The pathophysiology of respiratory chain dysfunction

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Stockholm 2005
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ISBN 91-7140-234-9
Printed by Repro Print AB, Stockholm, Sweden
To my parents
Abstract

Mutations of mitochondrial DNA cause a variety of clinical syndromes. It is unclear whether impaired oxidative phosphorylation on its own is the main cause of pathology or whether other factors such as secondary metabolic alterations, enhanced formation of reactive oxygen species (ROS) and induction of apoptosis also may contribute to the clinical phenotype. This thesis focuses on several topics relevant to the pathophysiology of mitochondrial disease, i.e. a mouse model for mitochondrial diabetes, analyses of ROS formation and cell death in mouse strains with tissue-specific respiratory chain (RC) deficiency and regulation of uncoupling protein (UCP) activity and RC function by superoxide.

We studied the pathogenesis of mitochondrial diabetes by tissue-specific (Cre-loxP mediated) inactivation of the gene encoding the mitochondrial transcription factor A (Tfam) in insulin producing pancreatic β-cells. Inactivation of Tfam resulted in mtDNA depletion, severe RC deficiency and abnormally appearing mitochondria in mutant islets. The β-cell specific Tfam knockout (KO) mice were diabetic with lowered pancreatic insulin release. Studies of isolated islets showed the lowered insulin release was caused by altered Ca\(^{2+}\) signaling. The RC deficient pancreatic β-cells eventually died as concluded from histological studies. This study thus demonstrates 2 phases in the pathogenesis of mitochondrial diabetes. Impaired glucose-stimulated insulin release is observed at an early stage and is later followed by β-cell loss. This study provides the first genetic in vivo evidence for a critical role of the RC in glucose-stimulated insulin release (Paper I).

We next examined whether RC deficiency caused ROS formation and cell death. We found increased cell death in homozygous germline Tfam KO embryos and in mice with tissue-specific inactivation of Tfam in cardiomyocytes and pyramidal forebrain neurons. Tfam KO embryos showed massive induction of apoptosis at embryonic day 9.5, Tfam KO cardiomyocytes showed a moderate increase in apoptosis and Tfam KO in forebrain neurons caused massive cell death. The majority of the neurons affected by the knockout died by necrosis as reflected by absence of apoptosis markers and presence of an inflammatory reaction in brain sections. We found only a moderate induction of antioxidant defenses in the Tfam KO cardiomyocytes and almost no induction in Tfam KO neurons. The activities of several iron-sulphur-cluster enzymes sensitive to ROS damage were essentially
normal in mtDNA-depleted heart and brain cortex. This result suggests that mitochondrial ROS production is normal or only moderately increased in tissues with RC deficiency and that any increase in ROS formation is fully compensated for by induction of antioxidant defenses (Paper II and III).

Superoxide has been reported to activate mitochondrial UCPs thus providing a feedback control to lower generation of superoxide by uncoupling respiration. The superoxide-effect is expected to significantly affect energy metabolism due to widespread tissue distribution of UCPs. We generated transgenic mice harboring P1 artificial chromosomes with the human SOD2 gene to investigate whether superoxide regulates UCP activity and energy expenditure in vivo. The human SOD2 protein was ubiquitously expressed and SOD2 enzyme activities showed an overall increase in transgenic mice. There was a linear correlation between SOD2 enzyme activity and mitochondrial oxidative capacity. Mitochondria with increased SOD2 activity were also resistant to induction of mitochondrial permeability. However, despite these obvious effects on mitochondria, SOD2 overexpressing mice exhibited normal UCP activities and adapted normally to cold. They also displayed normal metabolic rates and no change in mitochondrial mass or mitochondrial gene expression. These results suggest that superoxide does not regulate UCP activity and energy expenditure in vivo (Paper IV).

Keywords: mitochondrial disease, mitochondrial DNA (mtDNA), mitochondrial transcription factor A (Tfam), Cre-loxP, conditional knockout, reactive oxygen species (ROS), superoxide, uncoupling protein (UCP), mitochondrial bioenergetics, energy expenditure

List of publications

This thesis is based on the following publications, which will be referred to in the text by their roman numerals.

I Impaired insulin secretion and β-cell loss in tissue-specific knockout mice with mitochondrial diabetes
Silva JP, Koehler M, Graff C, Oldfors A, Magnuson MA, Berggren PO, Larsson NG.

II Increased *in vivo* apoptosis in cells lacking mitochondrial DNA gene expression
Wang J, Silva JP, Gustafsson CM, Rustin P, Larsson NG.
*Proceedings of the National Academy of Sciences USA* 2001, 98 (7): 4038-4043

III Late-onset corticohippocampal neurodepletion attributable to catastrophic failure of oxidative phosphorylation in MILON mice

IV Superoxide dismutase 2 overexpression: enhanced mitochondrial tolerance but absence of effect on uncoupling protein activity
Manuscript

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<th>Description</th>
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<tbody>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocase</td>
</tr>
<tr>
<td>bp, Kbp, Mbp</td>
<td>base pair, kilo base pair, mega base pair</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CSB</td>
<td>conserved sequence block</td>
</tr>
<tr>
<td>Da, kDa</td>
<td>Dalton, kDa</td>
</tr>
<tr>
<td>DNA pol γ</td>
<td>DNA polymerase γ</td>
</tr>
<tr>
<td>DFF</td>
<td>DNA fragmentation factor</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>EndoG</td>
<td>Endonuclease G</td>
</tr>
<tr>
<td>FAD</td>
<td>flavine-adenine dinucleotide</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HSP</td>
<td>Heavy strand promoter</td>
</tr>
<tr>
<td>IMS</td>
<td>mitochondrial intermembrane space</td>
</tr>
<tr>
<td>LHON</td>
<td>Leber’s hereditary optic neuropathy</td>
</tr>
<tr>
<td>LSP</td>
<td>Light strand promoter</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes</td>
</tr>
<tr>
<td>MERFF</td>
<td>myoclonic epilepsy and ragged-red fibres</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>mtSSB</td>
<td>mitochondrial single stranded protein</td>
</tr>
<tr>
<td>MIM</td>
<td>mitochondrial inner membrane</td>
</tr>
<tr>
<td>MOM</td>
<td>mitochondrial outer membrane</td>
</tr>
<tr>
<td>MM</td>
<td>mitochondrial matrix</td>
</tr>
<tr>
<td>NRF-1/NRF-2</td>
<td>nuclear respiratory factor-1/nuclear respiratory factor-2</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide</td>
</tr>
<tr>
<td>OH⁻</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>PAC</td>
<td>P1 artificial chromosome</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>PPARγ coactivator-1α</td>
</tr>
<tr>
<td>POLRMT</td>
<td>human mtRNA polymerase</td>
</tr>
<tr>
<td>PT</td>
<td>permeability transition</td>
</tr>
<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>Q</td>
<td>ubiquinone</td>
</tr>
<tr>
<td>Q⁻</td>
<td>ubisemiquinone</td>
</tr>
<tr>
<td>QH₂</td>
<td>ubiquinol</td>
</tr>
<tr>
<td>RC</td>
<td>respiratory chain</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STP</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>TAS</td>
<td>termination associated sequence</td>
</tr>
<tr>
<td>TFAM</td>
<td>mitochondrial transcription factor A</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage dependent anion channel</td>
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Background

