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MOLECULAR MECHANISMS OF AGING IN MTDNA MUTATOR MICE

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Do not go gentle into that good night.
Rage, rage against the dying of the light.

Dylan Thomas

ABSTRACT

Mitochondria are intensely studied in the field of aging. mtDNA mutator mice have a proofreading deficiency in the mitochondrial DNA polymerase POLG, which causes a large amount of point mutations to be accumulated in mitochondrial DNA. These mice have mitochondrial dysfunction and experience a range of premature aging phenotypes.

In this thesis we examine the molecular mechanisms behind the mitochondrial dysfunction and premature aging phenotypes of the mtDNA mutator mice. The main reason for the mitochondrial dysfunction is the inability to assemble respiratory chain complexes. This is due to point mutations in the mitochondrially encoded protein subunits of the electron transport chain. As a consequence of this, these proteins are rapidly broken down and lower levels of respiratory chain complexes I, III and IV are assembled. The vicious cycle hypothesis predicts that an increased load of mtDNA mutations would cause increased reactive oxygen species (ROS) production. In contrast to this, there is no general increase in ROS production in mtDNA mutator mitochondria. Rather, we observed a large decrease in ROS production from reverse electron transfer and a moderate increase in ROS production generated from forward electron transfer. On a combination of complex I and II substrates, mtDNA mutator mitochondria produced significantly less ROS mainly as a consequence of reduced ROS production from reverse electron transfer. Although ROS production was not increased in mtDNA mutator mice, oxidative stress poses a threat. Tissues exposed to ambient oxygen could not defend against oxidation to the same extent as wt tissues. When grown in cell culture at 20% oxygen tension mouse embryonic fibroblasts from mtDNA mutator mice tend to immortalize. This effect is eliminated when cells are grown in only 3% oxygen or on low glucose or galactose. This could possibly be due to altered sensitivity to ROS or a growth enabling effect of a glycolytic metabolism.

As a consequence of the mitochondrial dysfunction mtDNA mutator mice have increased levels of UCP2. One proposed function of UCP2 is to uncouple the electron transport chain from the ATP synthase, by increasing proton conductance through the inner mitochondrial membrane. In contrast to its proposed uncoupling function, UCP2 does not seem to mediate proton conductance in mtDNA mutator mice. However UCP2 appears to mediate a switch to fatty acid metabolism. One major effect of UCP2 depletion in mtDNA mutator mice is early heart pathology. This indicates that UCP2 has a protective role in the mtDNA mutator hearts, perhaps through allowing better utilization of fatty acids. The mtDNA mutator mice provide insight into how mtDNA mutations affect mitochondria and how defective mitochondria affect the aging process.

LIST OF PUBLICATIONS

I. Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice.

II. Low capacity for mitochondrial reactive oxygen species production but increased susceptibility to lipid peroxidation in mtDNA mutator mice.

III. Random mtDNA mutations modulate proliferation capacity in mouse embryonic fibroblasts

IV. Physiological upregulation of UCP2 protects from early heart dysfunction in mtDNA mutator mice

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LIST OF ABBREVIATIONS

COX	Cytochrome c oxidase
CR	Control region
DDR	DNA damage response
DM	Double mutant
FAD	Flavin adenine dinucleotide
FCCP	carbonylcyanide-p-trifluoromethoxyphenylhydrazone
FMN	Flavin mononucleotide
GSIS	Glucose stimulated insulin secretion
mtDNA	Mitochondrial DNA
O_H	Origin of replication for the heavy strand
O_L	Origin of replication for the light strand
POLG	Polymerase gamma
Q	Ubiquinone
QH_2	Ubiquinol
ROS	Reactive oxygen species
SAHFs	Senescence associated heterochromatic foci
SASP	Senescence associated secretory phenotype
UCP	Uncoupling protein

1 INTRODUCTION

1.1 MITOCHONDRIA

A long, long time ago when our planet was young, an ancestor to the α-proteobacteria entered a eukaryotic cell (Gray, Burger et al. 1999). This union turned out to be beneficial for both parties. Slowly the genes were transferred from the bacteria to the nucleus of the eukaryote leaving in the case of mammals only 37 genes. The bacteria turned into what today are the mitochondria. The mitochondria are a network of double membraned organelles. One major function of the mitochondria is to produce ATP, the energy currency of the cell (figure 1). This is done by the respiratory chain, which works across the inner membrane pumping protons out of the matrix (the space enclosed by the inner membrane) to create an electrochemical gradient. This gradient is then used by the ATP synthase to produce ATP from ADP and Pi.

ATP is used in all of the body's processes that require energy. ATP is also produced outside the mitochondria, however this is a far less efficient energy producing system. This is because mitochondrial respiration utilizes much more of the chemical energy stored in fat, sugar and protein that we consume. In fact it is a breakdown of all of the highly energetic chemical bond previously produced by the reverse reaction that takes place in plants, photosynthesis. This combustion process is broken down into several steps allowing for the generation of useful energy limiting the production of heat. I addition to providing the cell with ATP, mitochondria perform several other essential functions. These include fatty acid metabolism, Ca^{2+} signalling, iron sulphur cluster assembly, apoptosis signalling and reactive oxygen (ROS) production.

1.1.1 Mitochondrial DNA (mtDNA)

The mammalian mitochondrial genome is composed of ~16.5 kilobases of circular, double stranded DNA encoding for 2 ribosomal RNAs (rRNAs), 22 transfer RNAs and (tRNAs) 13 protein subunits of electron transport chain, namely ND1-ND6, ND4L, Cyt b, COX I-III, ATPase6 and ATPase8. The nuclear DNA encodes the rest of the 1000-1500 mitochondrial proteins. The two strands of the mitochondrial genome differ in their G and C content. There is a G rich strand called the heavy strand and a C rich strand called the light strand. The control region (CR) contains promotors for both the heavy and the light strand. Both rRNAs, and all of the polypeptides except ND6 are encoded by the heavy strand. The genome is transcribed as a polycistronic RNA and tRNAs, rRNAs and mRNAs are cleaved out. In contrast to nuclear DNA there are many copies of mitochondrial DNA in each cell. If all of the mtDNA molecules haves the same sequence the state of the cell is homoplasmic. However if the molecules are different perhaps as the result of a mutation we have a heteroplasmic cell. The level of the mutation can drift to higher or lower percentages and can be inherited from the mother at different levels. If the mutation is deleterious a high level of the mutation may cause disease.

1.1.2 A trip through the electron transport chain

The mammalian electron transport chain is composed of four complexes. Its main function is to pump protons out of the matrix into the intermembrane space to create an electrochemical gradient that can be utilized by the ATP synthase to produce ATP. Below follows a sequence of events that initiates at complex I or II and culminates at complex IV in the reduction of oxygen to H_2O (figure 1).

Complex I: Complex I or NADH dehydrogenase is the largest complex in the electron transport chain. Here two electrons are transferred from NADH to flavin mononucleotide (FMN) reducing it to form $FMNH_2$. From $FMNH_2$ the electrons jump to an iron-sulphur cluster in complex I. The electrons are then transferred to ubiquinone (Q). This is done in a two-step process. First the free radical semiquinone is formed, then the addition of the second electron results in ubiquinol (QH_2). Through this process four protons are transferred from the mitochondrial matrix into the intermembrane space.

