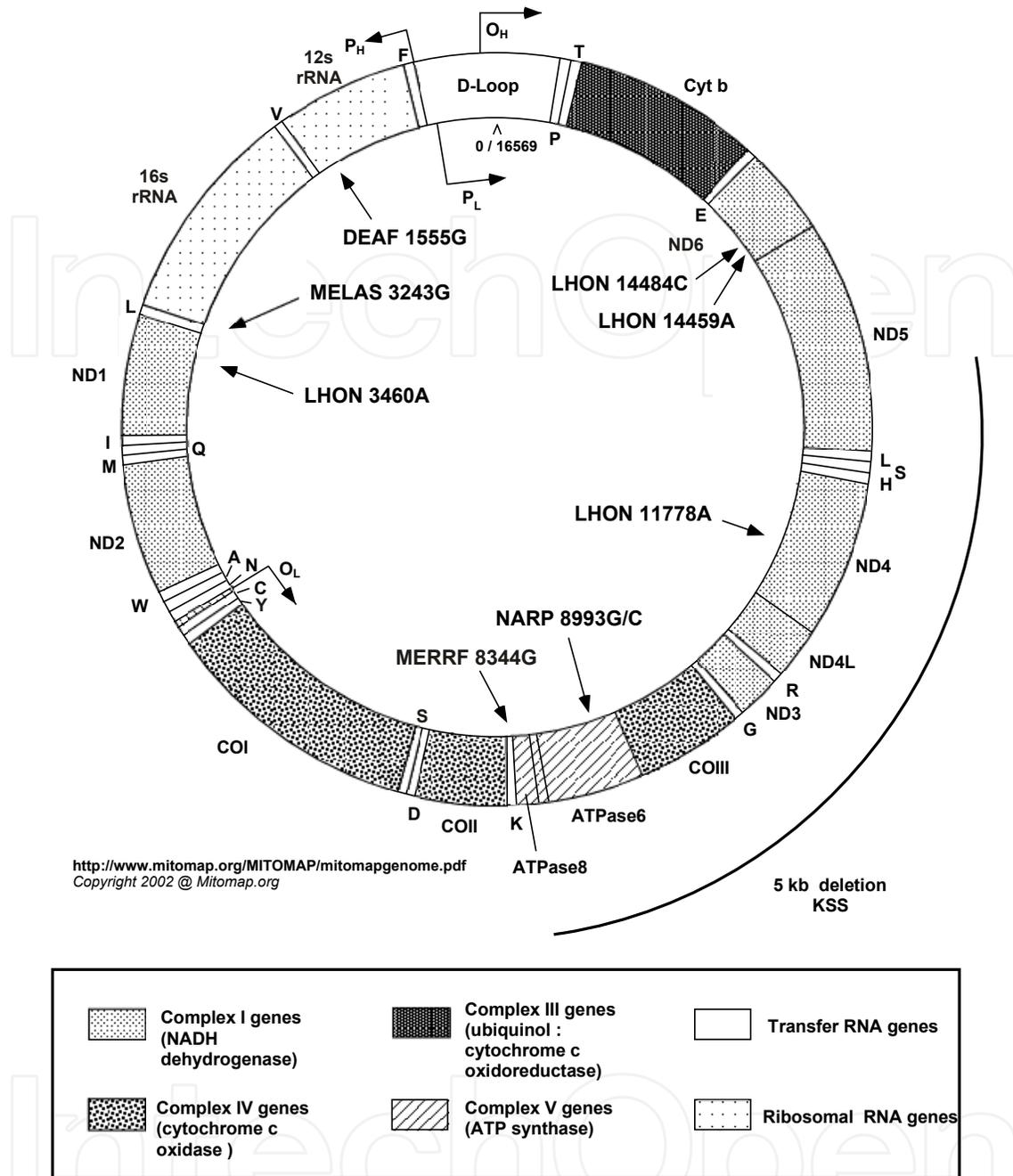
Researchers who are unfamiliar with mitochondrial genetics may find mtDNA to be a peculiar molecule, as it differs from nuclear DNA (nDNA) in almost every way. Some of the most important differences between mtDNA and nDNA are summarized in table 1. First, mitochondrial DNA is a very short DNA molecule, which is approximately 16.5kb in length in most mammalian species[9, 10]. And on top of that, it is circular in nature, not unlike a plasmid. However, even though mtDNA is relatively short, it still makes up a significant portion of the genetic content of a cell because it is typically present in hundreds, or even thousands of copies per cell. These molecules are more or less randomly dispersed across the mitochondrial population of a cell, so that each mitochondrion contains approximately 2-10 mtDNA molecules. This is especially important in the context of mutagenesis, because this multiplicity ensures that, if a mutation occurs in one of these copies, multiple WT molecules can complement the acquired defect.

Remarkably, this complementation is not limited to a single mitochondrion, but encompasses the entire cell. The expansion of this safety net is made possible by the dynamic nature of mitochondrial biology. Mitochondria are highly mobile organelles, which frequently change their position inside a cell. To do this, they travel along the cytoskeleton with the help of numerous proteins, of which Milton and MIRO are two key members. These proteins form a complex with a third protein, Kinesin-1 heavy chain, to mediate

mitochondrial transport[11]. Because mitochondria can travel in either direction, they frequently collide in an end-to-end fashion. During these collisions, mitochondria fuse together to form a single, continuous organelle and mix their contents in the process. This newly formed mitochondrion will eventually undergo a fission event, splitting it back into two smaller organelles. Since all the bio-molecules are randomly distributed across the emerging organelles, each mitochondrion contains a completely new mixture of DNA, RNA and proteins. In fact, each mitochondrion contains an “average” of all the contents that were present in the two mitochondria that initiated the fusion event. Because mitochondria undergo constant cycles of fusion and fission, mtDNA is constantly shared across the entire mitochondrial population. A WT genome can therefore always complement a mutation, even if it is harbored by a mitochondrion that is present elsewhere in the cell[12]. In a sense, you might even say that an “individual mitochondrion” does not really exist. Each mitochondrion is part of a fluid, interconnected network, whose components are in a constant state of flux[13].

Because of this cell-wide safety net, most mutations are thought to be harmless. As long as mutant genomes are outnumbered by WT genomes, mitochondrial fusion and fission ensures that any deficiencies are consistently complemented. However, if a mutation is replicated extensively inside a cell, it can eventually become very harmful. This occurs once a mutation is present in the majority of a cell’s mtDNA, and insufficient WT genomes left for complementation. This “threshold” is typically reached when 60-90% of the mtDNA molecules carry an identical mutation[14]. Such mutations are said to be heteroplasmic (figure 7), because they are present in only a fraction of the mitochondrial genomes in a cell. A mutation that is present in all genomes it is said to be homoplasmic. One of the most remarkable things about mitochondrial mutagenesis is that all the mutations that have been found thus far require clonal expansion before they cause cellular dysfunction. This means that every pathological mutation in mtDNA is recessive. One might argue then, that if we understood this expansion process in greater detail, and learned to control it, we could combat every mtDNA disease using a single intervention.

Another feature of mitochondrial genetics that is important in the context of mutagenesis is the density of the mitochondrial genome[9] (figure 1). MtDNA encodes 13 proteins that are all essential components of the electron transport chain (ETC). In addition, mtDNA encodes 2 rRNA molecules and 22 tRNA particles that help express these proteins. This leaves room for only a short region of non-coding mtDNA. This region is called the displacement loop, or D-loop for short, which was named after its peculiar triple helix structure with one “displaced” DNA strand. The D-loop contains an origin of replication and multiple sites for transcription initiation, which makes it the most important sequence for mtDNA metabolism. When you combine the lack of non-coding regions in mtDNA, with its short length and abundance of genes, it becomes clear how compact the mitochondrial genome is. It is so compact in fact, that some genes, like the ATP6 gene, do not even encode a complete stop codon. Instead, the final base of this codon, an adenine, is added to the mRNA during polyadenylation[9]. This gene density means that almost every mutation is bound to impact mtDNA in a non-trivial way.

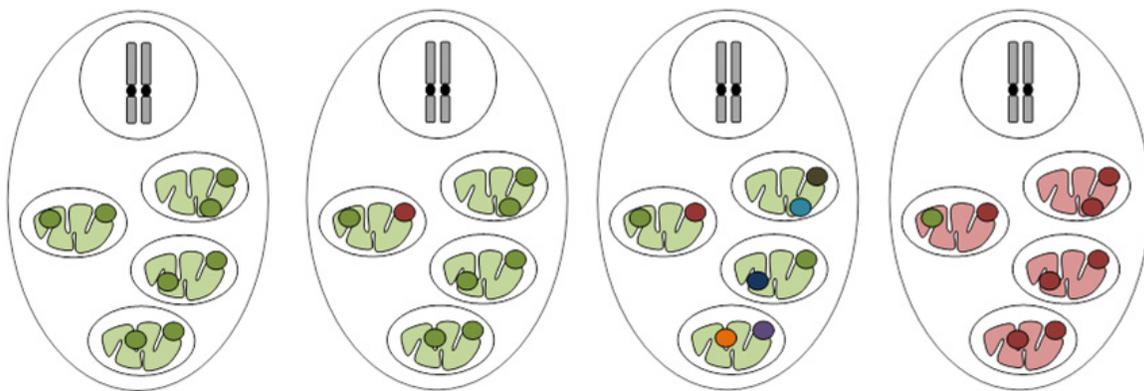


**Figure 1. Organization of the mitochondrial genome.** The mitochondrial genome contains 13 protein coding genes, 2 rRNA genes, and 22 tRNA genes. The mitochondrially encoded proteins are components of complex I, III, IV, or V. Notice how the tRNA genes punctuate the rRNA genes and proteins, which marks the sites where the multicistronic transcript is processed. Several mutations that are responsible for inherited mitochondrial diseases are shown.