Mitochondrial Genetics

Mitochondria

Mitochondria exist in most eukaryotic cells and form a dynamic inter-connected, tubular network in which they constantly divide and fuse. Mitochondria consist of two membranes, the outer and inner membrane separated by the intermembrane space (IMS). The interior part of the mitochondria is called the mitochondrial matrix (MM). The mitochondrial inner membrane (MIM) forms invaginations called tubular cristae that extensively increase the surface area of the MIM. The MIM is impermeable to most molecules. Transport of small molecules across the MIM depends on specific carriers. The MIM harbors the respiratory chain (RC) and the adenine nucleotide translocase (ANT) whose main function is to exchange mitochondrial ATP for cytosolic ADP. The mitochondrial outer membrane (MOM) is permeable to solutes up to 1500Da through the voltage dependent anion channel (VDAC). Most of the mitochondrial proteins are nucleus-encoded and imported into mitochondria by a mitochondrial protein import machinery. This import machinery sorts the protein to the corresponding mitochondrial compartment. Mitochondria also have their own genome (mtDNA) encoding several essential subunits of the respiratory chain (RC). Mitochondria have a central role in energy metabolism (Figure 1). They are the main cellular site of ATP generation through the process of oxidative phosphorylation whereby electron transport along the RC is coupled to ADP phosphorylation. Mitochondria harbor the tricarboxylic acid cycle (TCA), a pathway that generates reducing equivalents, NADH and FADH$_2$ to feed electrons to the RC. The TCA cycle is connected to the cytosolic glycolytic pathway and to the mitochondrial fatty acid β-oxidation cycle. The TCA cycle is also a source of amino acid precursors and porphyrin/heme biosyntheses. In specific cell-types, mitochondria are also a site of steroid biosynthesis and carry out some steps of the gluconeogenesis and urea synthesis pathways. Mitochondria also participate in regulation of cellular Ca$^{2+}$ homeostasis. The high Ca$^{2+}$ buffering capacity of mitochondria protects the cell from high cytosolic Ca$^{2+}$ concentrations. Transient cytosolic Ca$^{2+}$ peaks can act as a metabolic signal and activate Ca$^{2+}$ sensitive enzymes of the TCA cycle. The mitochondrial intermembrane space harbors several apoptosis-inducing factors that
become active once released from the mitochondria. Finally, mitochondria are the main cellular source of reactive oxygen species (ROS) that are produced as a byproduct of respiration and may determine the rate of ageing.

**Figure 1** Mitochondria have a central role in energy metabolism. ADP denotes adenosine diphosphate, ATP adenosine triphosphate, ANT adenosine nucleotide translocator, CACT carnithine-acyl carnitine translocase, CPT-I/-II Carnithine palmitoyltransferase-I/-II, CoQ coenzyme Q, carnithine palmitoyltransferase DIC dicarboxylate carrier, TCA tricarboxylic acid cycle, ETF electron transfer-flavoprotein, ETF-DH electron transfer-dehydrogenase, I complex I, II complex II, III, complex III, IV complex IV (DiMauro and Schon, 2003).
Evolution of Mitochondria

Mitochondria probably originate from a single ancient invasion of an Archea-type host by a \( \alpha \)-proteobacterium-like ancestor over 2 billion years ago. The symbiosis between the host and the proto-mitochondrial ancestor was probably driven by metabolic requirements. One theory assumes the host was a methanogenic archaean that associated with a methanotropic proteobacterium to obtain essential compounds, for example hydrogen (Martin and Muller, 1998), about 2.7 billion years ago under anaerobic conditions well before the rise in atmospheric oxygen tension. A second theory proposes that symbiosis was driven by the rise in atmospheric oxygen levels caused by photosynthetic cyanobacteria 2.2 billion years ago. Anaerobic life forms were exposed to the toxicity of oxygen and adopted \( \alpha \)-proteobacteria to detoxify oxygen by respiration. The genome sequence of the \( \alpha \)-proteobacterium *Rickettsia prowazeckii* is the most mitochondria-like genome. The genome of *Rickettsia prowazeckii* has a size of 1.1Mbp and encodes 834 proteins. It contains a complete set of genes for aerobic respiration, ATP production and ATP transport functions (Andersson et al., 1998). The mitochondrial genome (mtDNA) has the same fundamental role in all eukaryotes. It encodes a limited number of components of the RC, several ribosomal RNAs and a full or partial complement of tRNAs. MtDNA exhibits remarkable variation in conformation, size and actual gene content. MtDNA molecules are circular but linear mtDNAs exist as well. Mitochondrial genome sizes range from less than 6kbp (Plasmodium falciparum) to over 200kbp in land plants. Contemporary mitochondrial genomes contain 3 to 67 protein-coding genes which is much less than the 834 proteins encoded by the genome of *Rickettsia prowazeckii*. The evolution of the mitochondrial proteome displays both, reductive and expansive processes: (i) Many ancestral mitochondrial genes have been lost. The function of these genes are no longer required because these genes were needed by the \( \alpha \)-proteobacterium as a free-living organism, for example genes for bacterial cell wall synthesis; (ii) the coding sequences of a small number of mitochondrial proteins have been transferred to the nucleus; (iii) the largest number of mitochondrial proteins have no bacterial or archeal orthologues and have been adopted by the mitochondrion after the endosymbiotic event. They are encoded by the nuclear genome and imported into mitochondria (Andersson et al., 2003). Gene transfer from the mitochondrion to the nucleus is an ongoing process in eukaryotes, for example in angiosperms (Adams and
Palmer, 2003) and yeast (Thorsness and Fox, 1990). Numerous examples of mitochondrial pseudogenes have been documented in the nucleus of humans (Woischnik and Moraes, 2002). Furthermore, some nuclear genes appear to derive from edited mitochondrial transcripts (Nugent and Palmer, 1991) suggesting both, RNA- and DNA–mediated mechanisms that drive genome transfer and reduction. Nucleic acids could escape from the mitochondrion during mitochondrial fusion/fission or following damage to mitochondrial membranes (Adams and Palmer, 2003).

Several hypotheses have been proposed why mitochondria retain genes (Adams and Palmer, 2003). Firstly, some highly hydrophobic proteins may be difficult to import across the mitochondrial membranes and to sort to the correct location (Popot and de Vitry, 1990). The only two protein genes contained in every completely sequenced mitochondrial genome – cox1 and cob – encode the most hydrophobic proteins present in mitochondria (Claros et al., 1995). Secondly, mtDNA encoded protein genes may be toxic in the cytosol (Martin and Schnarrenberger, 1997). However this would only account for the cox1 and cob proteins that have been retained in all mitochondrial genomes. Thirdly, expression of genes in the mitochondrion may allow for quick and direct regulation by the redox state of the mitochondrion (Allen, 1993). However, there is no experimental evidence for this assumption. Fourthly, the use of the non-standard genetic code in the mitochondria prevents further gene transfer to the nucleus. In this context it is noteworthy that animal mitochondrial protein-coding gene content is almost completely constant at 13 genes (Boore, 1999).