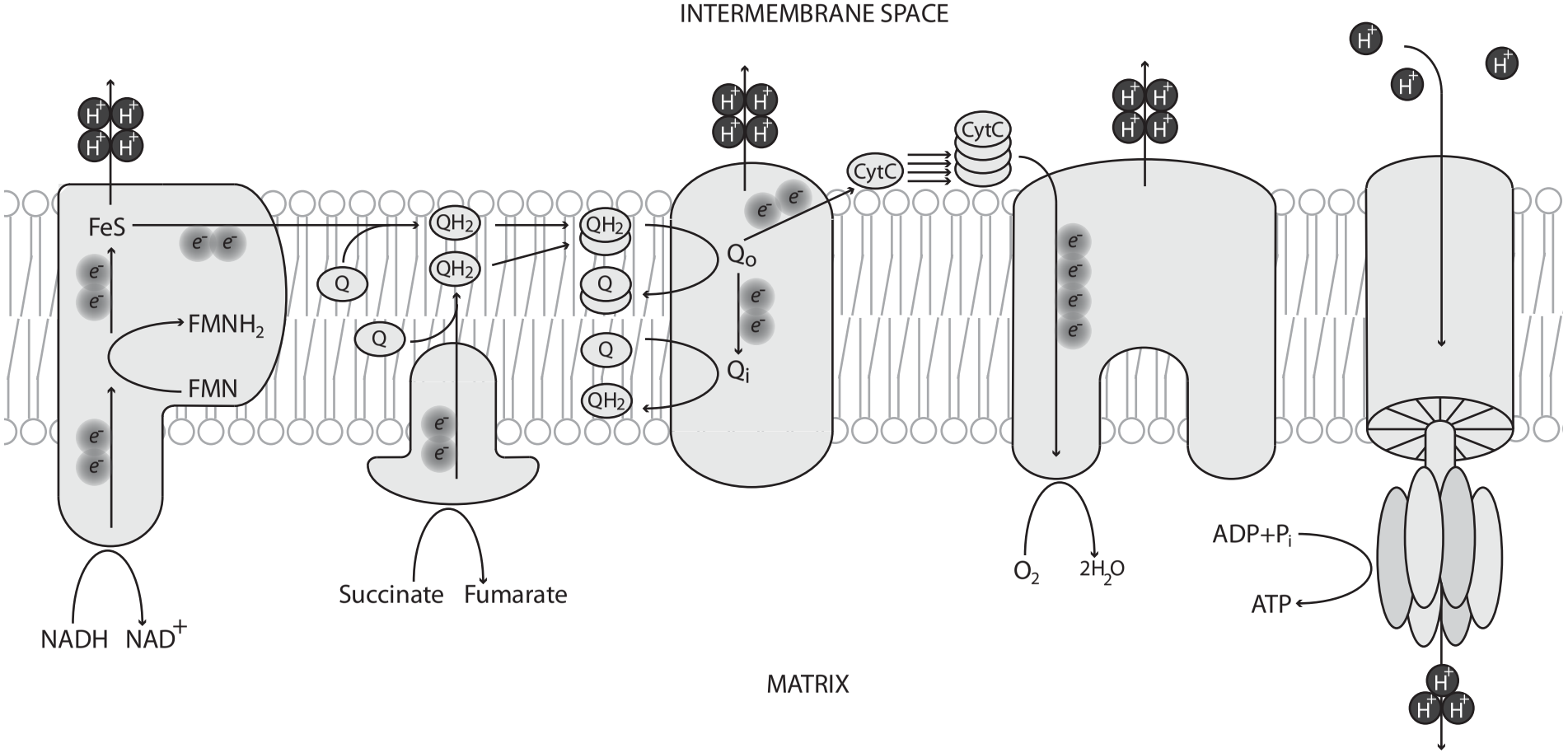
Complex II: Another entry point of electrons into the electron transport chain is complex II or succinate dehydrogenase. This is the only complex that is entirely encoded by the nuclear DNA. Succinate is oxidized via flavin adenine dinucleotide (FAD) to form fumarate, and electrons travel through iron sulphur clusters to ubiquinone.

Complex III: In complex III, or cytochrome bc_1, ubiquinols from complex I and II are oxidized at the Q_o site. This involves two electrons being transferred to the Q_o site. These electrons from the Q_o site are then transferred to cytochrome c via cytochrome c1. At the same time two other electrons are transferred from Q_o to the Q_i site where ubiquinone, is reduced to ubiquinol. The net result of these reactions is that four protons are removed from the matrix.

Complex IV: In complex IV or cytochrome c oxidase the cytochrome c transfers four electrons to O_2 producing two molecules of H_2O and at the same time four protons are transported across the inner membrane from the matrix.

The electron transport chain ends at complex IV but the electrochemical gradient, built up by the electron transport chain is used by another complex, complex V or the ATP synthase. Powered by the influx of protons into the mitochondrial matrix, the ATP synthase combines ADP and Pi into ATP, the energy currency of the cell (Nicholls and Ferguson 2002).

Figure 1. On the opposite page. A trip through the electron transport chain showing the five complexes and the flow of protons, electrons and production of ATP.



Complex I
(NADH dehydrogenase)

Complex II
(Succinate dehydrogenase)

Complex III
(Cytochrome bc_1)

Complex IV
(Cytochrome c oxidase)

Complex V
(ATP synthase)

1.1.3 Mitochondrial ROS production and oxidative stress

Reactive oxygen species (ROS) generally refers to molecules with an unpaired electron but also includes hydrogen peroxide H_2O_2 that does not have an unpaired electron. These are highly reactive molecules. Uncontrolled increases in ROS levels can lead to chain reactions, which indiscriminately target protein, lipids, polysaccharides and DNA. Superoxide is the precursor of most ROS within the cell. Superoxide can be turned into hydrogen peroxide H_2O_2, either spontaneously or by superoxide dismutase. This in turn can be fully reduced to water, or partially reduced to form hydroxyl radical OH^- (one of the strongest oxidants in nature) (Turrens 2003).

Superoxide production: The transfer of electrons between the different complexes is based on the fact that the new site is more easily reduced than the old one. However there is always the possibility that the electron will reduce molecular oxygen. When this happens superoxide is formed. How much superoxide is formed depends on various factors such as oxygen availability (if more oxygen is present there is a greater chance that an oxygen molecule will capture an electron), membrane potential or what substrates are used to initiate the electron transport chain. The main sites of ROS production in the electron transport chain are complex I and complex III. ROS can be produced from complex I by either forward or reverse electron transfer. Forward electron transfer can be achieved by adding glutamate + malate or pyruvate + malate to isolated mitochondria. This will produce NADH for complex I and ensures that no succinate will be produced for complex II. On forward electron transfer, superoxide will be produced by complex I. This production can be augmented by the complex I specific inhibitor rotenone. Rotenone blocks the quinone binding site causing electrons to back up and fully reduce upstream redox centres (Brand 2010). ROS can also be produced by complex I from reverse electron transfer from complex II. Succinate reduces quinone through complex II. If membrane potential is high, this drives the electrons “back” to complex I generating a significant amount of ROS. This reaction can be blocked by rotenone since it inhibits this electron transfer. It can also be inhibited by reducing membrane potential. If membrane potential is low it is thermodynamically more favourable to transfer the electrons to complex III. This can be achieved by uncoupling the mitochondria with carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Korshunov, Skulachev et al. 1997). Another site of ROS production is complex III. Complex III only produces superoxide at high rates when inhibited by antimycin A. Antimycin A is a Complex III inhibitor that blocks electron transfer from b hemes to quinone resulting in an increased formation of semiquinone at the complex III Q_o –site, which in turn can transfer an electron to oxygen yielding superoxide (Brand 2010). In addition to being harmful, ROS may also perform vital signalling functions within the cell. Targets of these endogenous ROS signals are the redox-sensitive catalytic Cys residues of Tyr and MAPK phosphatases, the oxidation of which reversibly abolishes enzymatic activity (D'Autreaux and Toledano 2007).

There seems to be an optimal redox range for the mitochondria to work within. Such that if the environment in the cell is too oxidized mitochondria produce little ROS but antioxidant systems function at a very low capacity. As the cell becomes more reduced mitochondria produce more ROS but antioxidant systems function at a much higher

level, leading to a decrease in ROS production. As the system becomes even more reduced antioxidant systems finally become overwhelmed and we again se a net increase in ROS (Aon, Cortassa et al. 2010).

1.2 UNCOUPLING

Uncoupling means that the primary proton pump (the electron transport chain) is uncoupled from the secondary proton pump, the ATP-synthase. This allows the electron transport chain to pump protons out of the mitochondrial matrix regardless of if the ATP-synthase is working (Nicholls and Ferguson 2002).

1.2.1 Uncoupling proteins (UCPs)

The UCPs are a subgroup of the mitochondrial protein carrier family. This family consist of 30 identified proteins. Mainly they transfer metabolites between the mitochondria and the cytoplasm. These proteins are all around 30 kDa and have two transmembrane domains. Some members of this family include the ATP/ADP translocator, the 2-oxoglutarate/malate and the citrate (tricarboxylate) carriers and UCPs. The first UCP found was uncoupling protein 1 (UCP1) reviewed in (Rousset, Alves-Guerra et al. 2004). UCP1 is exclusive to mammals. It is expressed in brown adipose tissue were it transports protons from the intermembrane space into the mitochondrial matrix without involving the ATP synthase. The main function of this process is to produce heat. Both UCP2 and UCP3 have 55% homology with UCP1 while being 80% identical to each other. Whilst the function of UCP1 is well characterized, the function of the other UCPs is less clear.