The gene composition of mtDNA also affects mutagenesis in a less obvious way: because mtDNA only encodes proteins that are involved in energy production, mitochondria must rely on proteins that are encoded by the nucleus for DNA maintenance. These proteins are transcribed in the nucleus, translated in the cytoplasm and ultimately distributed over the mitochondrial population. The proper distribution of these proteins is a daunting task,

because each cell contains hundreds of mitochondria, each of which should receive its fair share of DNA repair proteins. Ultimately, mitochondria solve this problem by undergoing continuous cycles of fusion and fission, which allows them to share DNA repair proteins and homogenize them across the mitochondrial population. Thus, mitochondrial fusion and fission promote mitochondrial function by enabling mitochondria to share proteins derived from mtDNA *and* nDNA (figure 8, 9).

Another feature of mitochondrial genetics that is important for our understanding of mutagenesis is the maternal inheritance of mtDNA[16]. This has highly complex consequences for human health. One of the more obvious of these consequences is seen in the clinic, where patients with mitochondrial disease receive genetic counseling for future pregnancies. Clearly, if mtDNA is inherited only through the maternal germ line, mutations are inherited maternally as well. Moreover, certain mutations, like large DNA deletions, are difficult to transmit, and counseling needs to take all of these factors in consideration. The more complex consequences go beyond a single human generation though, and affect us on much longer timescale.



**Figure 2. Heteroplasmy and homoplasmy of mtDNA in single cells.** Four cartoons of cells are depicted. Each cell contains 1 nucleus (circle with 2 chromosomes), and 4 mitochondria (ovals with light green coloring) that carry 2 genomes, which are either WT (dark green circle) or mutant (colored circles). The cell on the far left contains no mutant mtDNA, and is therefore homoplasmic for WT mtDNA. The second cell from the left contains 1 mutant molecule and is therefore 12.5% heteroplasmic. The third cell contains 7 unique mtDNA mutations in 7 molecules, and is therefore 12.5% heteroplasmic for each mutation. The cell on the right contains 7 identical mutations in 7 genomes, and is therefore 87.5% heteroplasmic. Although it contains strictly speaking the same amount of mutations as the third cell, only this fourth cell will display mitochondrial dysfunction.

One long-term consequence is that uniparental inheritance surrenders the benefits of sexual recombination. Sexual recombination gives evolution the opportunity to select against, and remove detrimental mutations from the germline. Without sexual recombination, detrimental mutations would simply accumulate in the germline and degrade our DNA until life becomes unviable, a process called Muller's ratchet. In the absence of sexual recombination, mtDNA must evade Muller's ratchet by some other mechanism. For instance, it is possible that some form of purifying selection occurs in the germline, which selects against oocytes that carry detrimental mtDNA mutations. Until recently though, no

direct experimental evidence had been found for this hypothesis. However, two recent studies have now documented powerful proof of such a mechanism in mice. In the first study, a mouse model was generated that exhibits a greatly increased mtDNA mutation rate. The authors discovered that newborn pups of these mutator mice carried significantly more synonymous than non-synonymous mutations than could be expected from random chance[17]. Thus, some form of selection against non-synonymous mutations must be occurring in the germ line. A second study generated two mouse models, which carry either a benign, or a severe mtDNA mutation in their germ line. These authors found that the severe mtDNA mutation was quickly removed from the germ line (in four generations), whereas the benign mutation lingered on for several more generations[18]. These experiments demonstrate that a form of purifying selection protects us from detrimental mtDNA mutations in our germ line, and that the strength of the selection depends directly on the severity of the mutation. The precise mechanism by which this purifying selection operates remains obscure.

A second potential consequence revolves around the co-evolution of the nuclear and the mitochondrial genome. The electron transport chain is composed of approximately a hundred proteins that are divided into 5 major complexes. Thirteen of these proteins are encoded by mtDNA, while the remaining proteins are encoded by nDNA. Because of this shared responsibility, mitochondrial and nuclear DNA have evolved together to optimize energy production. We now know though, that mtDNA differs slightly from person to person, based on their geographical heritage. Thus, the possibility arises that the fine-tuning between mtDNA and nDNA is geographically dependent as well. If so, the co-operation between mtDNA and nDNA may be disrupted by placing mtDNA from one region into the nuclear background of another region. This might cause subtle, but significant metabolic complications. This possibility is supported by experiments in *Drosophila*. Fruit flies are an outstanding animal model for this type of experiment, because different strains of *Drosophila* carry unique mitochondrial genomes. By mating female flies of one strain, with male flies from another strain (and extensively backcrossing their daughters into the nuclear background of their father), the mtDNA of the female fly can be placed in the nuclear background of the male fly. This breaks the evolutionary link between the mtDNA and nDNA of these strains. When mitochondrial function was examined in the experimental flies, it was found that new combinations of mtDNA and nDNA are indeed suboptimal when they are compared to control flies[19, 20]. Thankfully, this is unlikely to be applicable to humans, because even mtDNA from chimpanzees or gorillas function normally in cells with a human nucleus. It is not until mtDNA from animals with a greater evolutionary distance from humans is placed in human cells, like orangutans, that mitochondrial function becomes suboptimal[21].

Other features of mitochondrial genetics that may affect mutagenesis are mtDNA replication and transcription. For over 30 years, it was thought that mtDNA replication occurs through a unique, strand-displacement model[22]. This model stands in stark contrast to the symmetrical model of nuclear DNA replication, which involves coordinated leading and a lagging strand synthesis. If the asymmetric model of mtDNA replication is

correct, mtDNA must be partially single stranded for extended periods of time, which would affect the rate at which mtDNA spontaneously deaminates[23-25], or suffers single-strand and double strand breaks[25, 26].

It is important to note though, that a second model has been suggested for mtDNA replication[27, 28], which is more consistent with traditional leading and lagging strand synthesis. According to this model, enormous amounts of RNA are incorporated into the lagging strand during DNA synthesis[29]. If this is the case, then the ribonucleotide incorporation into mtDNA could have a substantial impact on mitochondrial mutagenesis and potentially explain the observed strand-asymmetry in mutagenesis[30]. Unfortunately, it is currently unclear which model is correct, and an important discussion about these models is currently ongoing[27, 28, 31-34]. Regardless of which model is correct, it is clear that the outcome would have an enormous impact on our understanding of mtDNA mutagenesis. After all, DNA replication is an important source for DNA lesions and the tool by which DNA damage is fixed into mutations.

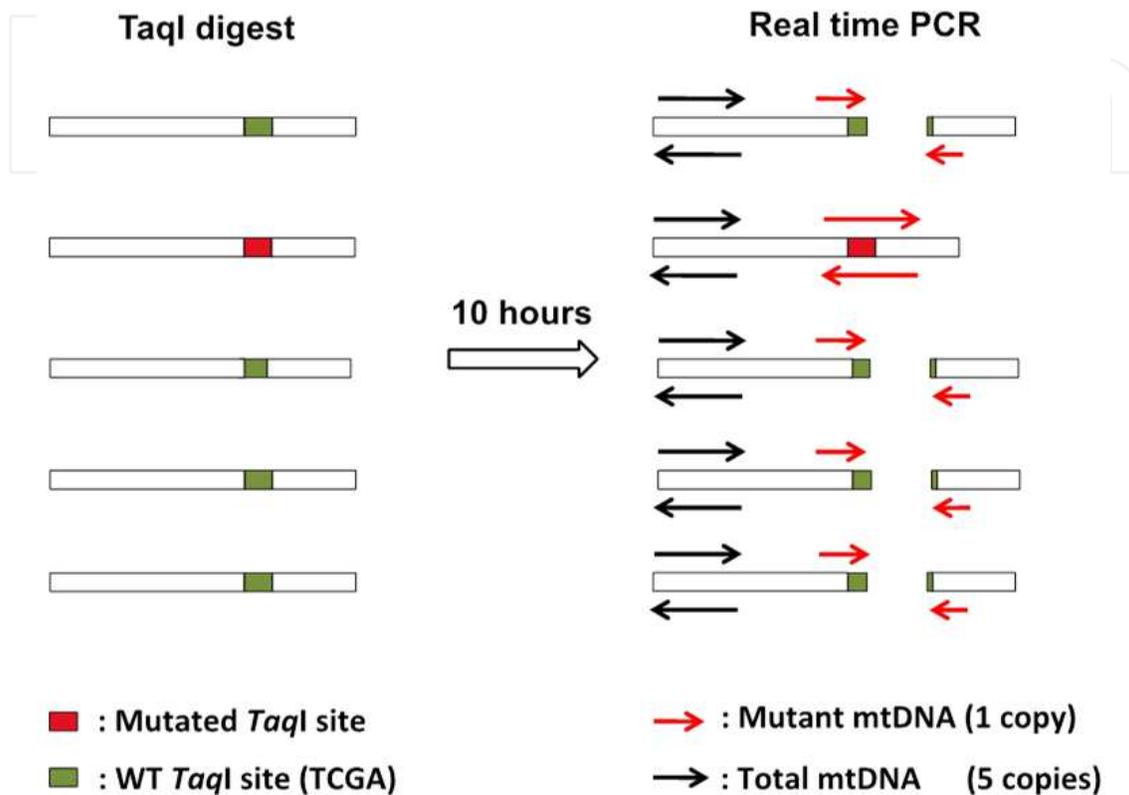
A final feature of mitochondrial genetics that could impact mutagenesis is mtDNA transcription. MtDNA is transcribed in a multi-cistronic manner, meaning that multiple protein-coding genes are transcribed during a single transcription event, similar to bacterial operons[35]. Nascent mRNA molecules are ultimately cleaved at appropriate sites to generate mature mRNAs, which are then handed over to the translation machinery in an orchestrated manner. Although this mechanism is very efficient, it is likely to be very sensitive to DNA damage, because a single DNA lesion could block transcription of multiple genes that are downstream from the lesion. In the nucleus, this problem is solved by transcription coupled DNA repair (TCR), which identifies transcription complexes that are stalled in front of DNA lesions, and recruits the DNA repair machinery with the help of the CSA and CSB proteins[36]. Although CSA and CSB have been localized to mitochondria[37], it is still unclear if, and how transcription coupled repair takes place. For instance, UV lesions are important targets for TCR in the nucleus, where it recruits the nucleotide excision repair machinery to excise the damaged DNA. However, UV lesions are not repaired in mitochondria, and nucleotide excision repair itself does not take place. However, it is possible that, instead of recruiting nucleotide excision repair to stalled transcription complexes, CSA and CSB recruit BER instead[36]. This would be consistent with the idea that oxidative damage is the most likely lesion to occur on mtDNA.