**Regulation of mitochondrial fusion and fission**

Mitochondria divide, grow and segregate independently from the cell cycle through mitochondrial fission and fusion mechanisms (Osteryoung and Nunnari, 2003). A group of proteins referred to as dynamin related proteins (DRPs) regulate mitochondrial fission. DRPs form a group of self-assembling GTPases. The mitochondrial fission DRPs in yeast, Dnm1, and its homolog in higher eukaryotes, Drp1, self assemble to form punctate structures that associate with the cytosolic face of the MOM at sites of mitochondrial constriction and fission. At least two more proteins interact with self-assembled Dnm1 structures, the integral MOM protein Fis1.
and Mdv1, a peripheral MOM protein. Fis1 targets assembled Dnm1 structures to the MOM and an interaction between Fis1 and Mdv1 transmits a signal for mitochondrial membrane fission by Dnm1. Fis1 homologs have been identified in animals and structural data suggest mouse Fis1 acts as an adaptor molecule similar to yeast Fis1. Mdv1 homologs have not been identified yet in mammals. All components required for mitochondrial fission to date associate with the outer membrane and it is at present unclear how fission of the outer and inner membrane is coordinated and what components of the inner membrane are involved in this process (Shaw and Nunnari, 2002).

The coordinated fusion of the mitochondrial outer and inner membranes is regulated by a group of proteins called the fuzzy onions (Fzo)/mitofusin (Mfn) family of mitochondrial outer membrane GTPases (Shaw and Nunnari, 2002). Yeast cells possess one Fzo-related gene, Fzo-1, that plays a direct role in mitochondrial fusion. In mouse and humans, two Mfn genes, Mfn1 and Mfn2, have been identified that are ubiquitously expressed and required for mouse embryonic development (Chen et al., 2003). In yeast, Fzo-1 interacts physically with the integral MOM protein Ugo-1 and the IMS protein Mgm1 but the exact nature of their interactions and their specific roles in mitochondrial fusion are largely unknown. Mutations of the human homologue of Mgm1, Opa1, result in autosomal dominant optic atrophy and cause defects in mitochondrial morphology (Alexander et al., 2000; Delettre et al., 2000).

Mitochondrial fission may allow segregation of mitochondria by transmission of their membranes and genomes without need for de novo synthesis and the exchange of mtDNA molecules between mitochondria. Mitochondrial fission may also participate in organelle remodelling during apoptosis as suggested by the colocalization of the pro-apoptotic BAX protein with assembled Drp1 structures on the MOM (Newmeyer and Ferguson-Miller, 2003).

**Structure, gene content and organization of mammalian mtDNA**

The structure, gene content and organization of mammalian mtDNA is strongly conserved (Fernandez-Silva et al., 2003). Human mtDNA is a double-stranded (ds) closed-circular molecule of about 16.6kb length (Figure 2). In most cells it represents about 0.5-1% of the total cellular DNA content. MtDNA is thought to be normally
present as a supercoiled molecule. In yeast, mtDNA is organized as supramolecular structures called nucleoids. Yeast nucleoids contain several (3-4) mtDNA molecules, about 10-20 different polypeptides (Kaufman et al., 2000; Miyakawa et al., 1987) and are associated with mitochondrial membranes (Hobbs et al., 2001). Very little is known about the organization of mtDNA molecules in mammalian cells. In most cells it forms monomeric closed double-stranded circles. In quiescent cultured cells, catenated structures have been observed. Studies indicate an association between mtDNA and the mitochondrial inner membrane in vertebrate cells (Albring et al., 1977).

Figure 2 The gene organization in human mitochondrial DNA showing the 13 protein coding genes (ND1-ND6, COXI-III, ATP6 and 8), the 22 tRNA genes (three letter amino acid symbols) and 2 rRNA genes (12S and 16S). The D-loop region controls replication and transcription of mtDNA. O_H denotes the origin of H-strand replication and O_L the origin of light strand replication (DiMauro and Schon, 2003).
The two strands of mtDNA are called the heavy (H-) and the light (L-) strand because they can be separated in density gradients due to their different Guanosine plus Thymidine content. A high proportion of mtDNA molecules in a metabolically active cell contain a triple-stranded structure called the displacement loop (D-loop) (Figure 3). In the D-loop, a nascent H-strand of about 700-800nt length, called 7S DNA, is annealed to the parental L-strand. The D-loop is about 1kb long in humans. It is the major non-coding segment comprising the origin of replication of the H-strand (O_H) and the promoters for H- and L-strand transcription. The D-loop also contains conserved sequences called CSB (conserved sequence blocks) and TAS (termination associated sequences). The D-loop sequence is also the most variable sequence between different species. The second non-coding region is around 30nt long and about two-thirds of the entire mtDNA molecule away from the O_H. It contains the origin of replication for the L-strand (O_L) and is located inside a tRNA cluster.

Figure 3 The D-loop region controls replication and transcription of mtDNA. L denotes the light strand promoter, H1 the Heavy strand promoter 1, H2 the Heavy strand promoter 2, Phe the tRNA^{Phe} gene, and Leu the tRNA^{Leu} gene, mtTFA the mitochondrial transcription factor A, TFB1M/TFB2M the mitochondrial transcription factor B1 and B2, mTERF the transcription termination factor, mtRNA pol the mitochondrial RNA polymerase (Fernandez-Silva et al., 2003).

Mammalian mtDNA encodes 37 genes, of these 22 are tRNA genes, 2 are rRNA genes (16S rRNA and 12S rRNA), and 13 are mRNAs encoding critical subunits of the RC (Figure 2). MtDNA encodes seven subunits of complex I (ND1-ND6 and ND4L), one subunit of complex III (cytochrome b), three subunits of
complex IV (COXI, COXII, and COXIII) and two subunits of complex V (ATP 6 and ATP 8). Of note, complex II is completely nucleus-encoded. The genes are asymmetrically distributed in mtDNA: The H-strand encodes 2 rRNA, 14 tRNAs, 12 mRNAs. The L-strand encodes the remaining 8 tRNAs and 1 mRNA (ND6).

Mammalian mtDNA has a very compact gene organization consisting of closely packed, intron-less genes. All the coding sequences are contiguous to each other or separated by a few bases. Some of the protein genes overlap (ATP 6 and ATP 8 by 46 nucleotides in humans; ND4 and ND4L by 7 nucleotides in humans). In several cases part of the termination codons are not encoded in mtDNA but are generated by the posttranscriptional polyadenylation of the corresponding mRNA (Ojala et al., 1981). The genetic code used for translation of genes encoded by mtDNA shows differences to the universal genetic code, for example in mammalian mitochondria, UGA specifies tryptophan instead of a termination codon.