1.2.1.1 Uncoupling protein 2

Many attempts to find the function of UCP2 have been based on its similarity to UCP1. However UCP2 does not seem to mediate thermogenic cold response (Nedergaard, Golozoubova et al. 2001) and the amount of UCP2 present in tissues calls into question if it is enough to mediate physiologically relevant uncoupling. Unlike UCP1, which is only expressed in brown adipose tissue UCP2 is expressed in many tissues. However UCP2 mRNA and protein expressions are not always correlated to each other (Pecqueur, Alves-Guerra et al. 2001). An explanation for this can be that UCP2 is regulated both on the transcriptional and translational level (Hurtaud, Gelly et al. 2006). This makes it likely UCP2 is involved in regulating rapid biological responses. Many phenotypes of the UCP2 KO mice could be interpreted as supporting the role for UCP2 as an uncoupling protein. UCP2 negatively regulates glucose sensitive insulin secretion (GSIS), this was initially seen in the UCP2 knock out mouse which displayed a higher GSIS (Zhang, Baffy et al. 2001). This could be a result of increased coupling in beta cells lacking UCP2 leading to higher ATP production and thus more sensitive insulin release in response to glucose. UCP2 KO mice have higher ROS production in macrophages (Arsenijevic, Onuma et al. 2000; Emre, Hurtaud et al. 2007). If UCP2 is an uncoupling protein, lack of UCP2 could give rise to higher membrane potential and thus higher ROS production. UCP2 has shown some role in neuroprotection were overexpression reduces seizures (Diano, Matthews et al. 2003), decreases brain damage

after stroke (Mattiasson, Shamloo et al. 2003) and protects dopaminergic neurons (Andrews, Horvath et al. 2005). These effects also seem to be mediated by decreased ROS production. Trenker et al. have shown that UCP2 and UCP3 are important for Ca^{2+} regulation into the mitochondria. Ca^{2+} extrusion from mitochondria is dependent on membrane potential. Thus this would be a mechanism to uncouple the mitochondria albeit through a more complex route (Trenker, Malli et al. 2007).

Another field of inquiry into UCP2 function is its ability to regulate substrate utilisation. UCP2 mRNA is upregulated in a variety of situations linked to increased fatty acids (Thompson and Kim 2004). UCP2 KO cells switch from fatty acid metabolism to glucose metabolism (Pecqueur, Bui et al. 2008). Another link to substrate utilization is glutamine, which can rapidly stimulate UCP2 translation (Hurtaud, Gelly et al. 2007). Since most of the family members in the mitochondrial carrier family transport metabolites between the mitochondria and the cytoplasm it has been proposed that many of the phenotypes seen in the UCP2 KO mice can be explained by a change in substrate utilisation, perhaps as a result of a transporting pyruvate out of the mitochondria (Bouillaud 2009).

1.3 AGING

Everyone feels they have an intuitive grasp of what aging is, but trying to define it is surprisingly difficult. There are several components to it. One is the age related increase in mortality rate. This is typically visualized by the Gompertz plot. In addition to the increase in mortality rate there is also functional decline and increased susceptibility to diseases that do not necessary result in death. There are several ways to define aging:

- The process of becoming older.

- The gradual changes in the structure and function of humans and animals that occur with the passage of time, that do not result from disease or other gross accidents, and that eventually lead to the increased probability of death as the person or animal grows older.

- A progressive, generalized impairment of function, resulting in an increased vulnerability to environmental challenge and a growing risk of disease and death.

Some of which are not useful for the purpose of this thesis. Biological aging is sometimes referred to as senescence in order to narrow the otherwise too broad meaning of the word. I choose to define aging as:

-The gradual accumulation of deleterious changes for which the body becomes progressively worse at compensating for or repairing resulting in an increased risk of death over time.

1.3.1 Evolutionary views on aging

Aging was first viewed as wear and tear. Just as inanimate objects wear out, so do living beings. This is superficially true. But living systems have an advantage over inanimate objects; by the influx of energy living systems are able to repair themselves and maintain their integrity. In essence, living systems are immortal by the fact that they can reproduce. Even if individual members of a species die the germline is immortal. Also it is evident that different species have different life spans and are able to fend of decline more or less effectively. Clearly there is a benefit to maintaining the body for a certain amount of time but is it sufficiently beneficial to attempt to do so indefinitely as with the germline? Are there other forces at play which antagonize this process?

The first thing to note is that the force of natural selection decreases over time. All animals have a certain statistical risk of dying despite the aging process. For animals that have many natural enemies they are statistically very likely dead before the deleterious effects of aging sets in. Thus there is very little that natural selection can act upon to slow aging. Indeed these animals are generally more short-lived.

In 1957 Williams (Williams 1957) proposed that genes which have a selective advantage early in life will be selected for even if the late life effects of these genes are deleterious. This might cause unbeneficial phenotypes late in life and thus aging. This theory is called antagonistic pleiotropy.

The production of long lived fruit flies by delaying the age of reproduction has revealed that it is relatively simple for natural selection to extend both lifespan and health span (Rose and Graves 1989). Rose (Rose 2009) argues that aging is due to the inability of the organism to sustain adaptation and thus health late in life. However if circumstances change making longer life and health a significant benefit, natural selection can adapt an organism to good health at any age.

Thomas Kirkwood proposed the disposable soma theory in 1977 (Kirkwood 1977). The main idea here is that the body has limited resources. With a finite amount of food not all processes can be optimized. It does not make sense for the body to allocate an inordinate amount of resources on maintaining a body that will likely only be used for a short amount of time.

Evolutionary theories of aging help inform us as to what to look for on the level of the organism. If aging is a simple program that is selected for we might expect to find non-aging mutants that have a disrupted aging program. Since we have not encountered any, this scenario does not seem very likely. Also there does not seem to be some intrinsic factor causing aging that natural selection cannot compensate for. Instead what we see in nature is that natural selection seems to be able to shift the lifespan of species with wildly different life styles toward a lifespan that makes sense in regard to current evolutionary pressures.

1.3.2 Cellular senescence

Most mammalian cells do not divide indefinitely. This was not always thought to be the case. In the dawn of cell culture research a French physician named Alexis Carrel was able grow cells from an embryonic chicken heart for over 20 years. This lead to the idea that cells could divide indefinitely if they were just given the right conditions. These experiments were however impossible to replicate outside of Carrels lab. Likely the serum used for the cell culture was not cell free and so new cells were added every time the media was exchanged.

In the 1960s Leonard Hayflick noticed that human fibroblasts predictably stop dividing after 40-60 population doublings (Hayflick and Moorhead 1961). To be sure of their results Hayflick and Moorhead sent their samples to several outside labs and asked them to give them a call when the cells stopped dividing. As expected, after 6 months the calls started dropping in. All of the cultures had stopped dividing and Hayflick and Moorehead were now sufficiently confident in their finding to publish the results. There seemed to be a counting mechanism that decided how many times the cells were able to divide. This counting mechanism was later found to be the telomeres (Yu, Bradley et al. 1990). Telomeres are the protective cap at the end of the chromosomes that gets shorter with each cell division. Cells can senesce when the telomeres are sufficiently deteriorated or when damage to other parts of the DNA invokes the DNA damage response (DDR). The DDR can either induce repair, apoptosis or senescence. Cells can also senesce through other stressors when they are grown in cell culture, such as serum, high oxygen tension or even cell plastic (Sherr and DePinho 2000).

What defines a senescent cell? Senescent cells cannot continue dividing, this is however not the same as a terminally differentiated cell. Senescent cells are bloated and swell up, sometimes to twice their size. On the chromatin level, cellular senescence is associated with a condensation of chromatin called senescence associated heterochromatic foci (SAHFs). The cells containing SAHFs also loose histone H1 (Funayama, Saito et al. 2006). In addition to morphological changes senescent cells have some additional defining characteristics. An important feature is their resistance to apoptosis (Wang 1995). The main biomarker of senescent cells is the activity of β-galactosidase at pH 6.0 which is normally only active at ph 4.0 (Dimri, Lee et al. 1995). This marker increases in both aging cell cultures and aging in vivo. Senescent cells generally express $P16^{INK4}$, a tumor suppressor that increases with age in both mice and humans.

Why does cellular senescence exist? There are strong arguments to be made that cellular senescence is a protective mechanism against cancer. Essentially all tumors have a mechanism for maintaining telomere length. Mice deficient for telomerase are protected against tumor formation (Feldser and Greider 2007). In many cases senescent cells have been found in pre malignant tumors suggesting that cellular senescence can restrict tumor progression, reviewed in (Collado, Blasco et al. 2007).

