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ABSTRACT

The cytoplasm of eukaryotic cells contains a dynamic network of double-membraned organelles, called mitochondria, which perform the process of oxidative phosphorylation (OXPHOS) that provides cellular energy in the form of ATP. The respiratory chain creates an electrochemical gradient across the inner mitochondrial membrane, which drives ATP synthesis by the ATP synthase. Mitochondria are indispensable for normal cell function and survival, and dysfunction of the OXPHOS system can lead to a variety of disease syndromes, collectively termed mitochondrial encephalomyopathies. Mitochondrial dysfunction has also been proposed to be involved in age-associated diseases such as diabetes mellitus, heart disease and neurodegeneration, as well as in the ageing process itself. Tissues with high metabolism seem to be particularly vulnerable to mitochondrial dysfunction and myopathy is one of the common phenotypes in mitochondrial disorders. However, the pathophysiological mechanisms linking respiratory chain deficiency to the various phenotypic manifestations are poorly understood. We therefore generated a mouse model for mitochondrial myopathy by tissue-specific disruption of the nuclear gene encoding mitochondrial transcription factor A (TFAM). These myopathy mice develop a progressive respiratory chain dysfunction in skeletal muscle with typical morphological changes consistent with mitochondrial myopathy. Surprisingly the overall mitochondrial ATP production rate was close to normal in the knockout muscles, likely due to the compensatory increase of mitochondrial mass in the affected muscles. Thus, other factors besides ATP deficiency are likely of importance in mitochondrial myopathy. There is a large number of correlative studies suggesting that mitochondrial dysfunction in skeletal muscle is causing the peripheral insulin resistance observed in patients with diabetes mellitus type 2 (DM2). Unexpectedly, the myopathy mice exhibited normal insulin sensitivity and increased glucose uptake in skeletal muscle, suggesting that reduced respiratory chain function in peripheral tissues may be protective against DM2. The mitochondrial theory of aging proposes that oxidative damage to mitochondrial DNA (mtDNA) leads to mutations and impaired respiratory chain function, which in turn, increases reactive oxygen species (ROS) production. ROS have been suggested to induce oxidative damage to various molecules of the cell and thereby cause the progressive decline seen in ageing. We generated mice expressing a proof-reading-deficient version of the mtDNA polymerase gamma. These mtDNA mutator mice accumulated mtDNA mutations at an increased rate and

developed a progressive respiratory chain deficiency. They also developed premature ageing phenotypes and exhibited a reduced lifespan, supporting the suggestion of a causative link between mitochondrial dysfunction and ageing. However, we found no differences in ROS production, no increased expression of ROS scavenging enzymes, and no or minor changes in levels of oxidative damage in cell lines and tissues from the mtDNA mutator mice. We instead propose that the accumulation of mtDNA mutations beyond a critical threshold leads to bioenergetic failure and loss of vital cells. This cell loss caused by respiratory chain dysfunction may lead to reduced organ function and eventually organ failure, giving rise to age-associated disease and important ageing phenotypes.

LIST OF PUBLICATIONS

I. **Increased mitochondrial mass in mitochondrial myopathy mice**
Wredenberg A, Wibom R, Wilhelmsson H, Graff C, Wiener HH, Burden SJ, Oldfors A, Westerblad H, Larsson NG.
Proc Natl Acad Sci U S A. 2002 Nov 12; 99(23):15066-71

II. **Respiratory chain dysfunction in skeletal muscle does not cause insulin resistance.**
Wredenberg A, Freyer C, Sandstrom ME, Katz A, Wibom R, Westerblad H, Larsson NG.
Biochem Biophys Res Commun. 2006 Nov 10; 350(1): 202-7

III. **Premature ageing in mice expressing defective mitochondrial DNA polymerase.**
Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly-Y M, Gidlof S, Oldfors A, Wibom R, Tornell J, Jacobs HT, Larsson NG.
Nature. 2004 May 27; 429(6990): 417-23.

IV. **Somatic mtDNA mutations cause ageing phenotypes without affecting reactive oxygen species production.**
Trifunovic A, Hansson A, Wredenberg A, Rovio AT, Dufour E, Khvorostov I, Spelbrink JN, Wibom R, Jacobs HT, Larsson NG.
Proc Natl Acad Sci U S A. 2005 Dec 13; 102(50):17993-8.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
adPEO	Autosomal dominant progressive external ophthalmoplegia
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
COX	Cytochrome *c* oxidase
CSB	Conserved sequence block
D-Loop	Displacement loop
DM1	Diabetes mellitus type 1
DM2	Diabetes mellitus type 2
ETC	Electron transport chain
GLUT	Glucose transporter
GPX	Glutathione peroxidase
HSP	Heavy strand promoter
KSS	Kearn-Sayre syndrome
LHON	Leber's hereditary optic neuropathy
LSP	Light strand promoter
MAPR	Mitochondrial ATP production rate
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes syndrome
MERRF	Myoclonous epilepsy with ragged-red fibres syndrome
MtDNA	Mitochondrial DNA
mTERF	Mitochondrial transcription termination factor
NARP	Neurogenic weakness, ataxia and retinitis pigmentosa syndrome
nDNA	Nuclear DNA
O_H	Origin of heavy (leading) strand replication
O_L	Origin of light (lagging) strand replication
OXPHOS	Oxidative phosphorylation
PEO	Progressive external ophthalmoplegia
POLγ	Mitochondrial DNA polymerase
POLRMT	Mitochondrial RNA polymerase
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SOD	Superoxide dismutase

TAS	Termination associated sequence
TCA	Citric acid cycle
TFAM	Mitochondrial transcription factor A
TFB1M	Mitochondrial transcription factor B1
TFB2M	Mitochondrial transcription factor B2
tRNA	Transfer RNA
kD	kilo Dalton
Kb	kilo base

1 INTRODUCTION

1.1 MITOCHONDRIA

The main energy currency necessary for function and maintenance of eukaryotic cells is adenosine triphosphate (ATP), generated through metabolism of chemical substances in food. ATP is predominantly generated in cytoplasmic double-membraned organelles, called mitochondria. The outer membrane separates the mitochondria from the cytosol and is permeable to small molecules up to 10 kD. The inner membrane is impermeable and maintains the proton gradient required for generation of ATP via a process termed oxidative phosphorylation (OXPHOS). The inner membrane is invaginated to form cristae, which is where the OXPHOS system is localised, and studies with immunogold labelling of bovine heart have shown that 94% of OXPHOS components are situated in the cristae (Gilkerson et al., 2003). The inner membrane with its cristae define an inner space of mitochondria called the matrix, which is where the mitochondrial DNA (mtDNA), mitochondrial ribosomes for protein synthesis, proteins necessary for mitochondrial replication, transcription, translation and enzymes important for different metabolic processes can be found (Fig.1). Although mitochondria contain their own DNA (Nass and Nass, 1963), most of the mitochondrial proteins are imported and encoded by nuclear DNA (nDNA). Based on phylogenetic studies of mtDNA sequences from various species, mitochondria are believed to originate from a prokaryotic organisms, most likely the α Proteobacteria, which developed an endosymbiotic relationship with an early eukaryotic cell (Gray, 1999; Lang et al., 1999). Mitochondria do not exist as isolated organelles but rather as a dynamic network, allowing for organelle reorganization and redistribution of its contents (Yaffe, 2003). The balance between mitochondrial fission and fusion is not only important for mitochondrial morphology, but also for the maintenance of mitochondrial function. For instance, cells lacking mitofusin 1 and 2, necessary for mitochondrial fusion, have a fragmented mitochondrial network and exhibit disturbed cell growth and decreased cellular respiration (Chen et al., 2005). Mitochondrial dynamics has also been proposed to be important in the fragmentation of mitochondria during apoptosis, and fission of mitochondria is an early event during apoptosis although fission itself cannot initiate apoptosis (Cheung et al., 2007).

In addition to producing cellular energy mitochondria are involved in many other metabolic processes such as lipid metabolism, the citric acid cycle (TCA), formation of

reactive oxygen species (ROS), apoptosis, assembly of iron sulphur clusters and calcium buffering. The mitochondrial proteome has been analysed by mass-spectrometry and at least 600 proteins targeted to the mitochondria were identified and could be divided into functional groups involved in the processes above (Taylor et al., 2003c).

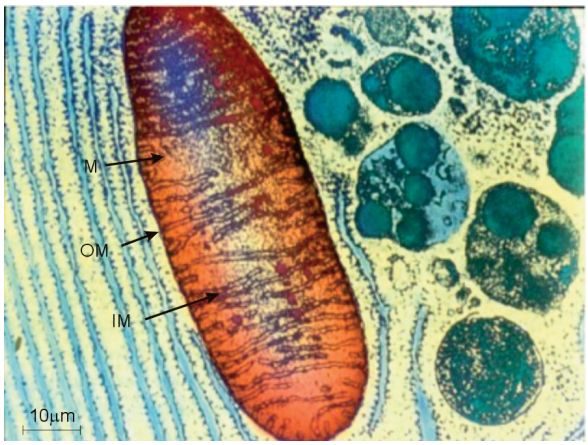

Figure 1: Electron micrograph of a mitochondrion.
The mitochondrion is stained red with an outer membrane (OM) and cristae of the inner membrane (IM) protruding into the mitochondrial matrix (M). From Jones 1995

1.2 THE OXPHOS SYSTEM

The OXPHOS system is composed of five enzyme complexes (complex I-V) and consists of approximately 90 subunits, of which only 13 are encoded by the mtDNA (Fig. 2). Complexes I-IV make up the electron transport chain (ETC). Oxidation of carbohydrates in the citric acid (TCA) cycle and lipids via β-oxidation generates the electron carriers NADH and $FADH_2$, which donate electrons to the ETC. In the ETC the transport of electrons is coupled to the generation of a proton gradient across the inner mitochondrial membrane, which is further used by the fifth enzyme complex to synthesize ATP from ADP+P (Saraste, 1999).