Multi-cistronic transcription also raises the chances of collisions between the transcription bubble and a replication fork. How often such collisions occur, and how they are resolved is still an unexplored question. In the nucleus, these events may be important sources of DNA breaks, which warrants future investigation in studies on mitochondrial mutagenesis.

### **3. Measuring mutagenesis in mitochondrial DNA**

One of the most important problems facing mtDNA researchers today is that there are only a few tools available to detect mutations. Several tools have been tried over the years, but it

was often difficult to draw conclusions from the data because different labs observed different results[38]. The source of this confusion is probably the methodology itself, which is inherently error prone[39, 40]. For the field to grow, it will therefore be essential to develop new tools that match the sensitivity and versatility of those used to detect nDNA mutations.



**Figure 3. Random mutation capture assay.** In this cartoon of the RMC-assay, 5 molecules of MtDNA are digested with TaqI. Four of these molecules contain a WT TaqI restriction site (green boxes) and one molecule contains a mutation in the TaqI site (red box). Using primers that flank the TaqI restriction site, real time PCR can then be used to count the number of copies of mtDNA that contain a mutation in the TaqI restriction site: WT molecules have been cleaved by TaqI, and thus do not provide a viable amplicon for PCR amplification. A second PCR reaction is then performed with primers that are near to, but not affected by the TaqI restriction site. This reaction counts the total number of molecules that were screened for mutations. The true power of this assay is revealed when it is used in a 96 well format, or droplet PCR, so that millions of molecules can be screened at once for mutated TaqI sites.

Most nDNA mutation assays rely heavily on transgenic technology[41], and custom-made mutation markers like fluorescent proteins[42] or the lacZ gene[43] are routinely inserted into a genome of interest. These markers can be genetically engineered to detect any type of mutation, including single base pair substitutions, insertions, deletions or gross rearrangements. Endogenous mutation markers such as the Hprt and Aprt locus can also be used for this purpose[44]. These assays have helped us identify genes that are involved in DNA repair[45], DNA sequences that are prone to mutagenesis[26], and identified environmental compounds that induce mutagenesis[26].

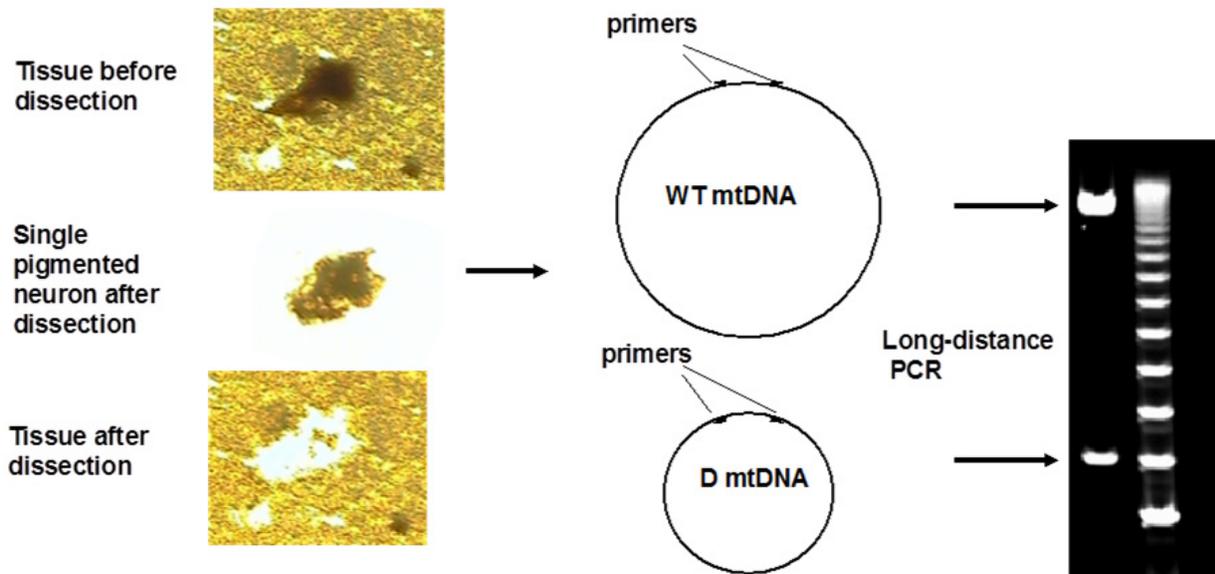
Because mammalian mtDNA cannot be transformed, it is not possible to repeat these experiments in a mitochondrial context. And the small size of mtDNA excludes the possibility of finding large, endogenous genes, such the Hprt or Aprt locus to detect mutations in. However, there are several mutations in the 16S rRNA gene that confer resistance to the drug chloramphenicol[46], which can be used in a mutation screen[47]. Only a handful of bases confer resistance though, which limits the scope of the screen. Another limitation of this assay is that, in order to acquire chloramphenicol resistance, most of the mtDNA molecules inside a cell need to carry the resistance mutation. Thus, mutations that are present in only one, or a few molecules will be missed, including most *de novo* mutations. This assay therefore detects primarily pre-existing mutations that have clonally expanded inside a cell. This extra requirement of clonal expansion introduces an unknown parameter into the assay that could confound the results.

It is important to understand that because of these technical limitations our knowledge of mitochondrial mutagenesis is still fairly limited. This is especially true when it is compared to our knowledge of nDNA, where we know of countless molecules that can induce mutagenesis[45]. We have detailed information about the lesions that are generated during exposure[45], identified the DNA repair proteins that repair these lesions[48], and we know the doses at which mutagenesis occurs. Moreover, we can predict the spectrum of mutations that will arise, and we know the time it takes for these mutations to be fixed. We even know how replication and transcription respond if they encounter these lesions, which signaling pathways are activated as a result, and what the ultimate physiological response of the cell is to these insults[49]. In contrast, we only know of a few chemicals that induce mtDNA mutations, and we have very little information about the proteins that repair these lesions, or how the cell as a whole responds to mtDNA damage.