It is not clear if or in what way cellular senescence is important for organismal aging. More senescent cells are found in older individuals compared to younger (Jeyapalan, Ferreira et al. 2007; Rodier and Campisi 2011). If cellular senescence is important in

vivo, it might be expected that cells from older individuals would undergo fewer population doubling than cells from younger individuals when grown in culture. However the evidence for this is ambiguous. Some studies indeed find that cells from older individuals have lower replicative capacity (Kaji, Ohta et al. 2009), whereas others have found only weak (Smith, Venable et al. 2002) or no correlation (Cristofalo, Allen et al. 1998). One problem with these types of studies is that they only select for cells that have replicative capacity. Even if old people have some cells that still have full replicative capacity the cells that cannot continue replication might pose a problem. One way senescent cells could contribute to organismal aging is due to a decrease in vital stem cell pools since senescent cells cannot divide and also not perform their initial function. Another way these cells can be detrimental is through their influence on other cells. Senescent cells secrete proinflammatory cytokines, this is called senescence associated secretory phenotype (SASP) (Campisi 2005). Older individuals have increased levels of proinflammatory cytokines such as IL-6 and TNF- α. These may contribute to chronic inflammation, which is associated with many age related diseases such as Alzheimers disease, osteoarthritis and diabetes. It has been observed that individuals who "age well" have a lower inflammatory profile (Franceschi, Capri et al. 2007). If these mechanisms are indeed important for organismal aging it can be seen as antagonistic pleiotropy. The mechanism that inhibits cancer growth early and throughout an individual's life does so at the cost of decreasing its fitness later when it is not as "visible" to the forces of natural selection.

1.3.3 Aging theories

There are many aging theories, which approach the phenomena from different angles. Damage is the common denominator of several aging theories. Organisms are constantly subjected to damage and have different ways of coping with it. Some damage is especially hard to deal with. One such type of damage is mutations. Since the DNA is the template from which the cell is built. Another form of DNA damage is damage to telomeres. Since nuclear DNA does not have the privilege of being circular like mitochondrial DNA, the end of the chromosome is not copied during DNA replication. To get around this issue the tips of the chromosomes are capped with TTAGGG repeats which are the DNA part of the telomeres. These get shorter for each round of replication but can be extended if the enzyme telomerase is activated. Telomeres have been an interesting candidate for causing aging since it was discovered that they limit the amount of times a cell can divide (Yu, Bradley et al. 1990). It remains unclear however how important this is for pathology and how many cells actually reach the point were they are inhibited by lack of adequate telomere length. Since mice have longer telomeres than humans it was thought that telomere length would not limit mouse life span since their telomeres would not become short enough to compromise chromosome integrity. A study overexpressing telomerase in cancer resistant mice (Tomas-Loba, Flores et al. 2008) shows that although mice have longer telomeres than humans, increasing their telomere length also increased their lifespan. Recently Jaskelioff, et al. (Jaskelioff, Muller et al. 2011) reintroduced telomerase into telomerase deficient mice and found that many of the premature aging phenotypes were reversed upon telomerase reactivation. In addition to being shortened at every cell division due to the polymerases inability to replicate the first bases, telomeres can also

be damaged by oxidative stress. This may in fact be the major cause of telomere shortening (Passos, Saretzki et al. 2007).

In addition to DNA damage waste accumulation can be problematic. Not all molecular damage will be repaired or degraded by the cell. Over time “garbage” builds up inside and outside the cell. In lysosomes this is easily visible as a pigment called lipofuscin. This is mainly found in non-dividing cells such as cardiomyocytes and neurons. These lipofuscin-loaded lysosomes perform poorly and cannot remove modified proteins, altered biomembranes, defective mitochondria and other organelles. This has been proposed to cause accumulation of defective mitochondria with increased ROS production (Terman and Brunk 2006).

The most widely accepted of all damage theories is the free radical and mitochondrial theory of aging, which will be discussed, in the next chapter.

1.3.4 Mitochondria and Aging

Mitochondria were first implicated in the aging process when it was discovered that they produce ROS as a by-product of respiration. Evidence from many paths of gerontological research suggests that mitochondria are one of the key players in aging. Several age-related changes occur in mitochondria. The number of mitochondria decreases in post-mitotic tissues like heart, skeletal muscle and brain during aging (Samorajski, Friede et al. 1971; Herbener 1976). Structural changes such as alterations of cristae structure, matrix vacuolization or densification and enlargement of mitochondria have been found to increase with age (Wilson and Franks 1975; Wilson and Franks 1975). Mitochondrial DNA (mtDNA) point mutations and deletions have been detected in aging humans, and have, in many cases been correlated to mitochondrial dysfunction. Deletions in mtDNA are found in postmitotic tissues e.g., skeletal muscle, heart and brain in contrast to kidney, spleen, skin and liver that are composed of dividing cells, indicating a tissue-specific "pattern" of deletion accumulation (Cortopassi, Shibata et al. 1992). The level of deletions is however usually very low (under 1% of deleted mtDNA in whole tissue homogenate) (Wallace 2005). This calls into question if they have a causative effect or if they are only correlated with aging. In mitochondrial diseases a threshold level needs to be reached before point mutations or deletions cause pathology. In dopaminergic neurons mtDNA deletions are related to Parkinson’s disease when they reach a level of around 52% (Bender, Krishnan et al. 2006). The same is true for point mutations. The threshold level varies depending on the point mutation but generally they need to reach a level of 90% before causing pathology (Trifunovic and Larsson 2008). However, point mutations can clonally expand and cause deficiency in single cells or a part of the tissue. For instance, analysis of mtDNA point mutations in five tRNA genes showed high levels of clonally expanded mtDNA point mutations (49%-94%) in COX-deficient skeletal muscle fibers isolated from aged individuals (Fayet, Jansson et al. 2002). Also, when stem cells in colonic crypts divide, mtDNA with point mutations can be unevenly distributed causing all the cells stemming from the crypt to have deficient respiration (Taylor, Barron et al. 2003).

Mitochondria are a key regulator for apoptosis through several pathways (Mayer and Oberbauer 2003). For example cytochrome c release from the mitochondria into the cytosol where it recruits adaptor proteins and initiates caspase signalling leading to apoptosis (Liu, Kim et al. 1996). There is evidence that mitochondrial dysfunction can cause an increase in apoptosis (Niu, Trifunovic et al. 2007). This might lead to the loss of critical cells, causing aging or premature aging phenotypes.

One of the first to implicate mitochondria in the aging process was Denham Harman (Harman 1972; Harman 1972). The theory attributes aging to damage caused by reactive oxygen species (ROS). Mitochondria produce ROS by the process of respiration in the electron transport chain. In addition, mitochondria are the only organelle that contain their own DNA, which is in close proximity to where ROS are produced. Potentially ROS could damage the mtDNA causing mutations, thereby leading to irreversible damage for the organelle. The vicious cycle hypothesis states that ROS causing damage to the mtDNA leads to mutations that in turn lead to faulty production of respiratory chain subunits for which the mtDNA encodes. This causes the respiratory chain, harbouring these faulty respiratory chain subunits, to produce more ROS. This cycle feeds on itself causing an ever-increasing speed of decline. The idea that ROS production would increase when respiratory chain subunits are damaged is true for certain mutations. An example of this is found in fibroblasts from patients presenting an isolated complex V deficiency due to mutation in ATPase 6 gene (Geromel, Kadhom et al. 2001; Baracca, Sgarbi et al. 2007). Studies of fibroblasts from patients suffering isolated complex I deficiency have shown both increase in ROS production (Iuso, Scacco et al. 2006) but also no increased ROS levels in fibroblasts with mutations in the NDUFA1 and NDUFV1 genes (Moran, Rivera et al. 2010). mtDNA mutations can cause increase in ROS production but it is not clear how common this is and if it is a significant contributor to the aging process.

A correlative link between ROS production and the aging process comes from a comparison between species longevity and the amount of ROS that is produced from isolated mitochondria (Ku, Brunk et al. 1993). They showed that longer-lived species produce less ROS from isolated mitochondria. Interestingly there is an inverse relationship between the level of antioxidant enzymes and longevity (Barja, Cadenas et al. 1994). This argues that if ROS damage is a cause of aging, low ROS production is more important in order to limit ROS damage than is antioxidant enzyme systems. In caloric restriction, an intervention that increases the lifespan of a range of animals, ROS production is reduced (Gredilla, Sanz et al. 2001). An alternative explanation could be that another aspect of mitochondrial function is selected for in long-lived species, which also decreases ROS production. If lower net ROS levels were not important for species longevity, then lower levels of antioxidant enzymes would be expected.

Studies overexpressing antioxidant enzymes have yielded conflicting results. Overexpressing copper zinc superoxide dismutase (CuZnSOD), catalase, or combinations of either CuZnSOD and catalase or CuZnSOD and manganese superoxide dismutase (MnSOD) does not increase the lifespan of mice (Perez, Van Remmen et al. 2009). However mice overexpressing catalase targeted to mitochondria

have a 20% increase in median lifespan and a 10% increase in maximum lifespan (Schriner, Linford et al. 2005).