NADH: ubiquinone oxidoreductase (complex I) is the largest of the enzyme complexes of the OXPHOS system consisting of 45 subunits in bovine heart, of which seven are mitochondrially encoded. The enzyme contains multiple prosthetic groups, one

flavomononucleotide (FMN) and eight iron-sulphur clusters (Carroll et al., 2006). NADH in the inner membrane space is oxidised to NAD+ transferring two electrons to the FMN moiety of complex I. The electrons are then further transferred via a series of iron-sulphur clusters to the matrix side of the inner membrane to reduce ubiquinone to ubiquinol. This transfer is coupled to the translocation of four protons across the inner membrane into the intermembrane space (Nicholls and Ferguson, 2002).

Succinate oxidoreductase (complex II), the only exclusively nuclear encoded complex, oxidises succinate to fumarate in the TCA cycle, and donates electrons to the ETC. Complex II consists of a catalytic subunit, succinate dehydrogenase, and two membrane subunits, anchoring the complex into the inner mitochondrial membrane (Capaldi et al., 1977). Electrons from succinate are donated to the covalently bound FAD of succinate dehydrogenase, reducing it to $FADH_2$. The electrons are then further transported via a number of iron/sulphur clusters to ubiquinone, reducing it to ubiquinol. Complex II feeds electrons to the electron transport chain without translocating protons across the membrane (Lancaster and Kroger, 2000; Saraste, 1999).

Ubiquinol-cytochrome *c* oxidoreductase or complex III catalyzes the transfer of electrons from ubiquinol, to cytochrome *c*. It consists of a homodimer, with each monomer composed of eleven subunits, of which one is encoded by the mitochondrial genome (Yu et al., 1998). Ubiquinol is a two-electron carrier, whereas cytochrome *c* is a single electron carrier. Ubiquinol donates its two electrons consecutively to complex III, releasing two protons at the inner membrane space. One electron is transferred to cytochrome *c* via the Rieske iron-sulphur protein, while the second electron is transferred back to the matrix side, to cytochrome *b* of complex III. Cytochrome *b* is able to accept two electrons, which in turn, are donated to ubiquinone at the matrix side, generating ubiquinol. The reduction of ubiquinone to ubiquinol via cytochrome *b* also requires the removal of two protons from the matrix side, thus adding to the proton gradient of the respiratory chain. Due to the recycling of ubiquinone this process is also termed the Q-cycle (Darrouzet et al., 2001).

Cytochrome *c* is a water-soluble protein that donates electrons on the cytoplasmic side of the inner mitochondrial membrane to cytochrome *c* oxidase, or complex IV, the final step in the respiratory chain. Complex IV is composed of 13 subunits of which three are encoded by the mtDNA. It catalyzes the transfer of electrons from the reduced cytochrome *c* pool to molecular oxygen, reducing it to water. In this step, four electrons have to be donated from complex IV to two molecules of oxygen, without generating

any reactive oxygen species. This is achieved by complex IV storing the four electrons on haem and copper atoms, before releasing them only in the presence of two molecules of oxygen and four protons at the matrix side of the mitochondrial inner membrane. Additionally, four protons are translocated across the inner mitochondrial membrane during this reaction (Schultz and Chan, 2001).

The overall outcome of the action of the ETC is the removal of protons form the matrix side, transferring them to the inner membrane space side; thus rendering the matrix side negatively charged, while storing protons on the cytoplasmic side of the inner mitochondrial membrane. This electrochemical gradient is finally utilised by the fifth component of the OXPHOS system, the ATP synthase (F_1F_0-ATPase) or complex V, which drives the generation of ATP from ADP and P (Saraste, 1999). Complex V is composed of a membrane-bound subcomplex (F_0), a large extra-membranous complex (F_1) that resides in the matrix space, and a stalk connecting the two complexes. Protons from the intermembrane space are allowed to enter complex V through the F_0 complex leading to subunit rotation within the enzyme complex. The energy from this rotation is then used for ATP synthesis, which takes place in the F_1 complex (Schultz and Chan, 2001).

Even though the structure of each individual OXPHOS complex is fairly well known, it is unclear how the complexes are organized in relation to one another. It has been suggested that the enzyme complexes of the OXPHOS system exists in supercomplexes, where several complexes are organized and function together. These supercomplexes have been reported in bacteria, yeast and mammals (Schagger and Pfeiffer, 2000; Stroh et al., 2004).

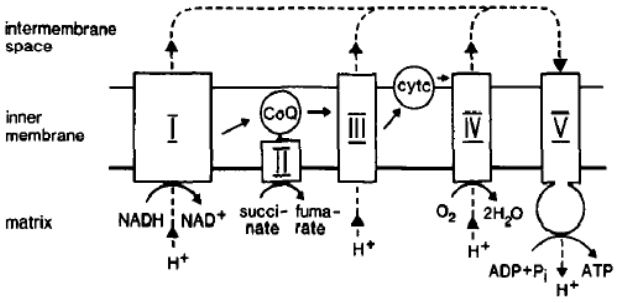

Figure 2: Schematic illustration of the structure and function of the respiratory chain.
The respiratory chain consists of five different enzyme complexes (complexes I-V), coenzyme Q (CoQ) and cytochrome *c* (cyt e). NADH and succinate are oxidized by complexes I and II, the electrons are transferred to coenzyme Q, complex III, cytochrome c, complex IV, and finally to molecular oxygen (1/2 O_2), which is reduced to water (H_20). Protons are pumped out of the mitochondrial matrix by complexes I, III, and IV and a proton gradient is formed across the inner membrane of the mitochondrion. The protons reenter the matrix through complex V (ATP synthase) and the energy of the proton gradient is used to synthesize ATP. From Larsson and Clayton 1995

1.3 GENERATION OF REACTIVE OXYGEN SPECIES

The OXPHOS system transfers electrons in close proximity to oxygen and is therefore an important intracellular source of reactive oxygen species (ROS), which can be harmful for the cell by causing oxidative damage. However, increasing data support the idea that ROS also may be important in normal physiology, e.g. in intracellular signalling (Dada et al., 2003; Nemoto et al., 2000). ROS are also produced at several other locations in the cell, e.g. by NADPH oxidases located in the plasma membrane or in the peroxisomes as a side product of lipid metabolism. The vast majority of ROS, approximately 90%, is generated by the ETC in the mitochondria. It has been estimated that about 0.2% of the basal molecular oxygen is converted into ROS (Balaban et al., 2005).

ROS include a variety of molecules and free radicals derived from molecular oxygen. The superoxide anion (O_2^- •) is the precursor of most ROS. Dismutation of O_2^- •, either spontaneously or by a reaction catalysed by superoxide dismutases, produces hydrogen peroxide (H_2O_2). H_2O_2 may then either be fully reduced to form water or partly reduced to a hydroxyl radical (OH•), which is one of the strongest oxidants in nature. The formation of OH• from H_2O_2 can be catalysed by reduced transition metals like ferrous or cuprous ions in the Fenton reaction (Turrens, 2003).

The majority of O_2^- • generated in the ETC can be attributed to complex I and III. In complex I both the iron-sulphur groups and the FMN site have been associated with ROS formation. In complex III, ubisemiquinone, an intermediate generated in the ubiquinone-ubiquinol redox reaction, donates electrons to O_2 and generate the O_2^- • (Balaban et al., 2005).

1.4 OXIDATIVE DAMAGE AND ANTIOXIDANT DEFENCES

ROS generated under basal conditions can normally be taken care of by the antioxidant defence mechanisms in the cell. However, during a state of altered ROS production, increasing ROS levels have been suggested to cause oxidative damage, which might compromise cell function. For instance, OH• can react with all cellular molecules including DNA (Valko et al., 2004). The most well studied oxidized DNA product is 8-oxo-deoxyguanosine (8-oxo-dG), which is potentially mutagenic and has been suggested to lead to base pairing of 8-oxo-dG with adenine (Cheng et al., 1992). Proteins can be modified by ROS directly due to oxidation of some amino acid residues (lysine, proline and arginine residues) leading to introduction of carbonyl groups. ROS can also react with sugars or lipids to generate products that in turn can react with amino acid side chains generating carbonyl derivatives (Stadtman and Levine, 2000). The activities of certain enzymes are particularly sensitive to ROS, especially proteins containing iron-sulphur centres, such as aconitase, complex I and succinate dehydrogenase, where O_2^- • oxidizes the iron of the iron-sulphur clusters, inactivating the enzyme (Flint et al., 1993; Li et al., 1995).