Transcription of mammalian mtDNA

Transcription of human mtDNA starts at 3 different promoters in the D-loop region (Figure 3), one in the L-strand (Light strand promoter; LSP) and two in the H-strand (Heavy strand promoter 1 and 2; HSP₁ and HSP₂) (Bogenhagen et al., 1984; Montoya et al., 1982). Transcription from HSP₁ starts 19nt upstream of the tRNA^{Phe} gene and yields the two rRNAs (12S and 16S rRNA), tRNA^{Phe} and tRNA^{Val} (Figure 2 and 3). Transcription from HSP₁ is subject to transcription termination immediately downstream of the 16S rRNA gene inside the gene for tRNA^{Leu} (Kruse et al., 1989; Montoya et al., 1983). Transcription from HSP₂ starts close to the 12S rRNA 5’-end and originates an almost full-genomic length polycistronic transcript of the H-strand that is further processed to release individual mRNAs and tRNAs. Transcription from HSP₂ operates 20 times less frequent compared with transcription from HSP₁. The presence of two transcription units, originating at HSP₁ and HSP₂, suggests a differential regulation of rRNA versus mRNA transcription (Montoya et al., 1983). Transcription from LSP results in a single polycistronic full-length genomic transcript from which the eight tRNAs and the ND6 mRNA derive.

HSP₁ and LSP have bipartite structure. Both consist of a 15bp consensus sequence surrounding the transcription start site and of an element immediately upstream of the initiation site (-15 to -39bp) containing a binding site for the
mitochondrial transcription factor A (TFAM). The third promoter, HSP₂, has only limited similarity to the 15bp consensus sequence and lacks the upstream TFAM binding site. Transcription termination at the 3’end of the 16S rRNA gene (following initiation at HSP₁) is dependent on a specific tridecamer sequence in the tRNA^{Leu} gene and a specific protein factor, mTERF (Christianson and Clayton, 1988; Hess et al., 1991). The termination promoting activity of mTERF is bidirectional (Shang and Clayton, 1994) and therefore it could also act to terminate L-strand transcription. The primary polycistronic transcripts originating from LSP and HSP₂ are endonucleolytically cleaved (Doersen et al., 1985; Montoya et al., 1983; Ojala et al., 1981). tRNA sequences located between each rRNA and mRNA (Figure 2) probably act as signal for the processing enzymes after acquiring a cloverleaf structure on the nascent RNA chain. The 5’ endonucleolytic cleavage is probably mediated by mitochondrial RNAse P (Doersen et al., 1985) or other unidentified RNases. Polyadenylation of the rRNAs and mRNAs occurs during or immediately after cleavage of the primary transcript by a mitochondrial poly (A) polymerase (Amalric et al., 1978).

Transcription of human mtDNA requires an organelle-specific RNA polymerase, human mtRNA polymerase (POLRMT). POLRMT is homologous to phage T7 RNA polymerase (Tiranti et al., 1997). Recombinant POLRMT together with recombinant TFAM and either recombinant human mitochondrial transcription factor B1 (TFB1M) or B2 (TFB2M) initiate transcription in vitro from an mtDNA template containing the mitochondrial promoters HSP₁ or LSP. TFB1M and TFB2M are not needed for transcription elongation (Falkenberg et al., 2002). TFB2M has at least two orders of magnitude more activity than TFB1M. TFB1M and TFB2M interact with POLRMT in a 1:1 ratio suggesting heterodimerization between POLRMT and TFB1M or TFB2M. TFB1M and TFB2M were identified through database homology searches for the yeast transcription factor Sc-mtTFB. Sc-mtTFB binds to the catalytic subunit of yeast mtRNA polymerase, Rpo41p, to confer specific promoter binding.

The rRNAs are methylated and contain a short poly(A) tail of 1-10 residues (Dubin et al., 1982). Mitochondrial tRNAs are smaller, have some structural differences and additional functions in replication and transcription regulation compared to nucleus-encoded tRNAs. Mitochondrial mRNAs start directly at the
initiation codon or have an extremely short untranslated 5’end (of 1-3nt) and have a poly(A) tail of about 55 residues immediately after the stop codon. Thus, they lack the typical features of cytosolic mRNAs, for example 5’ and 3’ untranslated regions, 3’ polyadenylation signal or the 5’ end cap structure (Montoya et al., 1981).

Replication of mammalian mtDNA

MtDNA replicates independently from the cell cycle phase and from nuclear DNA replication (Bogenhagen and Clayton, 1977). Two models have been proposed for mtDNA replication: (i) the strand-displacement and (ii) the strand-coupled replication model. The strand displacement model involves two unidirectional, independent origins. Replication starts at O_H located downstream of the LSP in the D-loop region (Figure 3) and proceeds along the parental L-strand producing a daughter H-strand. When H-strand replication reaches the O_L (Figure 2), the replication site of the L-strand is exposed and replication of the light strand in the opposite direction starts. H-strand replication is dependent on the generation of an RNA primer by transcription from the LSP. Consequently, replication of mtDNA is likely coupled to the mitochondrial transcription machinery, consisting of POLRMT, TFAM and the TFBM factors. The triplicate structure consisting of the newly synthesized RNA strand upstream of O_H, the parental H- and L-strand is called an R-loop. RNA processing activities, probably mediated by RNAse MRP or Endonuclease G (Cote and Ruiz-Carrillo, 1993), cut the RNA strand generating the mature RNA primer. The mtDNA polymerase (DNA pol γ) starts H-strand replication through extension of the RNA primer (Xu and Clayton, 1996). The R-loop is processed at the CSB I-III. The CSB are D-loop sequences of extreme sequence conservation between human, rat and mouse and are located downstream of the LSP and upstream of O_H (Figure 3). A major H-strand replication initiation point is almost always found near CSB I (Walberg and Clayton, 1983). After replication of the H-strand has started, most of the nascent DNA strand is arrested around the TAS (termination associated sequence) creating the 7S DNA strand and the characteristic triple stranded structure of the D-loop. At a much lower frequency however, replication proceeds over the entire length of the genome. The function of the 7S DNA strand and the mechanisms that
determine whether elongation proceeds beyond TAS are unknown. The initiation for replication of the L-strand at O_L occurs in a small non-coding region that is thought to assume a stable stem-loop structure once it is exposed as a single strand. Initiation of L-strand replication requires the activity of a specific primase to generate a short RNA primer with 5’ends mapping onto a T-rich portion of the loop in the stem-loop structure. Once initiation has started, elongation proceeds until completion of L-strand duplication (Wong and Clayton, 1985). The whole process of replication has been estimated to last 1 h implying a polymerization rate of 270nt/min (Graves et al., 1998). After completion of the synthesis of both strands, RNA primers must be removed and the gaps filled and ligated. The closed circular mtDNA adopts its tertiary structure following introduction of supercoils and interaction with DNA binding proteins.

The second model, the strand-coupled replication model, has been proposed to exist along with the strand-displacement model and corresponds to coupled leading and lagging strand synthesis of mtDNA (Holt et al., 2000).