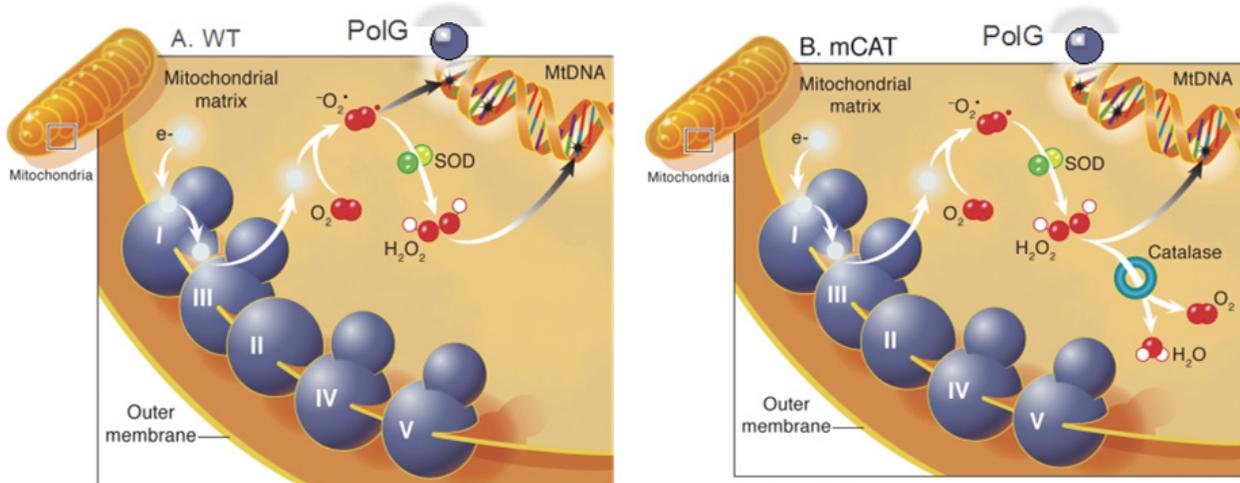
To fill in these gaps in our knowledge, we probably need to find a way to transform the mitochondrial genome first. Until that time comes though, we will have to rely on biochemical assays and sequencing techniques to detect mutations. These assays need to inform us about two important end points. One assay should help us detect mtDNA mutations in bulk samples. This assay will inform us about the overall mutation rate, frequency and spectrum of mtDNA of large amounts of tissue, so that the impact of diet, age, treatment or gene deletions on mitochondrial mutagenesis can be determined. The second assay should help us determine how rapidly mtDNA mutations are expanding within single cells under these conditions. MtDNA mutations must clonally expand before they affect a cell and this assay will therefore provide us with a direct read out of the physiological impact of mutations on an organism.

Currently, the most accurate assay to measure the mutation frequency in bulk samples is the “random mutation capture assay” (RMC-assay)[39, 50]. This tool measures point mutations, deletions or insertions within restriction sites, and can detect one mutation among  $1 \times 10^8$  WT bases (Figure 2). The RMC-assay is a broadly applicable tool, because it uses naked DNA as a template, so that any type of DNA can be interrogated. Moreover, it’s a very economic

tool, which can screen approximately 25 million bases for \$50 in a 4-hour time span. The development of this tool was an important step forward for the field, because it was the first assay to accurately document the spontaneous mutation frequency and spectrum of mtDNA in multiple tissues.



**Figure 4. Detection of mtDNA with large deletions in single cells by long-distance PCR.** To analyze mtDNA from single cells, a cell is first cut out of a tissue sections using a laser capture microdissection. MtDNA is extracted from the cell in a small scale lysis reaction, which is then used to in a PCR reaction. Primers that encompass the entire genome are used to amplify mtDNA, which can then be analyzed on a gel, or sequenced directly. In the example above, both WT molecules, and deleted mtDNA molecules were present in a single isolated neuron.



**Figure 5. MtDNA in mitochondria, with or without a human catalase.** MtDNA is located near the ETC, which exposes it to oxidative damage in the form of the superoxide anion  $O_2\bullet$ . Superoxide can be broken down into the less reactive  $H_2O_2$  by SOD1 or SOD2. Ultimately, this can react to form a hydroxyl radical,  $OH\bullet$  (not shown). Catalase targeted to mitochondria breaks  $H_2O_2$  down into water and oxygen, which decreases the amount of oxidative damage in mitochondria and lowers the mutation rate.

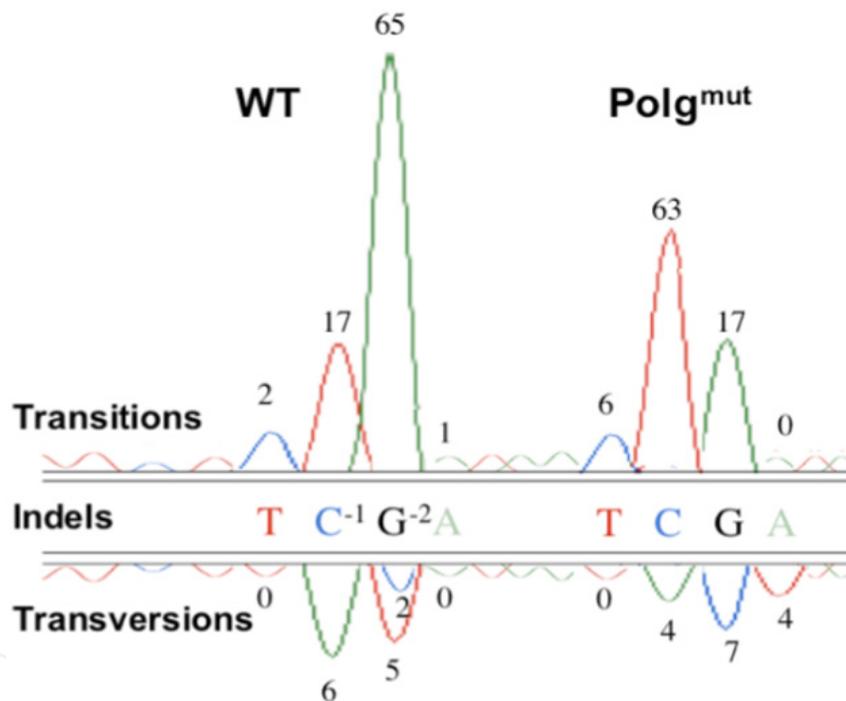
The most precise assay to measure the expansion rate of mtDNA mutations is single cell sequencing of mtDNA[51]. Multiple labs have now established single cell, and even single molecule techniques to do this. In these assays, single cells are collected from tissue slides using laser capture micro-dissection. These cells are lysed in a micro-reaction, after which the mitochondrial genome is PCR amplified and sequenced[51]. If the DNA is diluted to a single molecule level before PCR amplification, it is even possible to sequence single molecules, so that the number of WT and mutant molecules can be determined to estimate the level of heteroplasmy in a cell. This tool is especially powerful when it is used in conjunction with a histo-chemical staining technique to identify cells that have lost mitochondrial function[52].

Each assay does have its drawbacks though. For instance, an important limitation of the random mutation capture assay is that it only detects mutations in *TaqI* restriction sites. This makes it harder to extrapolate the results to the rest of the genome. Single cell sequencing on the other hand, is very costly and laborious. It is expected though, that modern sequencing techniques will greatly improve the cost-effectiveness of this assay in the near future.

#### 4. Mechanisms of mitochondrial mutagenesis

Accurate tools to measure mitochondrial mutagenesis in mammalian cells have only recently been developed. Thus, the mechanisms that cause mtDNA mutations in mammals are still being elucidated. However, several trends are already clear. First, mtDNA is anchored to the inner mitochondrial membrane, a structure that also harbors the electron transport chain. Along this chain, energy is produced in the form of ATP along 5 major complexes (complex I-V). Along this chain, electrons are shuttled from complex to complex, which stores up energy in the form of an electrochemical gradient that drives ATP synthesis. During this process, electrons can escape from the ETC, and react with oxygen to create a highly toxic form of reactive oxygen species. For instance, if the ETC becomes highly reduced, excess electrons from complex I or complex III can react directly with oxygen ( $O_2$ ) to generate the short-lived superoxide anion  $O_2^{\ominus}$ . This molecule can react directly with mtDNA, or be converted into another form of reactive oxygen species, hydrogen peroxide ( $H_2O_2$ ), by manganese superoxide dismutase (Sod2) or copper/zinc super oxide dismutase[53] (Sod1). Hydrogen peroxide is a less reactive than  $O_2^{\ominus}$ , which makes it safer, but it is also longer-lived, and can diffuse far enough into the cell to reach the cytosol or the nucleus.  $H_2O_2$  can be further reduced into a hydroxyl radical ( $OH^{\ominus}$ ), the most potent oxidizing ROS, when it encounters a reduced metal or  $O_2^{\ominus}$ . Under normal physiological conditions though, ROS production is kept at a low level. However, if ROS production increases (for instance if the respiratory chain is inhibited and electrons accumulate on the ETC carriers), it can exceed the antioxidant defenses of the mitochondria. If this happens, ROS can damage mtDNA, forming lesions on the genome that can be fixed into mutations. Thus, ROS is bound to be an important source of mitochondrial mutagenesis, and multiple experiments support this hypothesis. For instance, direct oxidative damage and drugs that induce oxidative damage cause homoplasmic mtDNA mutations in mammalian mtDNA[55].