Antioxidant defences in the cell, preventing the occurrence of oxidative damage, can scavenge most of the ROS. For instance, mitochondrial O_2^- • can be enzymatically converted into H_2O_2 by mitochondrial superoxide dismutase (SOD2 or MnSOD). H_2O_2 will then further be converted into water and glutathione by glutathione peroxidase (GPX) or deactivated by catalase into water and oxygen (Radi et al., 1991; Turrens, 2003).

1.5 MITOCHONDRIAL GENETICS

1.5.1 Gene organisation of mtDNA

The mammalian mitochondrial genome is a double-stranded, circular DNA molecule encoding 37 genes: 13 protein-encoding genes, 22 tRNA, and 2 rRNA genes. Seven of the 13 mitochondrial proteins are subunits (ND1-6 and ND4L) of complex I, one is a subunit (cytochrome *b*) of complex III, three are subunits (CO I- III) of complex IV and two are subunits (ATPase 6 and 8) of complex V (Anderson et al., 1981) (Fig. 3). Mitochondrial genes are encoded by a partly unique genetic code that differs from the universal nuclear code. The termination codon UGA in the universal nuclear code, encodes for a tryptophane in mitochondria, AUA codes for methionine instead of isoleucine and finally, the codons AGA and AGG do not encode for arginine, but are mitochondrial termination codons (Wallace, 1982). The two mtDNA strands can be separated on denaturing cesium chloride gradients, owing to a strand bias in GA content, and are thus designated the heavy strand (H-strand) and the light strand (L-strand), respectively. Most information is encoded on the H-strand, with genes for two rRNAs, 14 tRNAs and 12 polypeptides. The L-strand encodes for eight tRNAs and one single polypeptide. The mitochondrial genome is highly compact and contains no introns. Some of the open reading frames are overlapping, and certain stop codons necessary for proper mRNA translation are only generated after polyadenylation (Anderson et al., 1981; Montoya et al., 1981). There are only two longer non-coding regions in mtDNA. One is the ~ 1 kb long displacement loop (D-loop) region situated between $tRNA^{Phe}$ and $tRNA^{Pro}$, containing the origin of H-strand replication (O_H) and the promoters for H- and L-strand transcription. This D-loop region is often a triple-stranded structure where a 500-700 nt long nascent arrested H-strand remains annealed to the L-strand and the parental H-strand is displaced as a loop. The D-loop structure contains conserved sequences called conserved sequence blocks (CSB) and termination associated sequences (TAS) (Attardi and Schatz, 1988; Shadel and Clayton, 1997). The other non-coding region is about 30 nucleotides long and contains the origin of L-strand replication (O_L). This region is located in a cluster of five tRNA genes around two thirds of the mtDNA length from the O_H (Anderson et al., 1981).

1.5.2 Structure of mtDNA

Each cell contains multiple copies of mtDNA with up to 1000-5000 copies in somatic cells, organised in DNA-protein complexes called nucleoids (Legros et al., 2004; Wang

and Bogenhagen, 2006). Experimental studies with fluorescence in situ hybridization, DNA specific antibodies, DNA-binding dyes or visualization of incorporation of the thymidine analogue BrdU have shown that mtDNA molecules appear to be distributed in 300-800 nucleoids regularly scattered throughout the mitochondrial compartment (Malka et al., 2006). Yeast nucleoids contain one to two mtDNA molecules, whereas vertebrate nucleoids have been proposed to contain six to ten mtDNA molecules (Iborra et al., 2004). A number of proteins have been suggested to be part of the mitochondrial nucleoid, but the composition and structure of the mitochondrial nucleoid has not been fully elucidated. A recent study showed that nucleoids isolated from HeLa cells sediment into two fractions as determined by sedimentation velocity, one fast and one slow fraction, implying different composition of the two fractions (Wang and Bogenhagen, 2006). The slow fraction was analysed and around 20 different proteins were identified that can be divided into three different groups: (I) proteins involved in mtDNA maintenance, replication and/or transcription as previously reported (Bogenhagen et al., 2003; Garrido et al., 2003; Magnusson et al., 2003), (II) chaperon proteins and (III) proteins involved in intermediary metabolism, membrane transport, and the cytoskeleton (Wang and Bogenhagen, 2006).

In yeast the non-histone high mobility group (HMG) protein abf2 and the mammalian homolog TFAM have been proposed to be the core packageing proteins of mtDNA. Abf2 and TFAM can bend and unwind mtDNA in an unspecific manner (Fisher et al., 1992). In yeast abf2 is present at an estimated ratio of one molecule per 15-30 bp of mtDNA (Diffley and Stillman, 1992). In mammalian cells, TFAM has been proposed to coat mtDNA with an estimated 10-20 bp wrapping each protein (Ekstrand et al., 2004; Takamatsu et al., 2002). Recent data based on atomic force microscopy show that TFAM exhibit cooperative DNA binding capacity and appears to be the main nucleoid organizer (Kaufman et al., 2007). TFAM might therefore also regulate nucleoid number, this is further supported by experiments obtained from *Tfam* null mice, which results in the loss of mtDNA (Larsson et al., 1998) and *Tfam* overexpressing mice, which exhibit increased mtDNA levels (Ekstrand et al., 2004). However, TFAM is a bifunctional protein involved in both maintenance of the mtDNA and mitochondrial transcription. In fact, TFAM was initially identified as a transcription factor and not even abortive transcription is possible without TFAM (Fisher and Clayton, 1988; Gaspari et al., 2004).

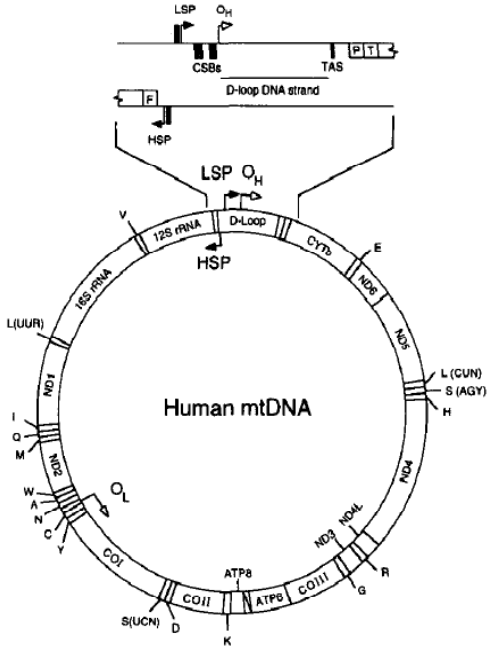

Figure 3: Map of human mtDNA
The following genes are indicated: NADH dehydrogenase subunits (ND1-6 and ND4L) cytochrome *b* (cyt*b*), cytochrome *c* oxidase subunits (COl-Ill), ATP synthase subunits (ATP8 and 6), rRNAs (12S and 16S), and tRNAs (indicated by the one-letter amino acid symbol). The arrows indicate the promoters for transcription of the heavy (HSP) and light (LSP) strand of mtDNA and the origins of heavy (O_H) and light (O_L) strand replication. The enlarged region shows the displacement loop (D-loop) with the nascent D-loop DNA strand, the conserved sequence blocks (CSBs), and the termination associated sequence (TAS).

From Larsson and Clayton, 1995

1.5.3 Transcription of mtDNA

The basal mammalian mtDNA transcription machinery was fully reconstituted *in vitro* with pure components in 2002 (Falkenberg et al., 2002). In addition to the phage-like mitochondrial RNA polymerase (POLRMT) and TFAM, the mitochondrial transcription factors B1 (TFB1M) and/or B2 (TFB2M), are required for transcription (Falkenberg et al., 2007). In addition to binding mtDNA in an unspecific manner, TFAM also binds mtDNA in a sequence-specific manner at the promoter regions (Fisher et al., 1987). The TFAM protein contains two tandem, HMG box DNA-binding domains separated by a 27 amino acid residue linker region, followed by a 25 residue

carboxy-terminal tail (Parisi and Clayton, 1991). Mutational analysis of the human TFAM identified that the tail region is important for recognition of specific DNA sequences and required for transcription activation (Dairaghi et al., 1995). Transcription is initiated from either the H-strand (HSP) or L-strand (LSP) promoters, generating polycistronic precursor RNA, encompassing all of the genetic information encoded on each of the specific strands. The primary transcripts are then processed by excision of the tRNAs to generate the individual mRNA, tRNA and rRNA molecules (Ojala et al., 1980; Ojala et al., 1981). HSP transcription is initiated from two distinct sites, H1 and H2 (Montoya et al., 1982). Transcription initiated from H1 generates a transcript starting 16bp upstream of $tRNA^{Phe}$ and terminating at the 3´end of the 16S rRNA. H2-derived transcription produces a polycistronic molecule corresponding to almost the entire H-strand, starting at the 5´end of the 12S rRNA gene and containing at least all genes until $tRNA^{Pro}$.