MtDNA polymerase, DNA pol γ, is composed of a catalytic α-subunit and a smaller β-subunit. The β-subunit is needed for processivity and primer recognition. DNA pol γ has a 3’-5’ exonuclease activity in the α-subunit (Longley et al., 1998). DNA pol γ requires an ATP-dependent mitochondrial helicase for unwinding mtDNA called Twinkle. Twinkle has a helicase domain that shares homology to the helicase domain of phage T7 primase/helicase. Replication of mtDNA requires mitochondrial single stranded (ss) binding protein (mtSSB) to stabilize single stranded DNA during replication and to increase the activity and fidelity of DNA pol γ (Farr et al., 1999; Hoke et al., 1990). A minimal mammalian mtDNA replisome has been reconstituted consisting of DNA pol γ, mtSSB and Twinkle in recombinant form. In combination, recombinant DNA pol γ and Twinkle form a processive replication machinery that can use dsDNA as a template to synthesize ssDNA of about 2 kb. Addition of recombinant mtSSB stimulates the reaction further generating DNA products of about 16kb. The observed DNA synthesis rate in this in vitro system is 180bp/min corresponding closely to the previously reported in vivo value of 270bp/min. The functional interaction between Twinkle and DNA pol γ explains why mutations in these two proteins cause an identical human disease, autosomal dominant external ophtalmoplegia (Korhonen et al., 2004)
Mitochondrial transcription factor A (TFAM)

TFAM is a nucleus encoded transcription factor of 24kDa. TFAM belongs to a group of small nuclear DNA binding proteins, the nuclear high mobility group box proteins (HMG box proteins). TFAM contains two high mobility group domains (HMG box) that confer DNA binding. TFAM has the ability to wrap, unbend and unwind the DNA (Fisher et al., 1992). The C-terminal tail is essential for the activation of mitochondrial transcription (Dairaghi et al., 1995). TFAM is required for mitochondrial transcription initiation (Falkenberg et al., 2002) and likely for mtDNA replication because an RNA primer must be synthesized to initiate H-strand replication. TFAM has a leader peptide sequence for protein import into the mitochondria.

TFAM appears to have a second, structural role besides its function as a transcription initiation factor. It appears that TFAM is regularly spaced bound to the D-loop probably allowing other transcription factors to interact with their target sequences (Ghivizzani et al., 1994). TFAM levels exceed the levels that would be expected for a transcription factor with regulatory functions. It has been proposed to have a histone-like function covering the entire mtDNA molecule (Fisher et al., 1992; Ghivizzani et al., 1994). In this sense, TFAM homologues of yeast, ABF2, and of Xenopus, wrap entirely the mtDNA molecule (Shen and Bogenhagen, 2001). In Xenopus, the TFAM homologue has been found to bind to mtDNA as a tetramer (Antoshechkin et al., 1997).

The yeast homologue of TFAM is ABF2p (sc-mtTFA). ABF2p also bends and introduces negative supercoils in DNA (Fisher et al., 1992) and functions as a histone-like protein with DNA packaging properties (Megraw and Chae, 1993). However, ABF2p lacks the C-terminal domain of TFAM and has only a minor effect on transcription (Xu and Clayton, 1992). Yeast strains lacking ABF2p lose mtDNA when grown on glucose indicating that ABF2p has a role in mtDNA maintenance. TFAM when expressed in an ABF2p deficient yeast strain can complement ABF2p function by partially rescuing the growth and respiratory defects of this strain (Parisi et al., 1993).

The level of TFAM is lower in cells depleted of mtDNA after treatment with Ethidium bromide and higher in tissues from patients with increased levels of mtDNA.
TFAM levels may follow the mtDNA levels by a feedback regulation or may be more stable in the presence of DNA targets. Heterozygous \textit{Tfam} KO mice have reduced mtDNA copy number and homozygous \textit{Tfam} KO embryos completely lack mtDNA and die at embryonic day 8.5-10.5 (Larsson et al., 1998) demonstrating that Tfam is required for mtDNA maintenance \textit{in vivo}. Overexpression of the human TFAM protein upregulates mtDNA copy number in mice (Ekstrand et al., 2004). TFAM likely controls mtDNA copy number by activating L-strand transcription and primer generation for H-strand replication (Ekstrand et al., 2004). TFAM may also enhance mtDNA stability by acting as a histone-like protein.

\textbf{Transmission and segregation of mammalian mtDNA}

The mtDNA copy number spans usually a range of 200-5000 in different cell types (Ekstrand et al., 2004)(Bogenhagen and Clayton, 1974; Robin and Wong, 1988; Veltri et al., 1990). Each mitochondrion contains several copies of mtDNA. In a given cell, all mtDNA copies are presumably identical, a condition called homoplasmy. Mutations of mtDNA can coexist with wild-type mtDNA and this condition is called heteroplasmy. As a consequence of this, the fraction of mutated mtDNA molecules has to reach a certain percentage, in order to cause disease. This usually happens when the fraction of mutated mtDNA is higher than 60-80\% (Lightowlers et al., 1997).

The mitochondrial genome is maternally inherited. The few mitochondria from the sperm cell that enter the oocyte during fertilization are actively eliminated by a ubiquitin-dependent mechanism (Sutovsky and Schatten, 2000). In some rare cases, paternal mtDNA can escape the mechanism of active elimination and be transmitted to the muscle tissues of affected patients (Schwartz and Vissing, 2002). The mature oocyte contains $10^5$ mtDNA molecules. The sperm cell has about $10^2$ copies and there is some evidence that downregulation of TFAM contributes to the observed downregulation of mtDNA copy number during spermatogenesis (Larsson et al., 1997b; Rantanen et al., 2001). MtDNA undergoes a bottleneck phenomenon during oogenesis by which a small subset of mtDNA molecules are amplified and transmitted to the offspring (Marchington et al., 1998). There is little mitochondrial proliferation despite massive cell proliferation between the primordial germ cell in the 3 week-old
fetus and the diplotene primary oocyte in the 20 week-old fetus resulting in a mitochondrial bottleneck (Poulton and Marchington, 2002). The reduction in mtDNA copy number is followed by amplification and selection of mtDNA later during oogenesis. This phenomenon probably explains the shift towards homoplasmy within one or a few generations. It appears that the number of transmitted mtDNA molecules (transmission units) is between 1-200 in the female germ line (Howell et al., 2000; Jenuth et al., 1997).

Studies of heteroplasmic mice demonstrate random genetic drift in some tissues but in others strong, tissue-specific and age-related, directional selection for different mtDNA genotypes (Jenuth et al., 1996; Jenuth et al., 1997). This trait showed linkage to loci on several chromosomes providing evidence for nuclear control of mtDNA segregation (Battersby et al., 2003).

Regulation of mitochondrial gene expression and biogenesis

Different cell-types have a rather constant mtDNA copy number (Moraes, 2001) and the ratio between mtDNA copy number and total DNA is cell-type specific (Shay et al., 1990). The mtDNA copy number in mammalian cells harboring wild-type mtDNA, mtDNA with partial deletions or mtDNA with partial duplications is inversely proportional to the size of the respective mtDNA suggesting that cells maintain a constant mass of mtDNA rather than a constant copy number of mitochondrial genomes (Tang et al., 2000). The above study also reported a correlation between mtDNA copy number and the levels of mtDNA-encoded RNA and polypeptides indicating that mtDNA copy number affects mtDNA expression (Tang et al., 2000). Genetic studies have addressed the role of transcription and replication factors in the regulation of mtDNA copy number. Homozygous Tfam KO mice display severe mtDNA depletion demonstrating the importance of Tfam for mtDNA maintenance (Larsson et al., 1998). Overexpression of human TFAM in transgenic mice upregulates mtDNA copy number without affecting RC function and mitochondrial mass, suggesting that regulation of mtDNA copy number can be dissociated from regulation of mtDNA expression and mitochondrial biogenesis (Ekstrand et al., 2004). Studies regarding the regulation of mtDNA transcription have been carried out in isolated mitochondria and have revealed some degree of autonomous regulation. External signals, for example a change in ATP concentration
or treatment with thyroid hormone activate mtDNA transcription. This may be relevant in the local (subcellular) tuning of mitochondria (Enriquez et al., 1999a; Enriquez et al., 1999b; Enriquez et al., 1996). However, no evidence has been provided yet that thyroid hormone receptors can use the mitochondrial transcriptional machinery to direct mitochondrial gene expression.