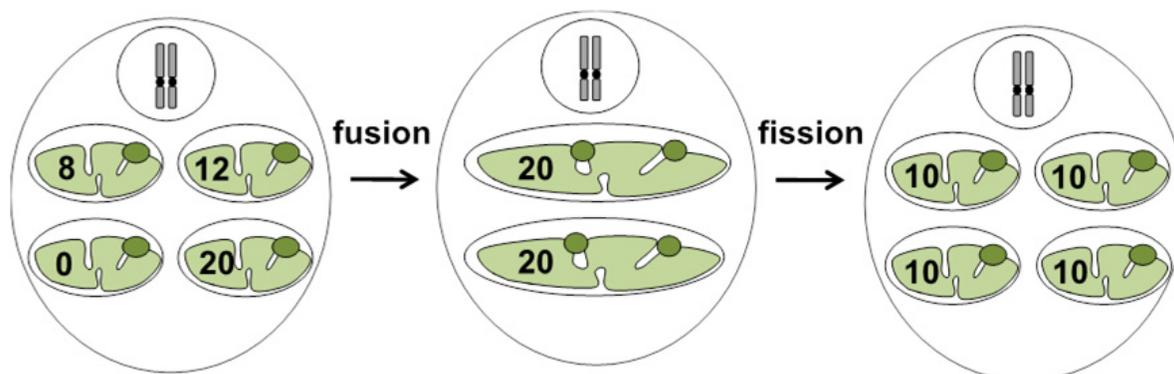
And the reverse was also shown: reducing ROS by expressing a human catalase (an enzyme that breaks hydrogen peroxide down into oxygen and water) that is targeted to mitochondria lowers the mutation rate (figure 3). Mice expressing mitochondrial catalase were shown to display a 2.5-lower mutation burden than normal mice[40, 56]. These, and many other experiments show not only that ROS *can* cause mutations during periods of oxidative stress, but also that ROS drive the endogenous mutation rate in the absence of stress. This hypothesis is further supported by the mutation spectrum of WT mtDNA (figure 4), in which the predominant mutation is a GC::AT transition, which is the most common mutation caused by reactive oxygen species[23, 57]. These GC::AT transitions are generated by cytosine deamination through cytosine glycol and uracil glycol intermediates. Ultimately, uracil will pair with adenine during replication, causing the observed GC::AT transitions[23, 57]. Interestingly, GC::AT transitions are also the predominant mutations found in phylogenetic analyses[30] and many congenital mtDNA diseases[58], suggesting that these mutations may also be the result of oxidative damage.



**Figure 6. Mutation spectrum of WT mice and mitochondrial mutator mice.** The mutation spectrum across 3 TaqI sites is shown in the form of electrophoretograms. The numbers above the peaks indicate the percentage of mutations found for that substitution. For instance, 65% of the mutations that occur at the TaqI sites in WT mice occur at the 3<sup>rd</sup> base pair (G), and are G to A transitions (green peak, corresponding to an adenine base). The mutator mice primarily display GC to AT transitions as well, but at a different base.

Reactive oxygen species are an unavoidable by-product of ATP synthesis[59]. Thus, it can be expected that the rate of ATP synthesis itself will affect mtDNA mutagenesis. Indeed, in a recent study in human cells, a strong correlation was found between mitochondrial respiration and mitochondrial mutagenesis. Cells that relied on mitochondrial respiration displayed higher mutation frequencies than cells that used glycolysis for ATP production.

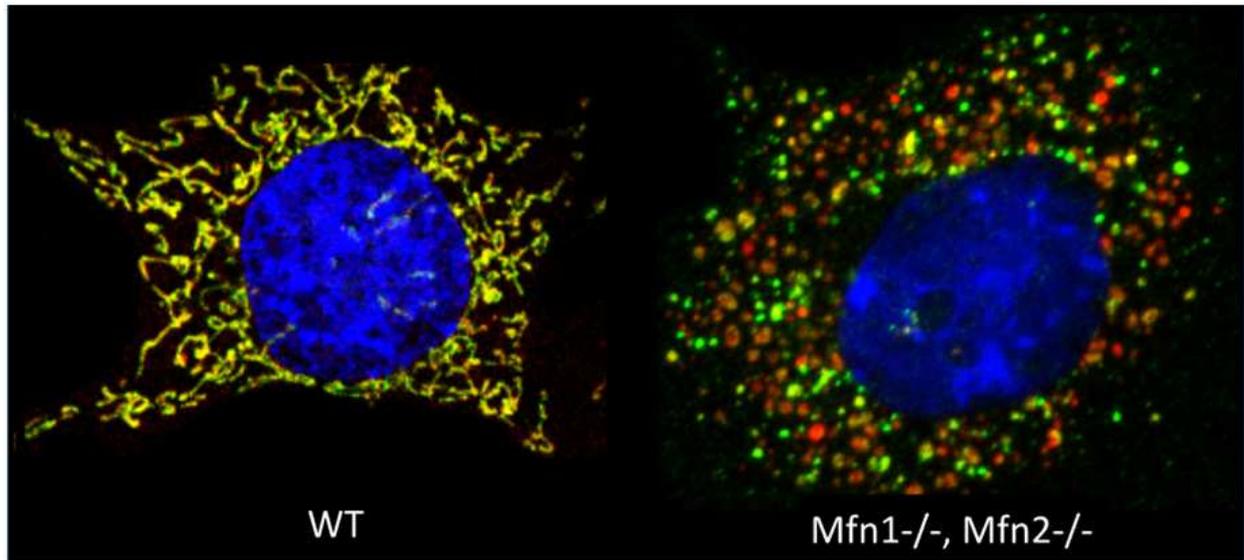
This included cancer cells, which are known to prefer glycolysis for ATP production over respiration, a phenomenon referred to as the Warburg effect[60]. This observation is so far just a correlation though, and more mechanistic experiments will need to substantiate this finding.



**Figure 7. Mitochondrial fusion and fission homogenize protein content.** Three cartoons of cells are depicted. Each cell contains one nucleus (circle with two chromosomes), and 4 mitochondria, which carry a variable number of DNA repair proteins (numbers inside matrix). After a cycle of fusion and fission, these proteins are homogenized.

Taken together, these experiments suggest that any mechanism controlling the production of oxygen radicals will impact mtDNA mutagenesis in some way. Such mechanisms could include homoplasmic mtDNA mutations[61]), proteins that control how tightly the electron transport chain is coupled, such as UCP1-3[62], or associated proteins that control major biological processes inside mitochondria such as ANT1[63].

Another potential source of mtDNA mutations is DNA polymerase gamma, the enzyme that replicates the mitochondrial genome[64]. DNA polymerase gamma is the enzyme that fixes DNA damage into mutations during DNA replication, but it is also possible that PolgA makes spontaneous errors on undamaged templates, which could contribute to the mutation rate as well. How large this contribution is, is difficult to say though, because DNA polymerase gamma has such a low error rate that it cannot be determined with current *in vitro* assays[65]. Thus, creating a mutation spectrum *in vitro*, and searching for traces of that spectrum *in vivo* is impossible. What is possible though, is to increase the error rate of DNA polymerase gamma by removing its proofreading domain. This proofreading domain has a 3'-5' exonuclease activity[64] which corrects errors that are made during DNA synthesis. If this activity is disabled with a point mutation, the spontaneous error spectrum of the enzyme can be exposed[65]. Interestingly, a proofreading deficient DNA polymerase gamma induces predominantly GC::AT transitions *in vivo*[66], similar the spectrum found in WT cells. However, a more careful comparison of these spectra at specific sites shows that the errors made by a proofreading deficient DNA polymerase gamma occur at different bases than those found in WT cells[40]. This suggests that spontaneous errors by DNA polymerase gamma on undamaged templates is not a major contributor to the mutation rate of WT cells. Its contribution may be greater under certain conditions though, for instance when rapid expansion of mtDNA is required, as is the case during development.



**Figure 8. Mitochondrial fusion and fission homogenize protein content.** One WT cell, and one fusion deficient cell line is depicted. In each cell, the nucleus is labeled with DAPI (blue) and immunocytochemistry has been used to label cytochrome C green and hsp60 red. Mitochondria in the WT cell contain approximately the same amount of cytochrome C and hsp60, resulting in a yellow coloring. Mitochondria in fusion deficient cells on the other hand carry highly variable amounts of these proteins.

A third source of mitochondrial mutagenesis is protein heterogeneity. Mitochondria typically contain 700-1400 proteins[67], which support a wide variety of functions. MtDNA only encodes 13 of these, all of which contribute to energy production. The proteins that safeguard the integrity of mitochondrial genome are therefore all encoded by the nucleus and imported into mitochondria from of the cytoplasm. These proteins include DNA polymerase gamma, the mitochondrial helicase Twinkle, and a wide variety of DNA repair proteins. Ultimately these proteins must be delivered in the proper stoichiometry in each mitochondrion to enable DNA repair[68]. As far as we know though, there is no way for the nucleus to orchestrate the distribution of these proteins over hundreds of mitochondria in such a precise manner. As a result, the distribution of DNA repair proteins across the mitochondrial population is likely to be uneven. This type of protein heterogeneity could put mitochondria at risk for mutagenesis. Ultimately, this problem is resolved by mitochondrial dynamics. While mitochondria travel along the cytoskeleton, they frequently collide in an end-to-end fashion. When they do, they fuse their membranes together to form a single, continuous organelle. This fusion process is mediated by 3 GTP-ases. Mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) help to fuse the outer membranes together, while OPA1 fuses the inner membranes together[13]. Fusion of the inner membrane creates one uninterrupted matrix, which contains all the molecules of the original fusion partners, including any DNA repair proteins. This newly formed mitochondrion will eventually be split back into two smaller organelles during a fission event, which is mediated by at least 3 proteins, Drp1[69], Fis1[69] and Mff[70]. The emerging organelles receive a random distribution of DNA, RNA and proteins, so that each mitochondrion contains an “average” of all the molecules that the initial fusion partners shared, including any DNA repair

proteins. Because mitochondria undergo constant cycles of fusion and fission, DNA repair proteins are continuously homogenized over the mitochondrial population, which results in a more equal distribution of protein content (see fig 8,9). This idea is supported by mouse models, as well as cell lines, in which mitochondrial fusion has been disabled by deletion of either Mfn1 or Mfn2. In these cases, proteins are no longer homogenized, resulting in greater protein heterogeneity, which increases mtDNA mutations in tissues and cultured cells[15].