Although the exact mechanism of mitochondrial transcription initiation remains unknown, it has been proposed that the binding and bending of DNA by TFAM allows a partial sequence-specific unwinding of the promoters. This in turn would promote the interaction with TFB1M and/or TFB2M, allowing the recruitment of the transcription machinery to the mitochondrial promoters (McCulloch and Shadel, 2003). On the other hand, it is has also been suggested that the structural changes induced by TFAM at the promoter sequences allows for TFB1M or TFB2M to bind the single stranded DNA and recruit POLRMT to the promoter (Asin-Cayuela and Gustafsson, 2007). Transcription termination for H1 transcripts has been proposed to occur at a termination site at the 3' end of the 16S rRNA gene (Christianson and Clayton, 1988) and is believed to be dependent on the mitochondrial termination factor (mTERF now termed mTERF1) (Fernandez-Silva et al., 1997; Kruse et al., 1989). An H2 transcription termination site has been suggested to be just beyond the control region immediately upstream of the $tRNA^{Phe}$ coding gene, dependent on two unknown proteins (Shang and Clayton, 1994). Interestingly, bioinformatic analyses identified three novel genes encoding protein homologues of mTERF all with predicted mitochondrial localization (Linder et al., 2005). Although the functions of mTERF2 and 4 remains to be determined, recent data indicate that mTERF3 is a negative regulator for mitochondrial transcription (Park et al., 2007).

1.5.4 Replication of mtDNA

Replication of mtDNA takes place in the mitochondrial matrix and is independent of the cell cycle. In contrast to nDNA, mtDNA can be replicated several times or not at all during a cell cycle (Bogenhagen and Clayton, 1977). The minimal mitochondrial replisome was recently reconstituted *in vitro* using recombinant purified proteins and found to consist of the mtDNA polymerase γ (POLγ), the mitochondrial helicase Twinkle and mitochondrial single-stranded binding protein (mtSSB) (Korhonen et al., 2004). The functional POLγ holoenzyme is composed of a heterotrimer composed of one molecule of the catalytic subunit (α-subunit) and two molecules of processivity subunits (β-subunits) (Yakubovskaya et al., 2006). The catalytic subunit has separate polymerase and 3´-5´exonuclease domains. The association of the accessory β-subunits with the catalytic subunit leads to increased affinity to DNA, resulting in increased processivity (Carrodeguas et al., 1999; Lim et al., 1999). The exonuclease domain of POLγ contains three highly conserved exonuclease motifs (EXO 1-3) with conserved aspartate and glutamate residues in their active sites. The 3´-5´exonuclease activity prefers mispaired 3´-termini and is believed to be responsible for the high fidelity of the POLγ (Longley et al., 2001). Purified POLγ from porcine liver and chicken embryos can accurately replicate mtDNA with error frequencies at less then 1 error per 500000 and 1 per 260000 bases, respectively (Kunkel and Mosbaugh, 1989; Kunkel and Soni, 1988). *In vitro* studies using recombinant human POLγ with or without exonuclease activity found mutation frequencies of $12x10^{-4}$ in the presence of exonuclease activity, whereas it was $22x10^{-4}$ without exonuclease activity (Longley et al., 2001). By substituting the conserved active site aspartic acid residues to alanine or glycine in EXO 1-3 in the yeast POLγ gene (MIP1), the strains exhibited a 100-fold increase in mutation frequency without affecting polymerase activity (Foury and Vanderstraeten, 1992).

1.5.5 Models of replication

Analyses of replication intermediates (RI) performed by electron microscopy, atomic force microscopy, biochemical isolation, pulse and pulse-chase labelling experiments has provided a generally accepted model for mtDNA replication, describing replication as an asynchronous displacement mechanism involving two unidirectional independent origins (Berk and Clayton, 1974; Bogenhagen and Clayton, 2003; Brown et al., 2005).

In this model replication starts from the origin of H-strand replication, proceeds along the parental L-strand to produce a nascent H-strand (leading strand). When H-strand synthesis has reached two thirds of the genome, the origin of light strand replication is exposed, and lagging strand synthesis initiates and proceeds in the opposite direction producing the L-strand (Shadel and Clayton, 1997). Initiation of replication requires an RNA primer, generated during transcription, processed to span the region between the LSP and the CSBs within the D-loop (Chang and Clayton, 1985). A site-specific endonuclease, RNase MRP, cleaves RNA at CSB I and II *in vitro* and has been proposed to process the RNA primer required for replication (Bennett and Clayton, 1990). An alternative mechanism for primer formation was recently proposed, where CSB II was found to be a transcription termination element. The RNA primer would then be generated by transcription termination rather then by enzymatic cleavage (Pham et al., 2006). Replication may continue from O_H to copy the entire genome or terminate ~ 700 bp downstream of O_H at the termination associated sequences (TAS) thus giving rise to 7S DNA, which is identical to the nascent H-strand of the triple stranded D-loop structure (Clayton, 1982). An additional origin of replication has been identified at position 57 in the D-loop and human cells have been suggested to exhibit two modes of mtDNA replication each associated with distinct origins of replication. One origin would be involved in regulating mtDNA copy number under steady-state conditions. The other mode associated with the previously known origin of replication in the D-loop may be more important for recovery after mtDNA depletion and/or for accelerating mtDNA replication in response to physiological demands (Fish et al., 2004).

The strand-asymmetric model of replication has recently been challenged by neutral/neutral 2D gel electrophoresis, which demonstrates the presences of RIs with properties of conventional coupled leading- and lagging strand synthesis (Holt et al., 2000). Coupled replication was first believed to initiate at or near O_H and proceed unidirectional. It was later suggested that mtDNA replication initiates from multiple sites in a broad zone extending into the coding region to then continue bidirectional (Bowmaker et al., 2003). Holt and colleagues characterized the RIs further proposing yet another model for mtDNA replication (Yasukawa et al., 2006). Two different types of RIs were found to exist in higher vertebrates. One kind of RIs contained few ribonucleotides and were compatible with coupled leading-and lagging strand synthesis initiated from multiple sites in a broad zone. The second kind was found to incorporate RNA over the entire length of the lagging-strand and replication was initiated strictly in

the D-loop region (Yasukawa et al., 2006). These findings led to the RITOLS (ribonucleotide incorporation throughout the lagging strand) model of replication, which suggests that replication of mtDNA initiates at or near O_H and proceeds unidirectional synthesising the daughter H-strand. The lagging strand would then initially be laid down as an RNA strand; the maturation of the RNA strand to DNA is then predominantly initiated from the O_L (Yasukawa et al., 2006). The function of this transient RNA strand is not known, but has been suggested to stabilize the single-stranded displaced DNA and protect it against damage during replication or to act as a roadblock to arriving transcription complexes and thereby inhibit transcription until replication has been completed.

1.5.6 Segregation and transmission of mtDNA

The fixation rate of mutations is ten times higher in the mitochondrial genome in comparison to the nuclear genome (Brown et al., 1979). Additionally, there is great sequence variation within a population due to the cell cycle independent replication and random drift, which can lead to the accumulation of acquired mtDNA mutations to high levels in some somatic tissues, or to their loss in others. The mtDNA copies within an individual can be identical, a state called homoplasmy, whereas a state where two or more mtDNA alleles coexist, is termed heteroplasmy. A pathogenic mutation can thus be present in all mtDNA copies or only in a subset of the molecules. A true homoplasmic situation with all mtDNA sequences within one individual being identical does probably not exist since mtDNA is constantly undergoing mutation with either clonal expansion or loss of deletions or point mutations (McFarland et al., 2007).

Mammalian mtDNA is inherited maternally with paternal mtDNA within the spermatozoa being actively destroyed in the fertilized oocyte (Sutovsky et al., 1999). Although one case of the transmission of paternal mtDNA has been identified in humans (Schwartz and Vissing, 2002), this seems to be a very rare event and occurred in a single disease state (Filosto et al., 2003; Taylor et al., 2003b).

A mother carrying a heteroplasmic pathological mtDNA mutation can transmit highly varying levels of that mutation to its offspring (Brown et al., 2001). This has been attributed to a bottleneck phenomenon, in which only a small subset of mtDNA molecules are propagated during early oogenesis to generate the many copies in the mature oocyte. This bottleneck is proposed to occur during maturation of the primordial germ cells with only a small number of mtDNA copies present in each cell (Jenuth et

al., 1996). However, recent data suggest that selective replication of a subset of molecules manifests the observed bottleneck (Cao et al., 2007).