Other studies have studies could be interpreted as mitochondrial ROS promoting longevity. Mice that are heterozygous for Mclk, a mitochondrial enzyme that is necessary for ubiquinone biosynthesis, (Liu, Jiang et al. 2005) (Lapointe and Hekimi 2008) have mitochondrial dysfunction with more oxidative damage in the mitochondria and less oxidative damage in cytosolic proteins. These mice also have increased lifespan despite having increased oxidative damage to mitochondria.

Many mutations that effect lifespan, as well as the most robust non-genetic intervention, caloric restriction, are linked to the insulin signalling pathway (Kenyon 2011). This pathway intersects with mitochondrial function at several junctions. In C.elegans the insulin/insulin like growth factor receptor is encoded by daf-2. This was one of the first longevity genes found. Daf-2 mutants have decreased insulin signalling and prolonged lifespan. This is accompanied by increased mitochondrial function higher membrane potential and higher ROS production but decreased oxidative stress in the form of protein carbonyls (Brys, Castelein et al. 2010). ISP-1 encodes a complex III subunit in C.elegans. ISP-1 mutants have increased superoxide production and longevity. Interestingly the effect on lifespan can be attenuated by antioxidants (Yang and Hekimi 2010).

Another possible link to the effect of mitochondria on the aging process comes from sirtuins. Sirtuins are NADH dependent deacetylases. It has been proposed that the effects of calorie restriction, a method of increasing lifespan in a variety of animals, are mediated by the SIR2 gene family that have been shown to extend lifespan in C.elegans, yeast and Drosophila (Donmez and Guarente 2010). SIRT1 a mammalian member of the SIR2 gene family targets PGC1α, which mediates mitochondrial biogenesis (Lagouge, Argmann et al. 2006).

Studies in mice that accumulate mutations in mtDNA (mtDNA mutator mice) (the mice which are the focus of this thesis) (Trifunovic, Wredenberg et al. 2004) argue against the vicious cycle hypothesis. Even though these mice have a large amount of point mutations they do not have increased ROS production (Trifunovic, Hansson et al. 2005). Rather it seems as if most point mutations cause the respiratory chain subunits to degrade (Edgar, Shabalina et al. 2009), causing decreased respiration but not increased ROS production. Still it is not inconceivable that ROS generating point mutations play a role in the aging process.

The role of mitochondria in normal aging is still unclear. Though it seems that some aspects of mitochondrial function are linked to longevity. Even though mtDNA mutations accumulate with age and mitochondrial function declines, we do not in normal aging accumulate the same levels of point mutations as the mtDNA mutator mice do. However mtDNA mutations can certainly cause aging phenotypes if present at sufficient levels and mitochondrial dysfunction can drive age related pathology. The fact that pathways associated with aging seem to affect and be affected by mitochondria, make them an attractive candidate for many of the pathological changes seen in aging. While it is obvious that very high levels of ROS are pathological, a

moderate increase in ROS is in some cases related to increased lifespan. One possible mechanism for this is hormesis. This is the notion that a small amount of a toxin, like ROS may promote a general stress resistance to ROS or even other stressors. Another possibility is that there are other aspects of mitochondrial function that are important for the aging process and that the effects on ROS are secondary to this. It might be fruitful to investigate if there are any common elements of mitochondrial function in different models were both aging and mitochondria are affected.

1.4 THE mtDNA MUTATOR MOUSE

To test the causative role of mtDNA mutations in aging, the mtDNA mutator mouse was developed. The mtDNA mutator mouse accumulates high levels of point mutations due to a proofreading deficiency of the mitochondrial DNA polymerase (POLG) (Trifunovic, Wredenberg et al. 2004). The estimated number of point mutations in the heart of 25-week-old mtDNA mutator mice is 14 mutations per 10.000 base pairs. The mice are born in Mendelian proportions, without any visible defects, but after 6-7 months they start to display a range of premature aging phenotypes, such as a weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anemia, reduced fertility, heart disease, sarcopenia, progressive hearing loss and decreased spontaneous activity (Trifunovic, Wredenberg et al. 2004; Niu, Trifunovic et al. 2007). Their lifespan is also greatly reduced compared with wild-type littermate controls and they die at around 46 weeks of age. In addition to the standard circular chromosome, mtDNA mutator mice harbour large linear mtDNA molecules caused by replication stalling (Bailey, Cluett et al. 2009). These molecules are around 11-12 kb in length, and encompass the region between the origins of replication for the heavy (O_H) and the light strands (O_L). Around 25-30% of the mtDNA consists of these linear molecules with deletion, and this ratio does not change as the animals age (Trifunovic, Wredenberg et al. 2004). As these molecules exist at quite high levels, it has been proposed that this may also contribute to the progeroid phenotype of the mtDNA mutator mouse (Trifunovic, Wredenberg et al. 2004; Bailey, Cluett et al. 2009). The POLG exonuclease activity may be involved in the resolution of replication intermediates at O_L, which can explain why exonuclease deficiency will lead to replication stalling, thus leaving the mtDNA molecule susceptible to breakage at the stall site (Bailey, Cluett et al. 2009). It has also been suggested that the time needed for the replication of mtDNA could be prolonged in the mtDNA mutator mouse, as evidenced by a high amount of replication intermediates (Bailey, Cluett et al. 2009). This prolonged replication is proposed to exhaust resources in the mitochondria. Although plausible, this hypothesis still requires direct experimental verification. One observation from the mtDNA mutator mice is that amino acid changing mutations in the protein coding genes are very poorly tolerated. Most mutations in the first and third codon positions disappear after only a few generations of backcrossing mtDNA mutator females to wt, whereas tRNA mutations do not seem to be strongly selected against (Stewart, Freyer et al. 2008).

One consequence of the compromised mitochondria is increased apoptosis. This was found in a study looking at progressive hearing loss in the spiral ganglion in the mutator mouse (Niu, Trifunovic et al. 2007) and a in a range of other tissues in a

similar mouse model (Hiona, Sanz et al. ; Kujoth, Hiona et al. 2005) including thymus, intestine, testis and muscle.

2 SPECIFIC AIMS

Paper I: To investigate how a defective polymerase causes mitochondrial dysfunction and premature aging phenotypes in the mtDNA mutator mice. More specifically, to examine weather point mutations or circular deletions are the driving force and to determine at what stage the mutations are causing a problem for the mitochondria i.e. transcription, translation or assembly.

Paper II: To examine how ROS production and oxidative stress is affected by having a large amount of mutations in the mitochondrial DNA. More specifically, to examine the sources of ROS production and the amount of ROS produced from forward and reverse electron transfer. As well as examining the amount and type of ROS damage produced.

Paper III: To examine the effect of mtDNA mutations on cell proliferation in vitro, more specifically, to investigate cellular senescence and immortalization of MEFs under various conditions. Also examining the pathways the cells take to immortalization or senescence.

Paper IV: To investigate the effect of UCP2 ablation in mtDNA mutator mice. Characterizing the bioenergetics of mtDNA mutator mice with and without UCP2. Also, looking at the phenotypic effects of UCP2 ablation in mtDNA mutator mice.

3 RESULTS AND DISCUSSION

3.1 ON POINT MUTATIONS AND CIRCULAR MOLECULES WITH LARGE DELETIONS

In paper I (Edgar, Shabalina et al. 2009) we state that point mutations are the driving force behind the progeroid phenotypes in the mtDNA mutator mice. We found that mtDNA mutator mitochondria have lower respiratory capacity compared to wt. We also directly measured the capacity of the electron transport chain by permeabilizing mitochondria with alameticin and feeding NADH directly into complex I, and found that it was reduced. With BN-PAGE we measured a reduction in the level of assembled complex for complex I, III, and IV. Reduction in complex levels could in principle be the result of impaired transcription, translation or assembly/protein stability. We measured steady state levels of RNA with northern blot. There was no general reduction in mitochondrial RNA levels, rather an up regulation of some transcripts. We directly measured protein synthesis from mitochondria by an in organello translation assay. Protein synthesis was normal, but after a 3h and 5h chase with cold methionine we found rapid breakdown of the mitochondrially transcribed proteins. We believe the best explanation for this is that point mutations that cause amino acid substitutions lead to the increased turnover in these defective subunits.

Another group has published a paper with a similar mouse model claiming it was circular molecules with deletions that were the main driving force behind the premature aging phenotype.