However, whether protein heterogeneity also contributes to spontaneous mutagenesis in WT cells remains unclear. If it does though, its contribution is likely to differ between cell types and conditions. For instance, in neurons, mitochondria are located far away from the cell body where fewer fusion partners may exist, which could result in increased protein heterogeneity. This problem may be exacerbated in older cells, where mitochondrial motility is further compromised due to excessive traffic jams inside axons[71]. Another sensitive cell type may be muscle fibers, which carry tens of thousands of mitochondria, and countless genomes. Some of the most active mitochondria in muscle fibers are placed in very rigid positions, in a pair wise pattern along the z-axis of the fiber. Their constant, regular shape and precise placement suggest that they undergo limited fusion and fission, and are restricted in their movement. As a result, these mitochondria may rely primarily on the import of DNA repair proteins to maintain mtDNA stability. This holds the inherent risk of increased protein heterogeneity.

## 5. DNA repair in mitochondria

Until recently, it was very difficult to measure mtDNA mutations in mammalian cells. For this reason, it remains unclear which *in vivo* DNA repair mechanisms suppress mutations in mtDNA. In contrast, mitochondrial mutation assays are easier to perform in yeast, and as a result our knowledge about mtDNA repair in mitochondria comes largely from yeast experiments. We now know that base excision repair (BER), mismatch repair (MMR) and recombination repair all occur in yeast mitochondria. However, nucleotide excision repair (NER) is conspicuously absent. Of these DNA repair pathways, BER understandably the most active pathway in mitochondria, given the proximity of mtDNA to the ETC. Accordingly, loss of either Ogg1p[72] (which repairs 8-oxo-guanine), Ung1p[73] (which excises uracil from mtDNA), or Ntg1p[74] (which excises oxidized pyrimidines) results in a 2-10 fold increase in mtDNA mutations in yeast. BER activity in mitochondria is further supported by Apn1[75] (an AP endonuclease), Pif1[74] (a helicase), MTH1[76] (an 8-oxo-dGTPase and 8-oxodATPase), and MYH1[77] (which removes adenine opposite 8-oxodG). These experiments in yeast have guided similar efforts to explore DNA repair in mammalian mitochondria, where a similar set of BER proteins has been detected. These experiments demonstrated that both the short and long-patch version of BER is present in mammalian mitochondria, with Dna2[78] and Fen1 removing the intermediate flaps[79]. Remarkably though, simultaneous loss of OGG1 and MYH (which removes adenine incorporated opposite 8-oxo-guanine) did not result in an increased mutagenesis in mammalian cells[80], in contrast to yeast. The reason for this discrepancy is unclear,

although it is possible that extensive back-up mechanisms are present in mammalian mitochondria in the form of NEIL1 and NEIL2[80, 81], two glycosylases whose functions overlap with OGG1. A second possibility is that 8-oxo-guanine is not a common a lesion in mammalian mitochondria. Historically, 8-oxo-guanine has been the most studied oxidative lesion in the DNA repair field because it is practically the only lesion that is easily detected. However, there are other oxidative lesions that are undoubtedly more mutagenic than 8-oxo guanine, and some of these may even occur more frequently, but because they are almost impossible to detect, they remain unstudied. Thus, our emphasis on this lesion may be the result of a bias in our studies. The fact that multiple DNA repair enzymes exist to remove this lesion clearly argues otherwise though. Regardless, it will be essential for our understanding of DNA repair in mitochondria to screen all available mouse models with deleted DNA repair genes for increases in mitochondrial mutagenesis using modern tools. This is the only way we will get a clear picture of the DNA repair mechanisms that are active inside mitochondria.

Although BER is now clearly defined in mammalian mitochondria, there is only limited evidence for other DNA repair pathways in mammalian mitochondria, including mismatch repair and recombination repair, two pathways that are active in yeast mitochondria. Nucleotide excision repair is absent in mammalian mitochondria though, as it is in yeast, since UV lesions are not repaired in mammalian mitochondria[82].

In the nucleus, mismatch repair is an extremely important DNA repair pathway, which corrects errors that are generated during DNA replication[83]. The mismatch repair (MMR) proteins are composed of heterodimeric polypeptides termed MutS and MutL, which differ in composition to match the type of lesion they repair (either single base mismatches or small insertion-deletion loops). These proteins orchestrate the DNA repair process by first detecting the mismatch, and then discriminating between the two DNA strands to identify the template strand (which is correct), and the newly synthesized strand (which contains the mistake). It is still unclear how strand discrimination occurs in mammalian cells though.

Mitochondrial MMR was first identified in yeast[84, 85]. And since then, numerous experiments have tried to detect MMR activity in mammalian mitochondria. Some of these studies have found that the lysate of isolated mitochondria can repair mismatched templates [86]. However, it is possible for these lysates to be contaminated with nuclear proteins, which could confound the results. Thus, numerous researchers have also tried to image cells, in order to detect fluorescently tagged MMR proteins in mitochondria. Most of these experiments failed to detect the MutS or MutL complexes in mitochondria. However, they did identify a new protein, YB-1, which seems to aid mitochondrial MMR[47]. This raises the surprising possibility that a different set of proteins governs mismatch repair in mitochondria compared to the nucleus. In contrast, BER in mitochondria is performed by the same set of proteins that are present in the nucleus. However, if it is true that mtDNA is replicated by a mechanism that is different from nuclear DNA, it may actually be expected that a different type of enzyme is required for MMR. For now though, it will be important to validate this observation further with mechanistic insight. It will be important to elucidate

the function of YB-1 is in mitochondrial MMR, determine its activity, and demonstrate how it aids in strand-discrimination. And finally, since loss of the proofreading activity of DNA polymerase gamma results in a >1000-fold increase in mutations, it will be important to understand how this increase can be so large in the presence of MMR.

Besides single base lesions, oxidative damage can also cause single strand and double strand breaks in DNA, as well as DNA cross-links. For this reason, it would make sense for recombination repair to take place in mammalian mitochondria alongside BER. The most direct evidence for mitochondrial recombination comes from sequence analysis of mtDNA molecules. For instance, multiple labs have found evidence for intra-, as well as inter-strand recombination events[87-91]. These experiments suggest that micro-homology between mtDNA sequences is used for repair activity. Although the mechanisms of homologous recombination in mitochondria are still unclear, it seems as though the proofreading domain of DNA polymerase gamma plays an important role in this process. When the proofreading domain is deactivated, mtDNA molecules seem to recombine without the need for homology, with breakpoints that are reminiscent of NHEJ events[88]. Thus, one possibility is that PolgA aids homology searching during DNA repair processes. Besides PolgA, it is unclear which molecules function in double strand break repair. For instance, RAD51 and RAD52 have not been found in mammalian mitochondria[79]. A third protein, Mre11, which is involved in nuclear DSB repair, does aid recombination repair in yeast mitochondria though, and may be present in mammalian mitochondria as well[92, 93].

The relatively sparse evidence for DNA repair pathways in mitochondria has even led some to suggest that rather than being repaired, damaged mtDNA may be targeted for destruction. Destroying an entire genome instead making a few repairs seems wasteful though, and more data is needed to build the case for this hypothesis. For instance, the nuclease that would destroy damaged mitochondrial genomes has not yet been identified[79]. Others have suggested that mitochondria containing damaged DNA or excessive mutations may be targeted for mitophagy. If this hypothesis is true though, one would expect that mutated genomes are removed from a cell until only the WT genome persists. Clearly this is not the case though, because congenital mtDNA mutations persist in mitochondrial patients, and are not selectively removed.

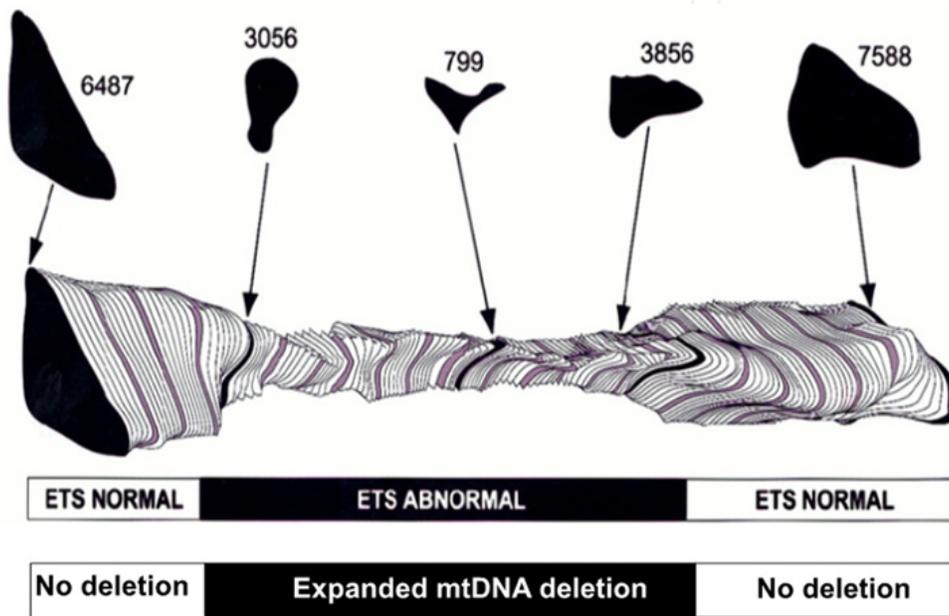
## 6. The effect of mtDNA mutations on human health

MtDNA encodes 13 proteins, all of which are essential to mitochondrial energy production. Accordingly, mtDNA mutations have the strongest impact on cells that have persistent, and high energy demands, including neurons, muscle fibers and the endocrine system. Despite the fact that only a limited set of cells is strongly affected by mtDNA mutations, it is very difficult to predict the effect a mutation will have on a patient due to the complexity of mitochondrial genetics. This is especially true in the case of inherited mtDNA mutations.