1.6 MITOCHONDRIA IN DISEASE AND AGEING

1.6.1 Mitochondrial disease

The mitochondrial diseases compose a group of genetically heterogeneous disorders presenting with a wide spectrum of different symptoms and clinical manifestations, engaging everything from a single organ/tissue to presenting as multi-system disorders (McFarland et al., 2002b). Mitochondrial diseases are more common than previously thought. In Finland a population of 245,201 individuals was analysed to estimate the frequency of the 3243>G "MELAS" mutation and the minimum point-prevalence was estimated to 1/6135 (Majamaa et al., 1998). In another study performed in northeast England, the prevalence of all mitochondrial diseases was analysed. It was estimated that 1/8031 individuals had a mitochondrial disease or were at risk of developing one (Chinnery et al., 2000). Based on two paediatric studies, one in Sweden (Darin et al., 2001) and one in Australia (Skladal et al., 2003) a minimum point-prevalence of about 1/20,000 was determined, making mitochondrial diseases amongst the most common inherited metabolic diseases (Schaefer et al., 2004). They are caused either by mutations in the mtDNA or in nuclear genes leading to disruption of the respiratory chain and can then potentially affect the cell in a variety of ways for example by reduced ATP synthesis, induction of apoptosis or production of ROS. Mitochondrial disorders can thus be inherited in any mode, autosomal recessive or autosomal dominant, X linked or maternal. Mitochondrial diseases often have skeletal muscle and central nervous system (CNS) manifestations and are therefore often referred to as mitochondrial encephalomyopathies. Mitochondrial myopathy may be present in disorders exclusively affecting skeletal muscle or as a part of a multi-system disease. The clinical features of mitochondrial myopathy include muscle weakness, exercise-induced cramps and myalgia. Due to its accessibility, skeletal muscle is often used for diagnostic screens of mitochondrial abnormalities. With the Gomorri-trichrome staining accumulation of subsarcolemmal mitochondria can be identified in muscle tissue as ragged-red muscle fibres (RRF). Double staining for succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX) activity, can reveal a mosaic pattern of COX activity, often with increased SDH staining indicating accumulation of subsarcolemmal mitochondria. This mosaic pattern is highly suggestive of a heteroplasmic mtDNA

disorder, and most RRFs are COX-deficient (Fig. 4). The mitochondria of RRFs often appear abnormal and may contain paracrystalline inclusions in EM (Taylor et al., 2004).

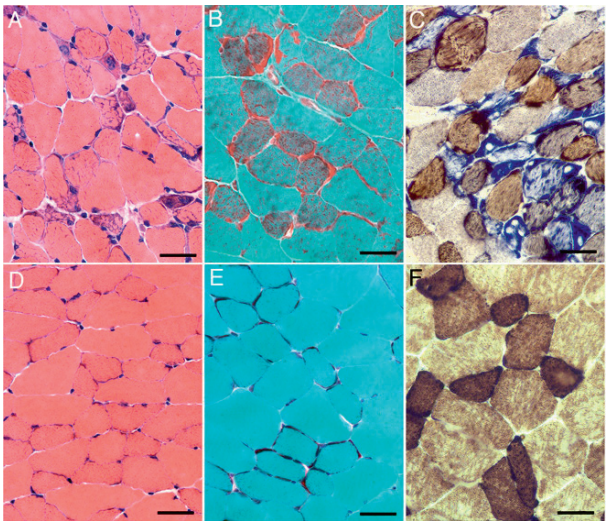

Figure 4: Histological findings in mitochondrial myopathy
Analysis of tissue sections from EDL muscle from a myopathy (A–C) and a control (D–F) mouse at 3 months of age. (Scale bar, 25 μm.) (A and D) Hematoxylin/eosin staining. There are scattered atrophic fibres in the myopathy mouse. (B and E) Modified Gomori trichrome staining. There are numerous RRFs in the myopathy mouse. (C and F) Staining to detect simultaneous COX/SDH activity. The muscle fibres that appear blue in the myopathy mouse are COX-deficient and contain accumulation of mitochondria.
From Wredenberg et al 2001

Similar clinical features can be caused by various mutations in mtDNA or nDNA. Surprisingly, a particular genetic defect in mtDNA can lead to different clinical manifestations (Schapira, 2006). This variability in clinical presentation can be explained in part by the specific features of mtDNA genetics; polyploidy, maternal inheritance and segregation. In a patient carrying a heteroplasmic point mutation, the fraction of mutated mtDNA molecules (also termed mutation load) can vary between different tissues and even between different cells in the same tissue. Therefore, mitochondrial dysfunction does not occur in every tissue, or even within every cell of a tissue and disease does not occur in every patient with a pathogenic mutation. This is due to a critical level the mutation load has to reach within a cell, before a biochemical effect is observed. This observation is also termed the threshold effect of mutations,

which can vary from 60% for some deletions (Hayashi et al., 1991) and can be up to 95% for some point mutations (Boulet et al., 1992; Larsson and Holme, 1992). Some tRNA mutations even seem to require a homoplasmic state, in patients with mitochondrial diseases (McFarland et al., 2002a).

1.6.1.1 MtDNA mutations

Mitochondrial dysfunction can be caused by mtDNA rearrangements, such as deletions and duplications, or point mutations. The first descriptions of rearrangements (Holt et al., 1988) or point mutations (Wallace et al., 1988) of mtDNA in association with disease were made in 1988. MtDNA rearrangements are usually sporadic, whereas point mutations can be inherited. The most common rearrangement is a 5 kb deletion, occurring between two 13 bp repeats, extending between the cytochrome *b* and cytochrome oxidase subunit II genes (Moraes et al., 1989). Single large-scale deletions have been associated with Progressive external ophthalmoplegia (PEO), Kearn-Sayre syndrome (KSS) and Pearson syndrome. PEO develops as a late-onset progressive external ophthalmoplegia, proximal myopathy and exercise intolerance (Graff et al., 2002). The main clinical features of KSS are onset before 20 years of age, progressive external ophthalmoplegia, pigmentary retinopathy and cardiac conduction defects (Larsson et al., 1990). The Pearson bone-marrow-pancreas syndrome, a rare disorder of infancy, is characterized by onset of a transfusion-dependent sideroblastic pancytopenia and in rare cases with severe exocrine pancreatic insufficiency with malabsorbtion (McShane et al., 1991). The infants surviving Pearson's syndrome may develop KSS later in life (Larsson et al., 1990).

There are several mitochondrial disorders caused by mtDNA point mutations, which can cause symptoms in either a heteroplasmic or homoplasmic state. Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes syndrome (MELAS) is defined by the presence of stroke-like episodes due to brain lesions most frequently localized to the parieto-occipital lobes and lactic acidosis and/or ragged red fibres (RRFs). MELAS has been associated with several point mutations in mtDNA, most commonly the heteroplasmic 3243 A>G transition in $tRNA^{Leu(UUR)}$ (Goto et al., 1990). Another pathogenic heteroplasmic point mutation identified is the 8344 A>G transition in the $tRNA^{Lys}$ gene, which has been associated with myoclonous epilepsy with ragged-red fibres syndrome (MERRF) (Shoffner et al., 1990). Mutations in protein coding genes, like the heteroplasmic 8993 T>G transversion in the ATPase 6 subunit gene,

resulting in neurogenic weakness, ataxia and retinitis pigmentosa syndrome (NARP) have also been identified, although mutations in protein coding genes seem to be more rare then mutations in tRNA genes (Holt et al., 1990). The level of heteroplasmy of the NARP mutation seems to be correlated to the degree of severity and when the level of mutated mtDNA is >90% patients show symptoms of Leigh syndrome, an often fatal, neurological disorder of infancy and early childhood (Santorelli et al., 1993).

One of the most common mtDNA disorders is Leber's hereditary optic neuropathy (LHON), characterised by subacute bilateral visual failure in young adults. Over 95% of patients with LHON carry one of three, usually homoplasmic, mutations in the mtDNA encoded subunits of complex I, namely 3460G>A, 11778G>A, and 14484T>C (McFarland et al., 2007; Zeviani and Carelli, 2003).

1.6.1.2 Nuclear mutations

The majority of pathological mutations identified leading to mitochondrial disorders are mtDNA encoded, despite the majority of OXPHOS subunits being nuclear encoded. However, mutations in nuclear encoded subunits of complex I and II have been associated with Leigh syndrome (Zeviani and Carelli, 2003). Additionally, OXPHOS assembly and regulation is dependent on the expression of nuclear encoded genes and defects of OXPHOS have been associated with a number of genes involved in OXPHOS function. For instance, mutations in the surfeit gene (SURF1) encoding a protein normally involved in complex IV assembly were found to lead to complex IV deficiency and Leigh syndrome (Tiranti et al., 1998). The inner mitochondrial membrane protein BCS1L is a chaperone protein necessary for complex III assembly. Mutations in this gene have been found to lead to infantile complex III deficiency and neonatal proximal tubulopathy, hepatic involvement and encephalopathy syndrome, also called GRACILE (growth retardation, aminoaciduria, cholestasis, iron overload, lactacidosis and early death) syndrome (Visapaa et al., 2002).

Nuclear encoded genes regulate maintenance and stability of mtDNA and mutations in any of these genes can potentially lead to mtDNA deletions or depletion. Accumulation of multiple mtDNA deletions is found in tissues of patients with autosomal dominant progressive external ophthalmoplegia (adPEO). Typical clinical features are progressive muscle weakness affecting most severely the external eye muscles. AdPEO has been associated with heterozygous mutations in one of three genes: *ANT1*, encoding the muscle-heart specific mitochondrial adenine nucleotide translocator (Kaukonen et al., 2000), *Twinkle*, encoding a putative mtDNA helicase (Spelbrink et

al., 2001) and *POLG1*, encoding the catalytic subunit of the POLγ (Van Goethem et al., 2001). Mitochondrial depletion syndrome (MDS) is characterised by a reduction in mtDNA copy number. MDS composes a group of disorders including fatal, infant congenital myopathy or hepatopathy leading to rapidly progressive liver failure (Moraes et al., 1991). It also includes a late infantile or childhood myopathy. MDS has been associated with mutations in two genes involved in dNTP metabolism, namely thymidine kinase 2 and deoxyguanosine kinase (Mandel et al., 2001; Saada et al., 2001).