Our finding that the dysfunction of the mitochondria was driven mainly by inability to assemble the complexes led us also to directly examine if we could detect circular molecules with deletions. We concluded that if they did exist the levels were very low and were unlikely the cause of the phenotype (Edgar, Shabalina et al. 2009). Our results were questioned by another group, who had previously claimed that it was these circular molecules with deletions that were the main driving force behind the premature aging phenotypes (Vermulst, Wanagat et al. 2008). This triggered a heated scientific discussion (Vermulst, Wanagat et al. 2009), (Edgar, Larsson et al. 2009) and (Edgar and Trifunovic 2009), that will be discussed in more detail below.

There has also been some discrepancy in the amount of point mutations found in the different mouse models and genotypes (hz, wt and mut POLG). These discrepancies are confusing and can lead to very different interpretations of the data. This following section attempts to clarify some issues regarding detection of point mutations and circular molecules with large deletions. I will first address the discrepancy in point mutations and then go on to the question of what type of mutations are most likely to cause the premature aging phenotype:

Genotype	Brain ~8 weeks	Heart ~25 weeks
WT	0,33 (CS) (Trifunovic, Wredenberg et al. 2004) 0,0066 (RMC) (Vermulst, Bielas et al. 2007)	4,58 (CS) (Trifunovic, Wredenberg et al. 2004) 2,11 (CS) (Kujoth, Hiona et al. 2005)
HZ	1,33 (CS) (Trifunovic, Wredenberg et al. 2004) 3,3 (RMC) (Vermulst, Bielas et al. 2007)	- -
MUT	9 (CS) (Trifunovic, Wredenberg et al. 2004) 14 (RMC) (Vermulst, Bielas et al. 2007)	14 (CS) (Trifunovic, Wredenberg et al. 2004) 10 (CS) (Kujoth, Hiona et al. 2005)

Table 1. The number of mtDNA point mutations/10000 base pairs detected by different groups using the CS and RMC approaches.

Different approaches have been tried to measure levels of somatic mtDNA mutations and the results often vary considerably depending on the method used, e.g. cloning-sequencing (CS) or random mutation capture (RMC) (Table 1). Both of these methods seem to detect comparable differences in levels of mutations in mice heterozygous ($POLG^{+/mut}$) or homozygous ($POLG^{mut/mut}$) for the mtDNA mutator allele. However, the RMC method detects a 500-fold less mtDNA mutation load in control mice, i.e. wild type animals obtained after intercrossing mice heterozygous for the mtDNA mutator allele (Table 1). The CS method is the most frequently used procedure to estimate the level of mtDNA mutations and involves PCR amplification of mtDNA, followed by cloning and sequencing of the obtained fragment. A problem with this method is that PCR might introduce mutations in the initial step, which are then carried through to the sequencing reaction. The RMC was developed by Vermulst et al. (Vermulst, Bielas et al. 2007) and is based on the fact that mtDNA mutations will alter a given restriction enzyme recognition site. After restriction enzyme digestion only mutated molecules should be amplified by PCR and those are subsequently quantified and sequenced. However, the target sequence analyzed by this method is only 4 bases long (*Taq I* recognition site) and may therefore not represent the prevalence of mutations over the entire molecule (Vermulst, Bielas et al. 2007). The RMC assay appears to work very well on large sample sizes when the mutations are evenly distributed throughout the genome (Kraytsberg, Simon et al. 2009). However, when existing mutations affect few sites but have been amplified, they might be missed. mtDNA mutations can clonally expand in certain tissues, i.e. when a stem cell gives rise to many cells. This can also happen randomly when mtDNA molecules are segregated or replicated unevenly. In human colonic mucosa (a tissue known to clonally expand mtDNA mutations) the RMC routinely detects between 10-1000 times less mutations than the CS approach and around 5 times less than with single-molecule-PCR based method (Kraytsberg, Simon et al. 2009).

The analysis of mtDNA mutation load in wild-type animals will be complicated when the animals are derived from a mother heterozygous for the mtDNA mutator allele. The reason is that the primordial germ cells of a heterozygous mother contain one copy of the mutant POLG allele and this could cause accumulation of a small amount of

mtDNA mutations during their proliferation and maturation into oocytes. A genetic bottleneck occurs after the oocyte is fertilized whereby a small subset of all mtDNA molecules are partitioned into the primordial germ cells of the next generation (Hauswirth and Laipis 1982). Subsequent rounds of replication in the wt embryo are then carried out by only wt POLG, whereas in the mtDNA mutator embryo proofreading-deficient POLG is used. As a result, the embryo with the mutator allele will continue to introduce new mutations, whereas the wt embryo will amplify only the pre-existing mutations with high fidelity. This could explain why wt littermates of mtDNA mutator mice may have a moderately increased mutation load in comparison with other wild-type mice whose mitochondria have never come in contact with the mtDNA mutator allele. Heterozygous animals from the same cross might continue to accumulate random mtDNA mutations, but to a much lesser extent than mtDNA mutator mice. The pedigree of the analyzed wild-type mice is therefore an important factor to consider when determining mtDNA mutation loads. In order to resolve this issue, we believe that a direct cloning and sequencing approach should be used. This would involve the cloning of large fragments or an entire mtDNA molecule into a vector, without the use of an intermediate PCR step (Battey and Clayton 1978). In this way PCR- induced errors will be avoided and a more complete picture of the distribution of mutations on the population of mtDNA molecules will be achieved. Direct cloning and sequencing has three major drawbacks. First, it is labour intensive. Second, it would require a large sequencing effort to sample the same magnitude of bases as a RMC assay, making sequencing costs prohibitive. Finally, cloning of small amounts of sample DNA is often inefficient and studying single cells is therefore not possible at the moment. Alternatively, the cloning step could be circumvented by usage of newly developed deep sequencing techniques that could be quantitative simply because the same region of the mtDNA would be sequenced over and over again. However, many deep sequencing methods have inherent error rates, which may obscure somatic mtDNA mutation loads.

In a recently published paper, Loeb and co-workers suggest that a third type of mtDNA mutation, besides the very abundant point mutations and linear deletions we have reported (Trifunovic, Wredenberg et al. 2004), are the main driving force behind the shortened lifespan in mtDNA mutator mice (Vermulst, Wanagat et al. 2008). This third type of mutation is circular mtDNA molecule with large deletions of several thousand base pairs. The main argument for this conclusion is that the RMC method detects a 500-fold increase of mtDNA point mutations in heterozygous, $POLG^{+/mut}$ in comparison with wild-type mice, despite the fact the heterozygous mice have a normal lifespan (Vermulst, Bielas et al. 2007). The authors also reported that circular mtDNA molecules with deletions are highly increased in frequency throughout the lifetime of the mtDNA mutator mouse. Similar molecules are also found to accumulate in the wt and $POLG^{+/mut}$ mice, but to a much lesser extent (10- 100 times lower). In light of this evidence they conclude that the circular mtDNA molecules with deletions correlate much better with the phenotype and therefore must be the driving force behind the premature aging of mtDNA mutator mice (Vermulst, Wanagat et al. 2008). However, their study is purely correlative and it does not take into account threshold levels that are the imperative in mitochondrial genetics. In their study, they have estimated only the relative amount of circular mtDNA molecules with deletions between genotypes.

To estimate the importance of these circular mtDNA molecules with deletions it is necessary to know what fraction of the total mtDNA they represent.

We attempted to detect the circular molecules with deletions using long extension PCR technique. As controls, we used two strains of mice with known amounts of deletions, the mito-mouse and the deletor mouse (Sato, Kono et al. 2005; Tyynismaa, Mjosund et al. 2005). These mouse strains carry circular mtDNA molecules with single (mito-mice) or multiple (deletor-mice) deletions in their mitochondria. Although we have robustly detected deleted mtDNA molecules in serial dilutions of these control samples we did not detect any deletions in mtDNA mutator heart or liver samples. Recently, Kraytsberg et. al used single molecule long extension PCR to detect deletions in mtDNA mutator samples provided by Loeb and co-workers (Kraytsberg, Simon et al. 2009). They did not detect deleted mtDNA among more than 320 amplified molecules from duodenum and 144 molecules from heart of mtDNA mutator mice (Kraytsberg, Simon et al. 2009). This completely agrees with our data obtained from heart and liver of mtDNA mutator mice. Kraytsberg et. al detected some mtDNA deletions in brain tissue, but estimated the relative levels to be around 1% and they argue that this could be an overestimation (Kraytsberg, Simon et al. 2009).