The vast majority of proteins required for mitochondrial biogenesis are nucleus-encoded including: (i) most of the about 100 subunits of the RC; (ii) the metabolic enzymes needed for fatty acid β-oxidation, the TCA cycle, the biosyntheses of certain amino acids and of heme; (iii) mitochondrial protein import and assembly factors; (iv) factors of the mitochondrial replication, transcription and translation machinery. These genes are under transcriptional control of the nuclear respiratory factors-1 (NRF-1) and -2 (NRF-2), thyroide hormone T3, stimulation protein 1 (Sp1), and others (Kelly and Scarpulla, 2004). NRF-1 and -2 regulate the expression of most nucleus-encoded subunits of all respiratory complexes, mtDNA transcription and replication factors, mitochondrial enzymes of the heme biosynthetic pathway, mitochondrial protein import and assembly factors, and ion channels of the MOM. The expression of the human TFAM and TFB1M/TFB2M genes was found to rely on functional NRF-1 and NRF-2 consensus sites in their promoters suggesting a regulatory link between nuclear and mitochondrial gene expression in mitochondrial biogenesis (Kelly and Scarpulla, 2004; Virbasius and Scarpulla, 1994). However, this contrasts with the presence of NRF-2 but absence of obvious NRF-1 sites in the human TFB1M/TFB2M promoters and rodent Tfam and Tfb1m/Tfb2m promoters (Larsson et al., 1997a; Rantanen et al., 2001) (Rantanen et al., 2003).

The transcriptional coactivator PPARγ coactivator-1α (PGC-1α) was cloned in a yeast two hybrid screen for brown adipose-specific factors that interacted with the adipogenic nuclear receptor PPARγ (Puigserver et al., 1998) and has proven to be a key regulator of mitochondrial biogenesis in various tissues. Forced expression of PGC-1α in adipogenic and myogenic mammalian cell lines induces the expression of NRF-1, NRF-2 and Tfam. PGC-1α can interact directly with NRF-1 to activate the mouse Tfam gene promoter (Wu et al., 1999). Overexpression of PGC-1α in primary cardiac myocytes and in hearts of transgenic mice upregulates the expression of genes involved in mitochondrial fatty acid β-oxidation (Lehman et al., 2000). Forced expression of PGC-1α in skeletal muscle of transgenic mice drives mitochondrial
proliferation and the formation of mitochondrial-rich type I, oxidative (slow-twitch) muscle fibres (Lin et al., 2002). PGC-1α interacts with a variety of nuclear-receptors and non-nuclear receptors (Kelly and Scarpulla, 2004). Several upstream signaling events activate PGC-1α and mitochondrial biogenesis in a tissue-specific manner. Upon cold exposure, the β-adrenergic/c-AMP pathway activates PGC1α- and UCP1-expression in brown adipose tissue (Puigserver et al., 1998). Exercise stimulates PGC-1α gene expression in skeletal muscle. Forced overexpression of Calcium/calmodulin-dependent protein kinase (CaMK) or of PGC-1α in skeletal muscle activates mitochondrial biogenesis (Lin et al., 2002; Wu et al., 2002). Overexpression of CaMK and calcineurin A induces PGC-1α expression (Handschin et al., 2003; Wu et al., 2002) suggesting that CaMK and calcineurin A are upstream of PGC-1α. Nitric oxide (NO) can activate PGC-1α and mitochondrial biogenesis in adipocytes and HeLa cells. The NO effect is dependent on cGMP activation and does not result from inhibition of oxidative phosphorylation by NO (Nisoli et al., 2003).

Mitochondrial bioenergetics and ROS formation

The respiratory chain
The RC consists of five distinct complexes (Complex I-V) that are bound to the mitochondrial inner membrane. Electrons are fed to Complex I or II and transferred to Complex III and IV. During the passage of electrons a proton gradient across the inner membrane is built up by proton translocation at Complex I, III and IV. The proton gradient is then used by Complex V to synthesize ATP from ADP and phosphate (Figure 4). This general mechanistic principle of oxidative phosphorylation explaining the coupling between respiration and ATP synthesis in mitochondria is referred to as the chemiosmotic theory and was proposed by Peter Mitchell in 1961 (Mitchell, 1961). Recent observations suggest that the different respiratory complexes are organized in supercomplexes (Schagger and Pfeiffer, 2000). The crystal structures of Complex II-V have been elucidated to some extent and important mechanistic conclusions have been drawn (Saraste, 1999).
Figure 4 The respiratory chain consists of 5 complexes, designated Complex I-V. Complex II does not translocate protons and is completely nucleus-encoded. Reprinted with permission from (Saraste, 1999). © AAAS.

Complex I (NADH:ubiquinone oxidoreductase) is the largest of all complexes, consisting of at least 46 subunits, one flavin mononucleotide (FMN), seven or eight different iron-sulfur centers, covalently bound lipids and at least 3 bound ubiquinol molecules. Seven subunits (ND1-ND6, ND4L) are encoded by mtDNA. Two electrons from NADH enter Complex I. The electrons are transferred along an electron transport chain consisting of FMN, iron-sulfur centers and bound ubiquinones to a mobile ubiquinone (Q) that takes up electrons to yield ubiquinol (QH$_2$) (Stryer, 1995). The flow of 2 electrons from NADH to ubiquinone leads to the pumping of 4 protons from the mitochondrial matrix side to the IMS. Electron microscopy (EM) studies demonstrated that Complex I has an L-shaped structure with two major domains separated by a thin collar (Grigorieff, 1998; Guenebaut et al., 1998).

Complex II, succinate:ubiquinone reductase (SQR) or succinate dehydrogenase (SDH), is completely nucleus-encoded and a component of the TCA cycle. It catalyzes the conversion of succinate to fumarate using FAD (flavine-adenine dinucleotide) as a cofactor. This reaction yields FADH$_2$. FADH$_2$ donates 2 electrons that are transferred to Q along several iron-sulfur centers. Mammalian mitochondrial and many bacterial SQR are composed of two hydrophilic subunits, a flavoprotein (SdhA) and iron-sulfur protein (SdhB), and two hydrophobic membrane anchor subunits, SdhC and SdhD (Figure 6). The crystal structure of *E.coli* SQR has recently been determined (Yankovskaya et al., 2003). SQR is composed of a trimer. Each monomer consists of one SdhA, SdhB, SdhC and SdhD subunit. The SdhA subunit contains the FAD cofactor and the substrate-binding site. The SdhB subunit contains
three iron-sulfur clusters for electron transfer from the substrate-binding site to the ubiquinone-binding site. The SdhC and SdhD subunits form a membrane-bound cytochrome b containing the ubiquinone-binding site that is sandwiched between the SdhC and SdhD subunits (Figure 5). The residues in the ubiquinone-binding site are strictly conserved among human, mouse and E.coli SQR. Importantly, mutations of the residues in the ubiquinone-binding site are associated with hereditary paraganglioma (Astuti et al., 2001a; Astuti et al., 2001b) and premature ageing in C. elegans mev-1 mutant (Ishii et al., 1998). The observed increase in ROS levels in the C. elegans mev-1 mutant is explained by leakage of electrons that are released from succinate but not accepted by ubiquinone because of the dysfunctional binding site.