1.6.2 Mitochondria in Diabetes mellitus

1.6.2.1 Insulin signalling and glucose-homeostasis

Insulin is a polypeptide-hormone produced in the β-cells of the islet of Langerhans in the pancreas. Insulin coordinates the utilization of fuels by different tissues; it has anabolic effects favouring uptake of glucose and synthesis of glycogen and proteins. Glucose enters the β-cells via glucose transporter (GLUT) 2, and the concentration of glucose in the β-cells will always be the same as in the peripheral blood. An increase in blood-glucose levels leads to increased ATP/ADP ratio and subsequent closure of ATP-sensitive potassium channels leading to β-cell depolarization and insulin secretion (Herman and Kahn, 2006). Upon release, insulin binds to the insulin receptor, which is located in the plasma membrane, and initiates a signalling cascade resulting in the translocation of GLUT4 to the cell surface, facilitating glucose uptake. There are several different types of GLUTs. GLUT1-4 can be distinguished based on their tissue distribution. GLUT1 is widely expressed, but highest levels have been found in brain, erythrocytes and endothelial cells. It is believed to be responsible for basal glucose uptake. GLUT2 is found in liver and pancreatic β-cells. GLUT3 is expressed in neuronal cells and GLUT4 is an insulin-responsive glucose transporter found in skeletal muscle, cardiac muscle and adipose cells (Joost et al., 2002). The main glucose transporters in skeletal muscle are GLUT1 and 4, responsible for basal versus insulin-stimulated glucose transport (Shepherd and Kahn, 1999). GLUT4 is also believed to be involved in the exercise-induced glucose uptake via signalling-pathways not yet completely elucidated but involving the stimulation of AMP-activated protein kinase (AMPK).

1.6.2.2 Diabetes mellitus

Diabetes mellitus (DM) comprises a group of metabolic diseases, all sharing the symptom hyperglycaemia. There are several different types of DM caused by a complex interaction of genetics, environmental factors and life-style choices. Disrupted insulin production/secretion, insulin resistance in liver, skeletal muscle and fat or a combination of both can cause DM. There are two main groups of DM, type 1 (DM1) and type 2 (DM2). DM1 is in general caused by destruction of β-cells, leading to the inability of the pancreas to secrete insulin in response to elevated blood glucose levels. DM2 on the other hand is a heterogeneous group of disorders with variable degrees of insulin resistance and impaired insulin secretion. Insulin resistance is the inability of peripheral tissues to respond to circulating insulin, and thus to stimulate glucose uptake. The initial step in disease progression is an insulin resistance in target tissues (liver and skeletal muscle). To maintain euglycaemia more insulin is secreted from the endocrine part of pancreas, but eventually the ß-cells of Langerhans islets cannot compensate anymore for the increasing insulin resistance, resulting in an impaired glucose tolerance. Finally, pancreatic ß-cell failure leads to decreased insulin secretion. (Braunwald, 2001; Mlinar et al., 2007; Petersen and Shulman, 2002). In addition to DM1 and 2 there are also a number of forms of DM caused by specific genetic defects leading to β-cell dysfunction including mitochondrial diabetes. Mitochondrial diabetes, also called maternally inherited diabetes and deafness, associates with the mutation A3243G in the mitochondrial $tRNA^{Leu,UUR}$ that also has been associated to the encephalomyopathy MELAS. Mitochondrial diabetes seems to be caused by reduced insulin production/secretion and not necessarily insulin resistance in peripheral tissues, as is the case in DM2 (Kadowaki et al., 1994; Maassen et al., 2004). Recently, mitochondrial dysfunction in skeletal muscle has been proposed to play a major role in the pathogenesis of DM2. Global gene-expression studies of skeletal muscle from DM2 patients revealed reduced expression levels of peroxisome-proliferation-activated receptor γ co-activator 1α and its down stream nuclear target genes, which include some mitochondrial proteins of the OXPHOS system (Mootha et al., 2003). Magnetic resonance spectroscopy (MRS) analyses of OXPHOS activity in skeletal muscle of aged individuals and lean insulin-resistant offspring of DM2 patients detected a 30-40 % reduction in OXPHOS activity in these individuals (Morino et al., 2006; Petersen et al., 2003; Petersen et al., 2004). Additionally, increased levels of intramyocellular lipids and decreased mitochondrial volume were found and it was proposed that some

unknown factors in patients with DM2 lead to reduced mitochondrial biogenesis, which in turn leads to reduced rates of OXPHOS, predisposing them to accumulation of intramyocellular lipids and insulin resistance (Morino et al., 2006). The notion that mitochondrial dysfunction might be involved in DM2 has also recently been supported by a group in Denmark, by demonstrating reduced mitochondrial respiration in skeletal muscle of patients with DM2 (Mogensen et al., 2007). In another study, low- and high-dose insulin infusions were administered to non-diabetic controls and DM2 patients prior to skeletal muscle analyses. The authors found no difference in mitochondrial ATP production rates (MAPR) between the two groups at low insulin infusion dosages. At high-dose insulin infusion the MAPR was elevated in the control group as expected, but there was no increase in the DM2 group. This finding combined with reduced glucose disposal and normal mtDNA levels in DM2 patients lead the authors to suggest that mitochondrial dysfunction in skeletal muscle of patients with DM2 is not the primary fault, but rather a functional defect due to impaired insulin response (Asmann et al., 2006).

1.6.3 Mitochondria and ageing

Ageing can be defined as the progressive, generalized impairment of function resulting in an increased vulnerability to environmental challenge and a growing risk of disease and death, usually accompanied by a decline in fertility. Ageing is a phenomenon that can occur in a protected environment. In many species of wild animal populations, individuals rarely survive to ages, when senescent deterioration becomes apparent; therefore it seems unlikely that genes specifically for ageing would have evolved. It would be more likely to believe that ageing is due to the failure of mechanisms necessary for survival. Such mechanisms could be maintenance and/or repair of proteins or DNA. As a result, ageing is believed to result from the random accumulation of unrepaired cellular and molecular damage throughout life (Kirkwood, 2005; Kirkwood and Austad, 2000). Several theories, explaining the processes leading to ageing, have been proposed; one of them is the mitochondrial theory of ageing. It is however most likely that ageing is due to limitations in a wide range of maintenance and repair mechanisms. Harman postulated in 1956 the free radical theory of ageing, suggesting that the production of ROS during life is the driving force behind the ageing process (Harman, 1956). The mitochondrial theory of ageing predicts that a vicious cycle contributes to the ageing process; (i) Normal metabolism produces ROS in the

ETC. (ii) ROS causes oxidative damage to lipids, proteins and DNA in the mitochondria. (iii) ROS-induced mtDNA damage leads to the synthesis of dysfunctional respiratory chain subunits, causing respiratory chain dysfunction and increased ROS production (Harman, 1972). This vicious cycle is proposed to lead to an exponential increase of mtDNA mutations over time resulting in ageing and associated degenerative diseases. A substantial amount of data supporting the involvement of mitochondria in ageing has subsequently been generated. Mitochondria have been shown to become larger and less numerous with age, accumulating vacuoles, cristae abnormalities, and intra-mitochondrial paracrystalline inclusions (Frenzel and Feimann, 1984). An age-related decline in respiratory chain function has been observed in mammals and COX-negative cells have been found to accumulate with age in many different tissues including muscle, heart, brain and colon (Cottrell et al., 2001; Cottrell et al., 2002; Muller-Hocker, 1989; Muller-Hocker, 1990; Taylor et al., 2003a). Deletions and point mutations of mtDNA accumulate with age in humans (Cao et al., 2007; Corral-Debrinski et al., 1992; Cortopassi and Arnheim, 1990; Cortopassi et al., 1992; Michikawa et al., 1999), monkeys (Schwarze et al., 1995) and rodents (Khaidakov et al., 2003). Oxidatively damaged molecules like the 8-oxo-dG accumulate with increasing age (Mecocci et al., 1993; Richter, 1995). However, the overall levels of individual point mutations in tissue homogenates have been found to be very low, ranging between 0.04 and 2.2% of all mtDNA copies (Liu et al., 1998), and the levels of the common deletion vary from 0.001% to 0.3% (Dufour and Larsson, 2004). Considering the level of specific mtDNA mutations required to cause pathology in the group of mitochondrial diseases, it is difficult to see how such low levels, as in ageing individuals, could lead to any critical respiratory chain dysfunction. However, the accumulation of COX-negative cells, even though less then found in patients with mtDNA disease, indicates that the level of mtDNA mutations in certain cells can be of substantial size to cause a respiratory chain dysfunction and therefore potentially have an impact on the function of the entire tissue. Thus, similar to patients with mtDNA mutations one can envision that individual mutations clonally expand to high levels resulting in the loss of individual cells in the ageing process. On the other hand, the total sum of different mtDNA mutations, albeit individually low, could potentially lead to a respiratory chain dysfunction within a cell, causing the observed tissue defect.