It is unclear how these rare circular molecules with deletions could be the major driving force behind the shortened lifespan in the mutator mice. The mito-mice with deletions in up to 30% of all mtDNA molecules do not die early, whereas those with deletions in 70% suffer from renal failure (Sato, Kono et al. 2005). The deletor mouse, although generating multiple deletions of the same range as proposed for mtDNA mutator mice, show no premature aging phenotype. The deletor mice have normal lifespan but develop mitochondrial myopathy late in life (Tyynismaa, Mjosund et al. 2005). Furthermore, deletions are not related to Parkinson's disease until they reach a threshold of around 52% in dopaminergic neurons of humans (Bender, Krishnan et al. 2006). The same is true for most diseases caused by mutations in mtDNA. There is always a threshold level of mutation that has to be reached before the mutation causes respiratory chain dysfunction. This threshold level will vary depending on mutation, but is generally between 60% (for deletions) - 90% (point mutations) (Trifunovic and Larsson 2008). In the mtDNA mutator mouse the mutations are spread out over the genome and a very high threshold level of a particular mutation might not be reached. However, if every molecule accumulates approximately 20 mutations, many of these will be in protein coding genes. For COXI for example there would be an 88% chance that the gene contains at least 1 mutation in a specific mtDNA molecule and a 61% chance that it contains at least 2 mutations of which 40% will cause amino acid change (and we know these mutations in mtDNA are very poorly tolerated). Then there are another 12 genes for which numbers are slightly different depending on the size of the genes. Assuming random distribution of the mutations, a threshold level will be reached easily, not by one specific mutation, but by the sum of all different mutations. Deletions, though affecting many base pairs, still need to be at significant levels to cause mitochondrial dysfunction. It has been estimated that there is about 1000 point mutations for every single deleted mtDNA molecule in mtDNA mutator mice (if there is >0.02 deletion per mtDNA molecule) (Kraytsberg, Simon et al. 2009). This also means that mtDNA mutator mice have about 800 additional point mutations for every

additional deletion when compared to $POLG^{+/mut}$ animals (Kraytsberg, Simon et al. 2009).

Loeb and co-workers do not report increased amounts of deletions in COX-negative cells (cytochrome c oxidase deficient cells – indicating mitochondrial dysfunction), only an increase in point mutations (Vermulst, Wanagat et al. 2008).

Large circular mtDNA deletions typically span over several protein coding genes and include a number of tRNA genes. Therefore, consequences of the large deletions could be detected as a reduction in mitochondrial transcript levels or as a decrease in translation of mitochondrially-encoded proteins if present at high levels (it is true that this might not be detected if these deletions are few and random but then the question of how they would cause pathology remains). This, however, does not seem to be the case. We found that the pattern of transcriptional change was more consistent with increased activity from the heavy strand promoter, rather than a loss of components via deletions. Translation was also normal, but we did observe increased protein turnover (Edgar, Shabalina et al. 2009). So the mechanism by which these rare, circular mtDNA molecules with deletions would be causing the premature aging phenotype remains unclear. We might not have conclusively proven that the point mutations are the cause of the premature aging phenotype, however I have tried to make the case that this is the most plausible explanation.

Finally the question of weather the mutator mice can be regarded as a model for normal aging. It is not normal aging, however it does answer several long-standing questions in the field of aging research. Mitochondrial dysfunction, as is seen in normal aging, can be the cause of many aging phenotypes. Mitochondrial mutations in protein coding genes are very poorly tolerated, often causing respiratory chain complexes not be assembled and they are strongly selected against. Also a large amount of mtDNA mutations does not seem to produce a vicious cycle of increasing amounts of ROS, but this will be discussed in more detail in the next chapter.

3.2 ON REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS IN MTDNA MUTATOR MICE

The vicious cycle hypothesis predicts that increasing point mutations in mtDNA would cause increased ROS production. This was not found in the mtDNA mutator mouse as measured in mouse embryonic fibroblast (Trifunovic, Hansson et al. 2005) and in isolated mitochondria (paper II). In paper II we see that ROS production changes in different directions depending on were what substrate is used. If mitochondria are energized by succinate mtDNA mutator mitochondria produce much less ROS than wt mitochondria. However if the electron transport chain is initiated at complex I with pyruvate or glutamate, mtDNA mutator mitochondria produce slightly more ROS. On a combination of complex I and complex II substrates, allowing mitochondria to work at their maximum capacity (Gnaiger 2009) maximum ROS production is substantially reduced in mtDNA mitochondria compared to wt mitochondria. This difference in ROS production between the genotypes is mainly a consequence of reversed electron transfer. This can be determined by the effect on ROS production by rotenone. Rotenone is a complex I inhibitor which increases ROS production from forward electron transfer but inhibits reverse electron transfer (Brand 2010). Succinate reduces quinone through complex II. If membrane potential is high, this drives the electrons "back" to complex I generating a lot of ROS. If membrane potential is low it is thermodynamically more favourable to transfer the electrons to complex III. It is not clear how much reverse electron transfer contributes to ROS production in vivo (Brand 2010). In many cases supraphysiological levels of succinate are needed to achieve reverse electron flow when mitochondria are respiring on succinate alone. Steady state concentrations of succinate under normal conditions, is low. In addition, succinate dehydrogenase is controlled in a subtle way by its potent inhibitor oxaloacetate. Therefore, elevated ROS generation accompanying succinate oxidation seems unlikely to be a major contributor to oxidative stress to mitochondria under normal metabolic conditions. However under conditions when glutamate is present very low levels of succinate are required for reverse electron flow to be achieved (Muller, Liu et al. 2008).

Oxidative stress is proposed to be a major contributor to the aging process. ROS production is not the only important factor determining the level of oxidative stress. The ability to counteract ROS is of critical importance. To determine the capability of the mtDNA mutator mouse tissues to defend against oxidative stress we exposed tissues to ambient oxygen pressure and measured malonyl dialdehyde (MDA) formation. MDA is a by-product of lipid peroxidation. Mutator tissues showed significantly decreased capacity to protect against oxidation.
This could be due to the redox state in the cell as a result of mitochondrial dysfunction. A more oxidized environment in the cell will not allow efficient elimination of ROS possibly due to lower pools of reduced glutathione and NADPH (Aon, Cortassa et al. 2010). I addition to measuring the capacity of the tissues to defend against oxidative stress, we attempted to determine the mtDNA mutator mouse suffered from increased oxidative stress in vivo. This was done by measuring 4-hydroxynonenol (HNE) adducts, a marker of oxidative modification originating from lipid peroxidation of proteins. In liver we detected a general increase in 4-HNE adducts however no such increase was found in the kidney. This could be due to a decreased antioxidant capacity

or possibly impaired energy demanding degradation and transport systems in the liver. In addition, the respiratory chain subunits produced by the mtDNA mutator mitochondria are likely misfolded (Edgar, Shabalina et al. 2009). This could increase their susceptibility to oxidative modification (Dukan, Farewell et al. 2000).

Mitochondrial dysfunction can lead to the opening of the permeability transition pore (PTP). This causes the mitochondria to depolarize completely and can lead to apoptosis. Also it has been proposed that complex I dysfunction, which is seen in mtDNA mutator mice, due to mtDNA mutations can, not only lower membrane potential but also shift the voltage threshold for opening of the PTP, so that less reduction in membrane potential is needed in order to open the PTP (Porcelli, Angelin et al. 2009). Since increased apoptosis, complex I deficiency, and mitochondrial dysfunction is seen in the mtDNA mutator mice, we measured the spontaneous and inducible high amplitude swelling of mtDNA mutator mitochondria. There are several effectors that can sensitize mitochondria to depolarization such as palmitate, ROS and Ca^{2+}. We assessed the sensitivity of liver and heart mitochondria to Ca^{2+}. The mitochondria of the mtDNA mutator mice were surprisingly not extra sensitive to Ca^{2+} induced swelling. This suggests that more detailed studies are needed to determine the mechanisms of how mitochondrial dysfunction sensitizes mitochondria to permeabilization.

Although the capacity to produce ROS is lower in the mtDNA mutator mice, the lack of sufficient ability to protect against oxidation may allow existing ROS to cause even greater harm. It is possible that under conditions were ROS production is exceptionally high this deficiency might lead to tissue damage and perhaps increased apoptosis as has been observed in our and a similar mouse model (Hiona, Sanz et al. ; Kujoth, Hiona et al. 2005; Niu, Trifunovic et al. 2007) This in conjunction with the cells inability to produce enough ATP when energy demands are high might very well conspire to overwhelm the capacity of the mtDNA mutator mice to maintain their tissues, leading to the observed premature aging phenotypes.