Figure 5 Three-dimensional structure of E.coli SQR. SQR is a trimer viewed parallel to the plasma membrane (top left), viewed from the cytoplasm normal to the plasma membrane (top right) or viewed as a monomer parallel to the membrane (bottom). One SQR monomer is composed of 2 hydrophilic subunits, SdhA and SdhB, and 2 hydrophobic subunits, SdhC and SdhD that are buried in the plasma membrane and provide the ubiquinone binding site. Reprinted with permission from (Yankovskaya et al., 2003). © AAAS.
Similarly, it has been proposed that mutations of the SdhB, SdhC and SdhD subunits associated with hereditary paraganglioma cause ROS formation and thereby predispose to cancer (Rustin et al., 2002).

Complex III (cytochrome $bc_1$) delivers electrons from $QH_2$ to cytochrome $c$. The transfer of electrons is coupled to the transfer of protons across the inner membrane by the so-called Q cycle (Figure 6). The mammalian Complex III is a dimer and each monomer contains eleven subunits but only three of them carry the redox centers for electron transfer. These key subunits are: (i) cytochrome $b$, the only mtDNA encoded subunit of Complex III; (ii) a membrane-anchored iron-sulphur protein (ISP) carrying a Rieske-type center ($\text{Fe}_2\text{S}_2$); and (iii) a membrane anchored cytochrome $c_1$. There are two active sites in Complex III (Iwata et al., 1998; Xia et al., 1997; Zhang et al., 1998b): one for oxidation of $QH_2$ and release of protons on the outer surface of the membrane, $Q_o$; and one for the reduction of $Q$ coupled to the uptake of protons from the inner side of the membrane, $Q$. In the first step of the Q cycle, one ubiquinol donates one electron to the to the Rieske-type iron-sulphur center and one electron to the $b_L$ heme group of the cytochrome $b$ subunit. The donation of both electrons is coupled to the release of two protons to the intermembrane space at the $Q_o$ site. The first electron passes from the Rieske-type iron-sulphur center to the heme $c$ group of cytochrome $c_1$ and from there to cytochrome $c$. The second electron passes from the $b_L$ heme to the $b_H$ heme group of cytochrome $b$ and is then transferred back to a new incoming ubiquinone molecule at the $Q_i$ site of cytochrome $b$. A subsequent Q cycle provides a second electron at the $Q_i$ site to reduce ubisemiquinone ($Q^\cdot$) to $QH_2$ that can again enter the Q cycle.

Complex IV (cytochrome $c$ oxidase) contains 13 subunits (Figure 7). The three major subunits are encoded for by mtDNA (COXI-III) and form the functional core of complex IV. This core is surrounded by 10 smaller nucleus-encoded subunits. Subunit II receives electrons from cytochrome $c$. These electrons are first transferred to cytochrome $a$ in subunit I and then to the bimetallic cytochrome $a_i$/Cu$_b$ active site in subunit I. Two hydrophilic channels, called D and K, connect the active site to the aqueous phase of the mitochondrial matrix. The reduction of oxygen at the active site in subunit I is linked to the translocation of four protons, two for the reduction of
Figure 6 Structure of the bovine mitochondrial cytochrome $bc_1$ and the Q cycle. Cytochrome $bc_1$ is a dimer and each monomer consists of 11 subunits (left). The functional core of the enzyme consists of three subunits: cytochrome $b$, Rieske ISP and cytochrome $c_1$ (middle). In the Q cycle, bifurcation of electron transfer occurs at the Q$_o$ site. One electron is transferred to the Fe$_2$S$_2$ center of Rieske ISP and from there to cytochrome $c_1$. The second electron is transferred to the heme $b_L$ and from there to the heme $b_H$ of cytochrome $b$ and delivered back to a new incoming ubiquinone at the Q$_i$ site. Reprinted with permission from (Saraste, 1999). © AAAS.

Figure 7 Structure of the bovine cytochrome $c$ oxidase. The functional core of the enzyme consists of subunits I-III (COXI-III) that are mtDNA encoded. Subunits I and II contain the metal centers and the active site (cytochrome $a_3/Cu_B$) resides in subunit I. Protons that are used for reducing oxygen to water or pumped to the IMS are transferred through two channels, D and K, from the matrix side. Reprinted with permission from (Saraste, 1999). © AAAS.
oxygen into water, and two for release into the intermembrane space (Tsukihara et al., 1995; Tsukihara et al., 1996; Yoshikawa et al., 1998).

Complex V \((F_1F_0\text{ATPase})\) synthesizes ATP using a proton-motive force across the inner mitochondrial membrane, but it can also hydrolyze ATP to pump protons against an electrochemical gradient. The bovine enzyme appears to contain 16 subunits (Lutter et al., 1993). It consists of a membrane bound part containing a proton channel, called \(F_o\), and of a catalytic part located in the matrix, called \(F_1\), which contains an ATP synthesizing or hydrolyzing activity (Figure 8). MtDNA encodes 2 subunits of the \(F_o\) part. The \(F_o\) and \(F_1\) parts are connected by two parallel structures, referred to as the “rotor” and the “stator” (Elston et al., 1998). The \(F_1\) part can be detached from the \(F_o\) part and acts as a soluble ATPase. The \(F_1\) part consists of 5 subunits \((\alpha, \beta, \gamma, \delta, \varepsilon)\). The \(\beta\) subunit contains the catalytic site and there are three active sites per one \(F_1F_0\text{ATPase}\) because each \(F_1\) part contains three \(\beta\) subunits. Each catalytic site passes through a cycle of open (unbound) state, loose (bound ADP and phosphate) state and tight (tightly bound ATP) state. The formation of ATP requires energy for substrate binding and ATP release but not for the phosphorylation reaction itself. The crystal structure of the \(F_1\) part of the bovine \(F_1F_0\text{ATPase}\) indicates that it operates as a rotational catalyst (Abrahams et al., 1994). A central structure inside the

![Structure of ATP synthase](image)

**Figure 8** Structure of ATP synthase. The \(F_1\) part corresponds to the crystallized bovine ATP synthase (Abrahams et al., 1994) and the remaining parts to the crystal structure of the bacterial ATP synthase (Lutter et al., 1993). A dodecamer of subunit \(c\), the \(\varepsilon\) and \(\gamma\) subunits form the rotor, while subunits \(b\), \(d\) and \(\delta\) form the stator. The active sites are present in the \(\beta\) subunits. Reprinted with permission from (Saraste, 1999). © AAAS.
F₁ ATPase rotates in 120° steps during catalysis (Noji et al., 1997; Sabbert et al., 1996; Yasuda et al., 1998). The membrane bound Fₒ part consists of three subunits called a, b and c. The subunit c forms a dodecamer and is thought to constitute the rotor together with the γ and ε subunits of the F₁ part (Figure 8). The subunit a and b form the stator together with the δ subunit of the F₁ part. The mechanism how ATP synthesis is coupled to proton transfer across the inner membrane has not been clarified yet. The current model proposes that proton movement through the interface between a-subunit and the subunit c dodecamer (Figure 8) causes the rotor and stator to move in opposite directions thereby causing a torque. The subsequent release of free energy by the torque may allow substrate binding and release of ATP, respectively (Elston et al., 1998; Wang and Oster, 1998). Complex V has recently been ascribed a role in the biogenesis of the mitochondrial inner membrane (Paumard et al., 2002).