First, it is possible for mutations in different genes to have nearly identical consequences. This is a direct result of the extensive functional overlap between different mtDNA genes.

One example of a mitochondrial disease that is caused by these types of mutations is Lebers hereditary optical neuropathy[94] (LHON), a disease that causes a sudden onset of blindness due to mutations in the mitochondrial ND1, ND2, ND3, ND4, ND5, ND6[95-97], COII, COIII[98], CYB[94], or ATP6 gene[58, 94] (see also figure 1).

Surprisingly though, there is also a second class of mutations, which occur in the same gene, but have completely different consequences. For example, missense mutations in the ND6 gene can either cause Leigh syndrome[99-101], generalized dystonia and deafness[101, 102], mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes[103] (MELAS), or Leber hereditary optic neuropathy[94].

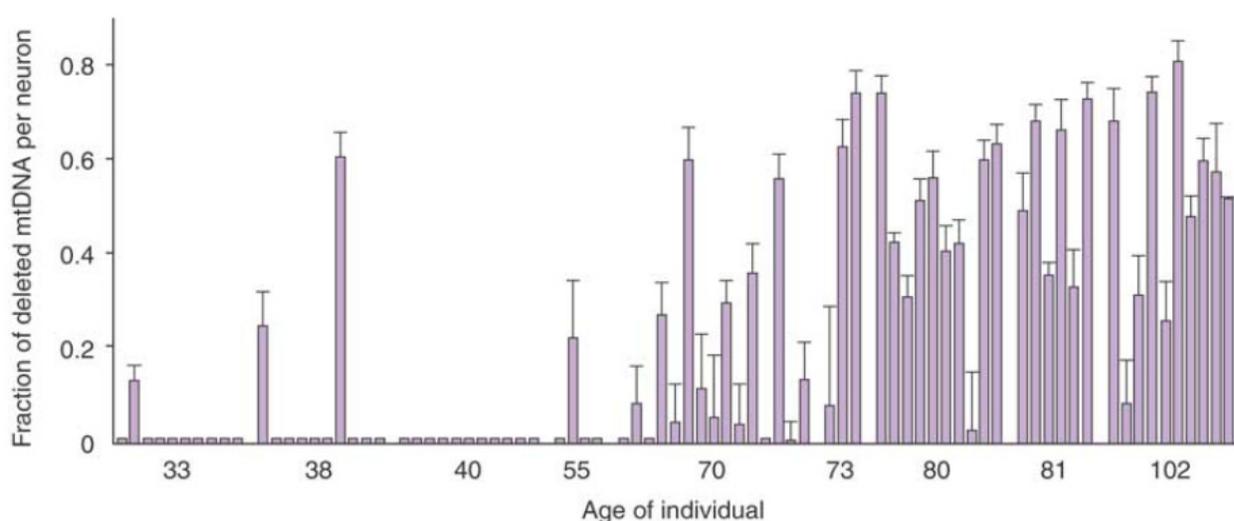


**Figure 9.** Data from Wanagat et al., FEBS 2000. A single muscle fiber is depicted in the cartoon above, which has been reconstructed from tens of sections that were taken across its length. Each slice represents one of these sections. Healthy sections of the fiber (at the edges) are larger than the sections that display mitochondrial dysfunction (center). The unhealthy sections contain excessive amounts of mtDNA deletions, whereas the healthy sections do not.

Finally, a third class exists, in which identical mutations in the same gene cause different phenotypes depending on the level of heteroplasmy at which that mutation is present in a patient's tissues. For instance, some of the most frequent mtDNA disorders are caused by mutations in the mitochondrial tRNA genes. The most common of these is an (A > G) mutation in the tRNA<sup>Leu</sup>(UUR) gene[104]. When present at low levels of heteroplasmy (10%–30%) a patient may present with type II diabetes, with or without deafness[105]. This is actually the most common inherited cause of type II diabetes, accounting for approximately 1% of all type II diabetes in the world[59]. By contrast, when this mutation is present in >70% of the patients mtDNA molecules, it does not cause diabetes, but presents itself with more severe symptoms[104], including short stature, cardiomyopathy, CPEO (a mitochondrial myopathy that is frequently associated with ophthalmoplegia and ptosis, referred to as chronic progressive external ophthalmoplegia), and the MELAS syndrome.

The diagnosis of these diseases is further complicated by the fact that the symptoms of different mtDNA diseases can overlap with each other. For instance, some mtDNA rearrangements cause CPEO, which is characterized by a slow, progressive paralysis of the extraocular muscles[59]. These features can be completely mimicked by Kearns–Sayre syndrome (KSS) though, which can present itself just like CPEO[106]. However, KSS is a much more devastating myopathy, which progresses to a multisystemic disorder manifested through cardiac dysfunction, mental retardation, and various endocrine disorders. Another example of clinical phenotypes melding into each other is Pearson’s pancreatic syndrome, which is caused by large mtDNA rearrangements that affect the bone marrow and frequently result in childhood pancytopenia. Pearson’s syndrome can progress to KSS if the pancytopenia is treated successfully[59].

Like congenital mutations, somatic mtDNA mutations tend to impact muscle fibers and neurons as well. However, they do so in a very different way. If a congenital mutation is present in the zygote, it is passed from cell to cell during development, so that a single mutation is dispersed across a patient’s tissues. Ultimately, the severity of the resulting disease is dictated by the amount of mutated mtDNA that ends up in a patient’s muscle fibers and neurons. However, each cell carries the same mutation.



**Figure 10.** Data from Kraytsberg, *Nature Genetics* 2006. MtDNA was collected from single neurons, derived from nine individuals aged 33-102. The mtDNA of these individuals was then PCR amplified and sequenced. The fraction of the mtDNA molecules in those cells that contained an mtDNA deletion is represented. Each peak corresponds to the analysis of one neuron.

Somatic mutations on the other hand, arise in neurons and muscle fibers that are already differentiated, post-mitotic cells. Thus, each mutation only affects the cell it arises in, and will not be passed on from cell to cell. For a tissue to be affected then, countless mutations must occur, resulting in a mosaic pattern of cells that either do, or do not carry a mutation. Moreover, since each mutation results from a unique event, each cell carries a unique mtDNA mutation. This causes an important problem, because if each mutation only occurs once, they are almost impossible to detect in bulk samples, where millions of cells are

monitored simultaneously. To find these mutations, a tissue must be screened one cell at a time. When single neurons, cardiomyocytes or muscle fibers with mitochondrial abnormalities are micro-dissected, and mtDNA is isolated from these single cells, it is indeed found that each cell carries a unique clonally expanded mtDNA mutation. These types of screens are very labor intensive, but they are the only way to measure the impact of somatic mtDNA mutations on a tissue.

These ground breaking single cell screens were first performed on muscle fibers[52, 107]. As we grow older, our muscle fibers tend to undergo age-related atrophy. This process is accompanied by segmental mitochondrial dysfunction, which can ultimately cause disruption of single fibers. If a dysfunctional muscle fiber is dissected, and mtDNA is isolated from different sections along the fiber, it is invariably found that dysfunctional sections carry a single mtDNA mutation that has clonally expanded to greater than 80-90% of all mtDNA molecules in the dysfunctional section of the fiber (figure 5). Healthy sections of the fiber on the other hand do not carry the mutation.

Similar experiments have been performed on neurons. As we grow older, our neurons tend to lose mitochondrial function, and the most prominent group of neurons to do so, are the dopaminergic neurons found in the substantia nigra[108, 109]. Like muscle fibers, these neurons can be recovered using laser capture micro-dissection, and their mtDNA can be sequenced. When the mtDNA is analyzed of patients with increasing age, it is indeed found that the substantia nigra of older patients carry more mtDNA deletions inside their cells than younger patients[109] (figure 6). Since the substantia nigra is the primary tissue affected by Parkinson's disease, it is thought that these mtDNA deletions may also play a key role in the etiology of this disease[110].