3.3 ON POINT MUTATIONS AND CELLULAR SENESCENCE

In paper III we investigated how mtDNA mutations affect cellular senescence (Kukat, Edgar et al. 2011). Since the mtDNA mutator mice have a progeroid phenotype perhaps also the cells would senesce at an accelerated rate. Alternatively, since there is a strong correlation between mtDNA mutations and cancer, reviewed in (Chandra and Singh 2011) the mtDNA mutations could cause a pro proliferation effect and let the cells escape from senescence. What we found was that cells grown in culture with ample access to glucose were able to escape senescence to a much higher degree than their wt counterparts. In fact almost all MEF cultures from the mtDNA mice went into spontaneous immortalization. This was only when the cells were grown in 20% oxygen in high glucose media. When cells were grown under more physiological conditions, in 3% oxygen, this was not observed. Neither did the mtDNA mutator cells immortalize when grown in low glucose or galactose. Only under conditions with ample glucose and increased oxygen tension did the cells immortalize.

In order for cells to continue to proliferate they must escape both apoptosis and senescence. That means they need to avoid the effects of the tumor suppressors pathways, which play an important role in both apoptosis and senescence. There are two major pathways involved in cellular senescence. The first involving p53, and the other involving pRB.

p53 guards the genome against damage and can initiate DNA repair cell cycle arrest or even apoptosis if the damage proves severe. It is mutated in most cancers and crucial in preventing cancer growth. p53 is regulated by MDM2 which targets it for degradation. MDM2 can be inactivated by p19ARF leading to increased p53 activity. Downstream of p53 is p21, which is believed to be the main target of p53 mediating cell cycle arrest.

RB mediates the transition from G1 phase to S phase when phosphorylated. RB phosphorylation is carried out by CDKs (Cyclin dependent kinases). P16 is a CDK inhibitor, which inhibits RB phosphorylation, and thereby the transition to S-phase (Lanigan, Geraghty et al. 2011). Earlier studies showed the importance of the p53/p19ARF pathway for the spontaneous immortlization of MEFs (Harvey and Levine 1991).

We analyzed the levels of the tumor suppressors p53, p19, p21, and $p16^{INK4}$ in early and late passages of wt and mtDNA mutator MEFs. p53 levels were significantly decreased in later passages in the mtDNA mutator MEFs whereas levels of p19 were decreased already at early passages. $p16^{INK4}$ levels were decreased at early passages but increased to wt levels again at the later passages. The decreases in tumor suppressor levels likely contribute to the ability of mtDNA mutator MEFs to escape senescence. Even though levels of p16 and p19 are reduced in early passages, they are not significantly reduced in later passages that coincide with immortalization. Since p53 is the only tumor suppressor that is significantly lowered at later passages it seems most likely that this is what is responsible for the immortalization of the mtDNA mutator MEFs. However as p21 levels are not changed it would seem that the reduction of p53 lets the cells escape senescence through a p21 independent pathway. This is possibly

due to that p21 is not essential for cellular senescence in MEFs (Pantoja and Serrano 1999). That changes in tumor suppressors affected the cells capability to escape senescence seems clear. So what was behind these changes?

One possibly explanation for the increased rate of immortalization is increased ROS production that might cause mutations in essential genes in the tumor suppressor pathways. No such increase in ROS production was found however, either using FACS analysis or confocal microscopy. Perhaps it is still possible that in a subset of cells there is increased ROS production causing the necessary mutations allowing immortalization. Alternatively mutations arise in both wt and mtDNA mutator cells but are not sufficient to induce immortalization (Fridman and Tainsky 2008). A glycolytic shift could provide the extra step needed for immortalization. It is known that cancer cells often have increased glycolysis, a phenomena first noted by Otto Warburg more than 80 years ago. Whether it is the causative or simply a side effect of cancer growth has been the subject of some debate. However, there are several theoretical proposals and experimental data that do point to the Warburg effect playing an active role in cancer progression. One example comes from colon cancer cell lines were, induction of oxidative metabolism by frataxin inhibits cancer growth (Schulz, Thierbach et al. 2006). However since no tumors were found in mtDNA mutator mice this might not be an important mechanism for them *in vivo*. Clearly cell culture conditions do not always accurately reflect what happens *in vivo*. However the study points to that aerobic glycolysis may have an effect on cellular proliferation, perhaps not only in cancer cells but also in normal tissues.

3.4 THE ROLE OF UCP2 IN MTDNA MUTATOR MICE

Various observations led us to examine what role UCP2 played in the phenotype of the mtDNA mutator mice. For example, at 25 weeks of age the mtDNA mutator mice weigh less than their wt littermates (Trifunovic, Wredenberg et al. 2004) however they eat 9% more. This discrepancy in food intake and weight gain could possibly be an effect of mitochondrial uncoupling. We also found an increase in UCP2 transcript levels in many tissues. In addition to this we saw an increase in UCP2 protein levels in spleen and lung. As UCP2 has been proposed to mediate proton leak, we measured membrane potential in wt and mtDNA mutator mitochondria. There was no difference in membrane potential in, spleen or liver mitochondria from wt or mtDNA mutator mice.

To further examine the role of high UCP2 levels on mtDNA mutator phenotypes, we crossed mtDNA mutator mice with a UCP2KO mice, producing a mtDNA mutator;UCP2KO double mutant (DM) mice. We measured basal, state 4 and uncoupled respiration in spleenocytes of wt, UCP2KO, mtDNA mutator and DM mice. This gave us mice with 3 different levels of UCP2 expression: mtDNA mutator (high), wt (normal) and UCPKO (none) DM (none). The results showed that state 4 respiration was independent of UCP2 level. This strongly argues against an uncoupling role for UCP2 in our cells, since state 4 respiration is dependent on proton leak.

Another proposed role for UCP2 is to mediate a switch from glucose to fatty acid metabolism (Pecqueur, Bui et al. 2008). If lower levels of CoA are generated from fatty acids, the cell will increase import of pyruvate into the mitochondria by increasing glycolysis. Increased glycolysis might be reflected on lactate production and blood glucose levels. We found that DM mice had high levels of lactate and low levels of glucose circulating in the blood. Also, DM spleenocytes showed a high level of lactate production. However, UCP2KO mice had very low levels of lactate circulating in blood and produced by spleenocytes. That UCP2KO mice have low levels of lactate could be explained by them using glucose instead of fatty acids for feeding the ETC. This would result in less pyruvate being available for lactate production. The fact that DM mice have high levels of lactate could be due to combination of trying to use glucose as a respiratory substrate while having damaged respiratory chain.

Analysing gene expression in mtDNA mutator hearts we found many genes associated with metabolism up regulated. We also found up regulation of several genes indicating a switch to fatty acid metabolism, such as carnitine palmitoyl transferase 1 (CPT-1) and PDK4. It has been shown that CPT-1 is the rate limiting step in lipid oxidation as it confines the level of fatty acids transport into mitochondria (Gower, Nagy et al. 2002). PDK4 is the enzyme that inactivates the pyruvate dehydrogenase complex, a crucial step for the inhibition of the whole oxidation of glucose (Sugden and Holness 2006). Also the mtDNA mutator mice loose their fat tissue preferentially while maintaining their lean body mass. In addition they have higher levels of circulating fatty acids early in the onset of symptoms. Later when their fat stores are gone these levels are reduced to half of their wt littermates (Trifunovic, Wredenberg et al. 2004). The DM mutant mice however do not have reduced levels of circulating fatty acids even late in their

life. These finding together point to a switch in substrate utilization towards fatty acids. Additionally since effects of UCP2 ablation can be detected on the systemic level the metabolic effects of UCP2 on the organism are not negligible.

If UCP2 is an uncoupling protein its effect on the mtDNA mutator mice could be either detrimental or beneficial. The same could be true if it regulates substrate utilization. If it is an uncoupling protein it might reduce ROS production by increasing proton leak. As was found in paper II, it seems as if mtDNA mutator tissues are more sensitive to oxygen exposure. Increasing proton leak might thus be a protective mechanism to reduce ROS produced from reverse electron flow. On the other hand damaged mitochondria already have trouble producing enough ATP. Letting part of the membrane potential dissipate as heat could exacerbate this problem leading to an energy crisis. Our data however does not support any of these hypotheses, since state 4 respiration and membrane potential was independent of UCP2. If UCP2 regulates mitochondrial substrate utilization, a shift towards fatty acid utilization could be beneficial allowing the cell to use glucose for glycolysis were it does not need to rely on mitochondrial respiration. Alternatively the reliance on glycolysis could worsen the problem by leading to a relative energy deficient state (Sebastiani, Giordano et al. 2007).

As a consequence of UCP2 ablation we observed an increase in mitochondrial deficiency in DM hearts compared to mtDNA mutator hearts. Mitochondrial mass was increased and enzyme activity was reduced in DM hearts compared to the other genotypes. We also found a greatly increased number of cox-negative cells in 25-week-old DM hearts. This cox negativity is not found in mtDNA mutator mice until 45 weeks of age. This suggests that up regulation or at the very least the presence of UCP2 does not worsen the situation but protects the mtDNA mutator heart from early dysfunction.

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