Several genetic models have been generated in order to study the involvement of ROS in mitochondrial function and therefore indirectly the involvement of mitochondrial ROS production in ageing. Nevertheless, the results of these studies have not managed

to elucidate the role of ROS in ageing, as they are not consensual and often contradictive. For example, disrupting the gene encoding for oxoguanine DNA glycosylase (OGG1), the enzyme responsible for the removal of 8-oxo-dG, in mice leads to a 20-fold increase in the levels of 8-oxo-dG in mtDNA (Stuart et al., 2005). However, these Ogg1 null mice did not show any phenotype, neither did they develop any respiratory chain dysfunction. Mice deficient of Sod 1, 2, 3 and Gpx 1 or 2 have been reported. Sod 2 null mutants die within the first 10 days of life and display dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle, and metabolic acidosis (Li et al., 1995). Sod 1, 3 and Gpx 1 or 2 null mice, on the other hand, appear healthy and have a normal lifespan. Additionally, the Sod 1 or 3 null mice display increased sensitivity to oxidative stress (Carlsson et al., 1995; Esworthy et al., 2000; Ho et al., 1998; Ho et al., 1997). The compound $SOD2^{+/-}/GPX1^{-/-}$ mice have a normal lifespan and appear phenotypically normal, despite exhibiting a severe sensitivity to oxidative stress (Van Remmen et al., 2004). Even though none of these mice strains displayed any compelling signs of ageing another mouse strain with overexpression of catalase targeted to mitochondria displayed a longer lifespan in comparison to control mice (Schriner et al., 2005), and thus favours the hypothesis of ROS involvement in ageing.

2 SPECIFIC AIMS

The specific aim of each paper was to:

Paper I: generate and study a murine model for mitochondrial myopathy by disrupting the *Tfam* gene in skeletal muscle

Paper II: study if progressive respiratory chain dysfunction in skeletal muscle leads to disturbed glucose homeostasis and insulin resistance

Paper III: test whether a progressive accumulation of mtDNA mutations leads to ageing

Paper IV: test whether a progressive accumulation of mtDNA mutations leads to premature ageing by the excessive production of ROS.

3 RESULTS AND DISCUSSION

3.1 STUDIES OF SKELETAL MUSCLE SPECIFIC DISRUPTION OF THE *TFAM* GENE (PAPER I)

In order to study the effect of a skeletal-muscle-specific respiratory chain dysfunction we generated skeletal-muscle-specific *Tfam* null mice (mitochondrial myopathy mice), by crossing homozygous $Tfam^{LoxP}$ mice to a mouse strain, expressing *cre* recombinase under the *mlc1f* promoter. The *mlc1f* promoter is expressed at day 10 post coitum and is specific for fast twitch muscle fibres. The myopathy mice displayed no phenotype until around 3 to 4 months of age, when weight loss and muscle weakness could be observed. The phenotype progressed and the mice had to be euthanized at 4 to 5 months of age because of weakness.

Knockout of the *Tfam* protein was confirmed by western blot, and the protein could not be detected in skeletal muscle of the myopathy mice at 1, 2 and 4 months of age. MtDNA levels were reduced to ~ 30% of control values in skeletal muscle of myopathy mice at 1, 2 and 4 months of age. Interestingly, the level of mtRNA decreased progressively from ~70 % of control levels at 1 month of age to ~ 30% of control levels at 4 month of age in the myopathy mice. Protein levels of mitochondrial COX II displayed a similar pattern with a 50% reduction at the age of 2 months and no detectable protein at 4 months of age in the myopathy mice. In accordance with this we also measured progressively deteriorating respiratory chain activities and mitochondrial ATP production rates (MAPR) per mitochondria.

The myopathy mice exhibited histological signs of mitochondrial myopathy, with a mosaic pattern of COX negative and ragged-red muscle fibres in sections of skeletal muscle. Electron microscopy revealed accumulation of enlarged and abnormal mitochondria, and increased mitochondrial volume was further supported by an increase in citrate synthase activity in skeletal muscle of myopathy mice.

In vitro force measurements of isolated fast twitch extensor digitorum longus (EDL) and slow twitch soleus muscles displayed reduced absolute muscle force in EDL and to some extent in the soleus muscle of 3-3.5-month-old myopathy mice. However, during fatiguing stimulation muscles from the myopathy mice and their controls showed the same trend in force decline, indicating that lack of ATP was not the limiting factor. Similar MAPR per kg of skeletal muscle, when comparing myopathy and control mice at 4 months of age, further suggested that ATP deficiency was not of major importance.

The myopathy mice developed a mitochondrial myopathy as determined by molecular biological analyses, enzyme activities and *in vitro* force measurements. However, our data indicated that an increase in mitochondrial mass in skeletal muscle of the myopathy mice could compensate for the respiratory chain deficiency to a certain degree. Thus, the reduced muscle force detected in the myopathy mice might be due to some other factor like dysregulation of calcium homeostasis or increased ROS levels.

3.2 RESPIRATORY CHAIN DYSFUNCTION IN SKELETAL MUSCLE DOES NOT LEAD TO INSULIN RESISTANCE (PAPER II)

Respiratory chain dysfunction in skeletal muscle has been proposed to lead to insulin resistance and eventually DM2. We analysed the mitochondrial myopathy mouse model with respiratory chain dysfunction in skeletal muscle in order to directly test this hypothesis. Insulin tolerance tests in myopathy mice and their littermate controls showed no difference in peripheral blood glucose clearance between the two groups. This indicates that the myopathy mice had normal insulin sensitivity and thus no insulin resistance. Glucose tolerance tests (GTT) at one month of age displayed no difference in glucose clearance from the peripheral blood, when comparing myopathy mice to their controls. Surprisingly, at the age of 2 months, the myopathy mice did show signs of increased glucose clearance and at 4 months of age there was a clear increase in glucose clearance from peripheral blood in the myopathy mice. This was confirmed by *in vitro* glucose uptake measurements in isolated EDL and soleus muscles of myopathy and control mice isolated at 4 months of age. Glucose uptake was increased in resting and insulin-stimulated EDL muscle of myopathy mice in comparison to control mice. However, no difference in glucose uptake in soleus muscle was observed. In EDL muscle of myopathy mice we found decreased levels of glycogen and phosphocreatine. Metabolic stress, like muscle contraction or hypoxia, is known to lead to decreased glycogen and phosphocreatine, which in turn can activate AMPK and stimulate glucose uptake via increased translocation of GLUT4 to the plasma membrane.

Real-time PCR revealed no increase in total levels of GLUT4 transcripts in skeletal muscle of myopathy mice, which was confirmed by Western blot analysis of GLUT4 protein levels. There was, however, an increase in total GLUT1 transcripts. In order to investigate whether there could be increased translocation of GLUT4 to the plasma membrane due to increased AMPK activity, we analysed AMPK activities and protein levels. There was no difference in AMPK activity in EDL muscle between the muscle-specific KO mice and control mice. Levels of phosphorylated or unphosphorylated

AMPK protein were also similar. The AMPK activation is not only dependent on phosphorylation, but can also be activated allosterically by AMP or glycogen. AMPK in turn phosphorylates, among other enzymes, acetyl CoA carboxylase (ACC). In our assay it is not possible to measure allosteric AMPK activation and thus we indirectly analysed AMPK activity by determining the phosphorylation state of ACC by Western blots. We detected increased phosphorylation of ACC in the muscle-specific KO mice in comparison to control mice, consistent with an enhanced AMPK activation. The levels of unphosphorylated ACC were similar in muscle-specific KO mice and control mice.

The muscle-specific KO mice have a progressively deteriorating respiratory chain dysfunction in skeletal muscle, they are not insulin resistant but rather have an increased glucose uptake in skeletal muscle from the peripheral blood. Insulin resistance and reduced glucose uptake are characteristics of DM2.

Due to the above findings it is suggested that respiratory chain dysfunction in skeletal muscle is not the primary cause but rather a secondary finding in DM2.

3.3 THE MTDNA MUTATOR MOUSE – LINKING ACCUMULATION OF MTDNA MUTATIONS TO PREMATURE AGEING IN MICE (PAPER III)

Knock-in mice, expressing a proof-reading-deficient version of POLγ, were created in order to investigate the effect of an increased mtDNA mutation load in mice. These mice were generated by substituting the aspartic acid codon (GAC) at position 257 with an alanine codon (GCC) of the exonuclease domain 2 in exon 3 of the POLGA gene encoding the catalytic subunit of POLγ. *In vitro* biochemical analyses of recombinant mtDNA polymerase and mitochondrial extracts confirmed that mutant POLγ had a reduced exonuclease activity and a normal polymerase activity. These mutator mice appeared normal and healthy until the age of 6 months, after which several different phenotypes appeared. These mostly resembled ageing associated phenotypes seen both in mice and humans, such as progressive weight loss, osteoporosis, anaemia, extramedullary haematopoiesis in liver, reduced subcutaneous fat, cardiomyopathy with a mosaic pattern of COX-negative cells and enlarged mitochondria with distorted ultrastructure. The mtDNA mutator mice had a maximum lifespan of 61 weeks of age, whereas the median lifespan was 48 weeks of age. Wild-type mouse strains housed in an animal facility live ~3 years.