Besides post-mitotic cells, mtDNA mutations can also affect dividing cells. For instance, mtDNA mutations are frequently found in aging colonic stem cells[111]. Most dividing cells do not require enormous amounts of ATP production though, so that the pathology caused by mtDNA mutations is relatively mild in these cell types. Tumor cells may be an important exception to this rule[112]. In recent years, countless tumors have been tested for mutations in their mitochondrial genome. These screens revealed that human tumors frequently carry clonally expanded mtDNA mutations. Interestingly, these mutations seem to be present in almost every cell of the tumor, suggesting that they either originated in the original cancer stem cell, or were acquired during one of the genetic bottlenecks that define cancer progression. Since then, an important debate has erupted on the role of mtDNA mutations in human cancers. This debate centers around a single question: do mtDNA mutations provide a selective advantage to cancer cells?[112]

Opponents of this idea pointed out that, if it is true that mtDNA mutations induce carcinogenesis, then the offspring of a mouse that carries such mutations should develop tumors as well[113, 114]. However, no bias toward maternal inheritance of carcinogenesis has been reported. The foundation of this hypothesis was further shaken when it was shown that several studies that initially reported the presence of mtDNA mutations in

human cancers[115-118] contained analytic or technical errors[119]. As a result, most researchers started to doubt whether mtDNA mutations had anything to do with carcinogenesis.

However, an unexplored possibility is that mtDNA mutations do not affect the initial oncogenic transformation of a tumor cell, but a later stage of cancer progression. One recent study tested this hypothesis by investigating whether mtDNA mutations control metastasis {!!!!Ishikawa, Hayashi, Science, 2008 please insert reference properly!!!!}, the final, and most deadly stage of cancer progression. To do this, the authors of this study replaced the mtDNA of a cell line with low metastatic potential, with the mtDNA of a cell line that has high metastatic potential. They discovered that the metastatic potential of the malignant cell line was transferred to the benign cell line, not with its nDNA, but with its mtDNA. Conversely, if the mtDNA from a benign cell line was placed in the cytoplasm of the malignant cell line, the malignant cell line lost its metastatic potential. Additional analysis then showed that the mtDNA of the malignant cell line contained an mtDNA mutation in the ND6 gene, which increased ROS production. The increased ROS production must have been responsible for the metastatic potential of the cells, because if the malignant cell line was treated with ROS scavengers prior to implantation into mice to test its metastatic potential, the malignant cell line displayed a benign phenotype. This experiment was extremely important for the field for three reasons. First, it explained how mtDNA mutations can be involved in cancer progression without causing oncogenic transformation. Second, it demonstrated how proper mechanistic studies can be conducted in order to probe the role of mtDNA mutations in cancer progression. And third, it provided the first true mechanistic link between mtDNA mutations and metastasis.

## 7. Mouse models of mitochondrial mutagenesis

Because it is currently impossible to transform the mitochondrial genome of mice, it has been difficult to generate mouse models that mimic mitochondrial disease. Despite this frustrating technical limitation, there has actually been tremendous progress in this area over the last decade. During this time, three classes of mouse models have been developed: one class of mouse models was generated to study hereditary mtDNA diseases, a second to study somatic mtDNA diseases, and a third class was generated to study alleles of genes that cause disease in humans.

Of these three mouse models, the most difficult class to develop was a model to study inherited mtDNA mutations. Such a model requires a change to be made to the primary sequence of the mitochondrial genome. To do this, researchers have started using cybrid technology[120, 121]. Cybrid technology allows researchers to generate cells that are cytoplasmic hybrids of each other, or cybrids for short. A typical cybrid is created by fusing an enucleated donor cell, which contains an interesting mtDNA mutation, to an embryonic stem cell that has previously been depleted of its own mitochondrial DNA with ethidium bromide treatment. If the fusion process is successful, the resulting cell line will contain the nDNA of the ES cell and the mtDNA of the donor cell. This cell line can then be injected into

blastocysts and placed in a foster mother to generate mice with an inherited mtDNA mutation.

Although this is technically challenging, the true task is to find a cell line that carries an interesting mtDNA mutation. One way to find such a cell line, is to exposing cells to drugs that interfere with the ETC, and then select for cells that become resistant. For instance, treatments with chloramphenicol[120, 122], antimycin A[123], or rotenone[124] have yielded several cell lines that contain harmful homoplasmic mutations in their mtDNA. These mutations can then be moved into the germ line of mice according to the protocol described above. Mice that carry the chloramphenicol resistance mutation for instance, exhibit pathology that is reminiscent of the MELAS syndrome, attesting to the usefulness of this approach.

Other “mito-mice” that have been generated carry either a 4.7kb DNA deletion[125, 126], or point mutations in the ND6, COI, or 16s rRNA gene[18]. These mice exhibit a spectrum of phenotypes that mimic mtDNA diseases and have become valuable tools to understand the natural history of inherited mtDNA mutations. So far, they have helped us understand how mtDNA mutations are transmitted through the germ line, how they are dispersed throughout the organism during development, and how they ultimately cause pathology in mature mice[18, 125]. There were also some surprises though. For instance, patients with large mtDNA deletions typically present with muscle weakness, exercise intolerance, abnormal mitochondria in their skeletal muscle fibers, diabetes, pancreatic dysfunction and hearing loss. However, mice that carry a 4.7 kb deletion have a very different phenotype. They do exhibit respiratory dysfunction in their muscle cells, but suffer no diabetes, hearing loss, exercise intolerance or loss of pancreatic function. Instead, these mice die overwhelmingly of kidney failure, which is not a typical symptom of mitochondrial disease.

A second class of mouse models was developed to study the effect of somatic mtDNA mutations on human health. To do this, researchers increased the somatic mutation rate of mtDNA, by generating error prone versions of DNA polymerase gamma, the enzyme that replicates the mitochondrial genome. DNA polymerase gamma contains a 3'-5' exonuclease domain, which corrects errors that are made during DNA synthesis, similar to the major DNA polymerases in the nucleus[127]. This proofreading domain can be knocked out with a single point mutation[128, 129], which vastly increases the mitochondrial mutation rate[40]. Numerous mouse models have been generated that express this error prone allele either as a tissue specific transgene[130], or systematically using gene substitution techniques[128, 129]. The most informative model has been the mice that express the error prone polymerase gamma from its native locus. Interestingly, both the heterozygous and homozygous carriers of the error prone allele display enormous increases in mutations. Thus, the error prone allele is partially dominant, which is consistent with previous results in yeast[131, 132]. It was found that homozygous carriers of the error prone allele display a 1000 fold increase in point mutations, whereas heterozygous carriers display a 100 fold increase in mutations[40], which is again comparable to experiments performed in yeast[131, 132]. These mouse models represent an extremely important contribution to the field because they are the first

to directly manipulate the fidelity of the mitochondrial genome in mammals. As a result, they are also the first models to show a true mechanistic link between mtDNA mutations and disease. Interestingly, it was found that the homozygous carriers of the error prone allele suffer from extensive mitochondrial disease, which manifests itself as a premature aging like syndrome. These animals exhibit symptoms of alopecia (loss of fur), kyphosis (arching of the spine), premature deafness, premature blindness, cardiomyopathy, loss of subcutaneous fat, anemia, osteoporosis, loss of fertility and numerous other problems that resemble human aging[128, 129]. Partially, these problems are caused by extensive apoptosis, especially in dividing tissues[128]. Whether mitochondrial mutations cause these symptoms normal mice is still being debated. In later experiments it was shown that the heterozygous carriers of the proofreading deficient PolgA allele (which also display increased amounts of point mutations compared to normal mice) do not display overt symptoms of disease, or features of premature aging[40]. However, one important distinction is that, in contrast to the homozygous mice, the heterozygous carriers do not display an increased amount of mtDNA deletions, or aborted replication intermediates[88, 133]. It will be important to further test whether these deletions or the replication intermediates contribute significantly to the phenotype of the homozygous mice with the appropriate tools.

Finally, a third class of mouse models has been developed that carry specific alleles of genes that cause disease in humans. These mutations interfere with the normal function of proteins that are part of the mitochondrial replication fork. This fork consists of four core proteins: the catalytic subunit of DNA polymerase gamma, two accessory subunits, and the mitochondrial helicase Twinkle. Mutations in all these genes are known to cause various neuromuscular diseases, including progressive external ophthalmoplegia (PEO), Alpers disease and ataxia neuropathy. Some of the mutations in Twinkle that are known to cause PEO in humans have now been reconstituted in mice[136]. These mouse models have provided us with fantastic new insight into the etiology of adult onset PEO. First, the muscles of these animals faithfully replicate all of the key histological, genetic, and biochemical features of PEO patients. Secondly, these alleles recreate a mitochondrial mutator phenotype by increasing the amount of mtDNA deletions that occur in somatic cells. Importantly, these mtDNA deletions do not result in a premature aging like syndrome, which is powerful evidence against a role for mtDNA deletions in aging. On the other hand though, it should be noted that these mice over-express transgenic copies of Twinkle. As a result, the expression pattern is likely to be mosaic, which may affect the severity of mitochondrial disease in these tissues. Regardless, these mouse models are extremely important successes on the road to battling mitochondrial disease. They provide a new system to test treatment options in, and to study the natural history of diseases that are notoriously difficult to track. As a result, these mouse models are our best chance to make a difference in the lives of afflicted individuals, and their families. They will eventually illuminate the great unknowns of mitochondrial genetics, and open the door to improved health care and a longer health span, courtesy of mitochondrial medicine.

## Author details

Marc Vermulst

*Department of Chemistry, University of North Carolina, Chapel Hill, USA*

Konstantin Khrapko

*Department of Medicine, Division of Gerontology, Beth Israel Deaconess Hospital, Harvard, Boston, USA*

Jonathan Wanagat

*Department of Medicine, Division of Geriatrics, University of California Los Angeles, Los Angeles, USA*

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