The molecular characterization revealed a widespread tissue distribution of a class of shorter mtDNA molecules, up to 12 kb in length in mtDNA mutator mice. The level of full-length mtDNA was 70% of wild-type levels and mtDNA transcript and protein levels were normal, indicating that the reduction in mtDNA did not affect overall expression of mtDNA. The cytochrome *b* gene and the non-coding control region was amplified by polymerase chain reaction (PCR) from wild-type and mtDNA mutator mice brain, heart and liver at 8 and 25 weeks of age. Cloning and sequencing of these products revealed an ~ 3-5 times increase of somatic mtDNA mutation load in the mtDNA mutator samples in comparison to wild-type mice. Already at 8 weeks of age there was a substantial increase in mtDNA mutations followed by a small additional increase between 8 and 25 weeks, suggesting that the majority of mtDNA mutations were generated at an early stage in mouse development. Further, all codon positions were affected equally, indicating that there were no mutational hotspots or codon bias.

Parallel to the increased mutation load in the heart of mtDNA mutator mice, respiratory chain activities in complexes containing mitochondrially encoded subunits progressively decreased with age, suggesting a causal link between mutation load and respiratory chain dysfunction.

The above data are thus fully consistent with a link between increased mtDNA mutations, respiratory chain deficiency and phenotypes associated with ageing in healthy mammals. Loss of vital cells, in which mtDNA mutations have accumulated beyond a critical threshold, may be the critical process that manifests as premature ageing and reduced lifespan. In a similar model presented elsewhere the authors identified signs of increased apoptosis and suggest that this was the central mechanism leading to tissue dysfunction and ageing phenotypes (Kujoth et al., 2005). However, recently published data, based on a new technique for estimating mutation frequencies that minimizes the risk of counting PCR-induced mutations, challenges the importance of mtDNA mutations in ageing (Bielas and Loeb, 2005). In this study wild-type and heterozygote mtDNA mutator mice were analysed, and heterozygote mice were found to carry a substantial amount of mutations already at 2.5 months of age in comparison to wild-type. The authors claimed that ageing is not associated with accumulation of mtDNA mutations, since the heterozygote mice never developed any phenotype or exhibited shorter lifespan despite the finding of a 500-fold higher mutation burden in these mice in comparison to wild-type mice (Vermulst et al., 2007). However, this method is based on the mutation frequency at a specific four basepair endonuclease restriction site, which, when mutated, is not cleaved and therefore can be amplified by

PCR. Although elegant, this method does not distinguish between unique mutations and the clonal expansion of a single mutational event. Thus, the authors counted every positive PCR amplification as a unique mutation, ignoring the possibility of clonal expansion and therefore most likely overestimating the mutation frequency of POLγ in their assay. The questionable interpretation of the authors' results is supported by the observation that, when sequencing mtDNA from tissue homogenates of mtDNA mutators, no individual mutations can be detected on the electropherogram, since an approx. 30% mutation load is required. However, in single cells, selected from a tissue section of heart from mutator mice, mtDNA mutations can be readily detected by sequencing, demonstrating that clonal expansion of individual mutations has occurred in single cells (unpublished data).

Additionally, the detection threshold of the method used has not been identified, questioning whether a linear detection of heteroplasmy can be assumed and thus underestimating the mutation frequency in wild-type samples.

It is thus still under debate whether accumulation of mtDNA mutations can be causative or part of the ageing process in mammals. However, taking into consideration the complexity of ageing, involving several systems and processes, it is likely that there will be several different mechanisms involved in driving the ageing process; mitochondrial dysfunction due to mtDNA mutations could be one of them.

3.4 TESTING THE MITOCHONDRIAL THEORY OF AGEING – ACCUMULATION OF SOMATIC MTDNA MUTATIONS IS NOT ACCOMPANIED BY AN INCREASE IN ROS PRODUCTION (PAPER IV)

The mitochondrial theory of ageing proposes that ROS is a major player in the ageing process. With the generation of the mtDNA mutator mice that accumulate somatic mtDNA mutations and develop premature ageing we were further able to investigate whether ROS and ROS-induced damage are important causative factors behind these phenotypes. The mitochondrial theory of ageing predicts that levels of mtDNA mutations should increase exponentially as a consequence of a vicious cycle by accelerating oxidative stress. Sequencing of the cytochrome *b* gene and the non-coding control region in tissues from mouse embryos at day 13.5 and 25 and 40 week old mice, demonstrated that the level of mutations were high in mtDNA mutators already at E 13.5 with 7.8±0.4 mutations/10 kb in comparison to 1.5±0.9/10 kb in wild-type embryos. In contradiction with the predicted exponential increase, mtDNA mutations

were found to increase in a linear manner after E13.5, reaching 15.7±1.8/10 kb in mtDNA mutator mice at the age of 40 weeks. Respiratory chain function as determined by polarographic measurements of O_2 consumption was severely reduced in isolated mutator mouse embryonic fibroblasts (MEFs) in comparison to wild-type MEFs. Considering the high mutation burden already at E13.5 together with the compromised respiration found in mutator MEFs, ROS levels should be high already at this time point if they are the causative factor. $O_2\bullet^-$ and/or $OH\bullet$ oxidizes dihydroethidium to fluorescent ethidium, which can readily be detected by FACS sorting. No difference in the capacity of mutator MEFs versus wild-type MEFs to oxidize dihydroethidium was found. Similar results were obtained, when using a carboxy-H2DCFDA probe that is oxidized into fluorescent DCF in the presence of particularly H_2O_2. Even in immortalised MEFs, where enough cell divisions would have taken place for mutations to accumulate, no difference in production of $O_2\bullet^-$ and H_2O_2 could be observed, when comparing mutator to wild-type MEFs. However, ROS production could still cause a problem if mutator MEFs were more sensitive to ROS and therefore more prone to go into apoptosis even though levels were indistinguishable between mutator and wild-type MEFs. Mutator and wild-type MEFs were treated with increasing levels of H_2O_2 in the cell media and incubated with the fluorescently labelled apoptotic marker annexin V. MEFs were then evaluated for viability by FACS sorting and there was no difference between wild-type and mutator MEFs in the fraction of apoptotic or necrotic cells.

In addition to these ROS measurements, ROS induced antioxidant defences and protein damage were analysed. As a marker of proteins damaged by oxidative stress, we measured the level of protein carbonyls in liver and heart of 40-week-old mutator and wild-type mice and found no difference between the groups in mitochondrial protein extracts of heart and liver. However, we did observe an increase in protein carbonyls in total liver extracts of mutator mice, which must be due to extra-mitochondrial protein carbonylation. No difference in aconitase activity was observed in the mutator mice at 12-, 25- and 40 weeks of age in comparison to age matched wild-type controls, further strengthening the absence of increased oxidative damage to proteins. Finally, increased ROS production could potentially lead to the induction of antioxidant defences and thereby making the ROS levels appear normal. The expression levels of SOD2 and GPX were normal in mtDNA mutator hearts at 40 weeks of age.

Therefore, in our model, which displays a progressive accumulation of somatic mtDNA mutations, resulting in a shortened lifespan and the development of a number of

phenotypes previously reported in ageing mice and humans, we found no indication of increased ROS production. Similar observations have been made in the mutator mouse model by Kujoth and colleagues (Kujoth et al., 2005), and it is therefore tempting to propose that ROS and oxidative damage are unlikely to be major pathophysiological events in the development of premature ageing in the mutator mice.

4 CONCLUDING REMARKS

The central function of mitochondria is to generate energy for the cell. Thus, mitochondrial dysfunction in disease has been proposed to lead to energy deficiency and a wide range of clinical symptoms. Our myopathy mice developed a skeletal muscle phenotype strikingly similar to mitochondrial myopathies seen in patients. Surprisingly, our data suggests that reduced muscle force might not primarily be caused by an ATP deficiency. Rather, it proposes that respiratory chain deficiency in skeletal muscle induces mitochondrial biogenesis partially compensating the loss of ATP. Therefore, the phenotype in the myopathy mice might be caused by some other factor, such as aberrant calcium signalling due to altered calcium storage capacity of the mitochondria or increased ROS production. Further, we were able to show for the first time in a genetic model that respiratory chain dysfunction in skeletal muscle does not lead to impaired insulin sensitivity, but rather to increased glucose clearance from the peripheral blood. This directly contradicts claims that respiratory chain dysfunction is the underlying cause for insulin resistance in humans. Thus, the increased mitochondrial biogenesis is accompanied by increased glucose uptake, presumably increasing the amount of substrate for ATP production.

Mitochondrial dysfunction due to accumulation of somatic mtDNA mutations has been proposed to cause ageing due to increased ROS production and a subsequent vicious cycle. We generated mice expressing a proof-reading-deficient mtDNA polymerase, which led to an increased accumulation of mtDNA mutations and a premature compilation of phenotypes resembling ageing. These data demonstrate that mtDNA mutations can cause phenotypes associated with ageing in mice, suggesting that mitochondrial dysfunction may be involved in the complex clinical presentation of ageing. However, this accumulation of mtDNA mutations and the progressive respiratory chain deficiency was not associated with increased levels of ROS or oxidatively damaged proteins.

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