**Generation of ROS by the respiratory chain**

O₂⁻ is constantly produced in respiring mitochondria at a rate of about 1-3% of all oxygen consumed (Boveris and Chance, 1973). O₂⁻ dismutates spontaneously to H₂O₂ and this reaction is accelerated by superoxide dismutase (SOD). H₂O₂ can react with reduced transition metals, for example with Fe²⁺ in the Fenton reaction (Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + OH⁻), and give rise to the highly reactive hydroxyl radical (OH⁻). O₂⁻, H₂O₂ and OH⁻ are collectively referred to as reactive oxygen species (ROS).

It is difficult to determine the exact site of O₂⁻ generation because O₂⁻ has a short lifetime due to its rapid conversion to H₂O₂ by SOD or because it immediately reacts with lipids of the mitochondrial inner membrane. H₂O₂ is more stable than O₂⁻ and can diffuse through the membrane lipid bilayer out of the mitochondrion. Most of the O₂⁻ is probably produced at the matrix side of the inner mitochondrial membrane because O₂⁻ generation is only found in submitochondrial particles, which are inside out with respect to mitochondria while in intact mitochondria only H₂O₂ is detectable extramitochondrially. However, a recent study using spin traps revealed O₂⁻ formation in mitoplasts (mitochondria devoid of the outer membrane) (Han et al., 2001).

O₂⁻ generation depends on whether mitochondria are actively respiring (low O₂⁻ production) or the RC is highly reduced (high O₂⁻ production). The rate of O₂⁻
generation increases when electron flow slows down or the concentration of oxygen increases. The proton gradient built up during respiration is dissipated through complex V to synthesize ATP. In the absence of complex V activity, for example following inhibition by oligomycin, the proton gradient builds up massively increasing the mitochondrial membrane potential and causing electron flow to slow down and the RC to become more reduced. This results in increased steady-state concentration of $O_2^\cdot$ (Boveris et al., 1972). Inhibitors of the RC result in $O_2^\cdot$ generation due to increased reduction of the carriers upstream of the site of inhibition and have revealed specific sites of $O_2^\cdot$ formation within complex I, II, and III. In complex III, $O_2^\cdot$ is formed when the electron flow between the $b_L$ and $b_H$ heme of cytochrome $b$ is blocked, for example by antimycin A (Figure 9). Reoxidation of ubisemiquinone ($Q^\cdot$) to ubiquinone ($Q$) at the outer side of the membrane and reduction of $Q^\cdot$ to ubiquinol ($QH_2$) at the inner side of the membrane are inhibited, leading to increased steady-state concentrations of $Q^\cdot$ (Figure 9). $O_2^\cdot$ formation by

**Figure 9** The Q cycle in complex III. Ubiquinone (Q) is reduced to ubiquinol (QH$_2$) either by electron transfer from Complex I or Complex II or by electrons transferred from cytochrome $b$ of complex III. QH$_2$ releases one electron to the Rieske ISP, cytochrome c1 (c1) and cytochrome c (c). The second electron is released at Q$_o$ from a ubisemiquinone ($Q^\cdot$) and transfers to the heme groups of cytochrome $b$ to reduce a new Q at the Q$_i$ site. Superoxide is probably produced at the two sites, Q$_o$ and Q$_i$, but the contribution of each site has not been clearly determined yet, Antimycin A blocks electron transfer between $b_L$ and $b_H$ heme of cytochrome $b$. 
antimycin A is blocked when electron flow between the Rieske protein and cytochrome c1 or oxygen is inhibited, for example by myxathiazol or cyanide, indicating that O$_2$· formation depends on generation of Q· by the Q-cycle (Figure 9) (Trumpower, 1990; Turrens, 2003; Turrens et al., 1985). In Complex I, O$_2$· production likely occurs in one of the iron-sulphur clusters, either N1 or N2 upstream of the binding site for ubiquinone and rotenone (Genova et al., 2001; Kushnareva et al., 2002). In Complex II, mutations affecting the ubiquinone binding site result in electron leakage and increased O$_2$· formation (Yankovskaya et al., 2003). Reduced FAD was also identified as an electron donor to oxygen in reconstituted complex II (Zhang et al., 1998a).

**Antioxidant defense mechanisms**

Antioxidant defenses are classified into non-enzymatic and enzymatic defenses. The non-enzymatic defenses are compounds that reduce oxidizing agents, for example the lipid soluble vitamin E and ubiquinone, or the water-soluble vitamin C, glutathione, uric acid and ceruloplasmin (Halliwell and Gutteridge, 1989).

The superoxide dismutases (SOD) are the first line of enzymatic antioxidant defense. SOD catalyze the reaction O$_2$· + O$_2$· $\rightarrow$ H$_2$O$_2$ + O$_2$. All members of the SOD family utilize different transition metals at their active sites. There are three different SOD isoforms (Fridovich, 1995). SOD1 (Cu/ZnSOD) is expressed in the cytosol and in the mitochondrial intermembrane space (Okado-Matsumoto and Fridovich, 2001). SOD2 (MnSOD) is expressed in the mitochondrial matrix and is closely related to the bacterial MnSOD. The expression of SOD2 is induced by agents that cause oxidative stress, for example radiation and hyperoxia through oxidative activation of the transcription factor NFκB and by various cytokines, for example TNFα, IL-1 and IFN-γ (Li and Karin, 1999). SOD2 is transported into the mitochondrial matrix where it assembles into an active homo-tetramer (Fridovich, 1995). Hydrogen peroxide, the product of the dismutation reaction is reduced to water and oxygen mainly by glutathione peroxidases and to a lesser extent by catalases. Glutathione peroxidases utilize glutathione (GSH) as a reductant according to the following reaction: H$_2$O$_2$ + 2 GSH $\rightarrow$ 2H$_2$O + GSSG. There are four GPx isoforms. The major GPx isoform, GPx1, is expressed in mitochondria and cytosol of most tissues (Esposito et al., 2000; Frampton et al., 1987). GPx2 is found in plasma
Glutathione is a tri-peptide consisting of L-\(\gamma\)-glutamyl-L-cysteinyl-glycine. Each GSH molecule provides one reducing equivalent to the conversion of \(H_2O_2\) into water and oxygen by the sulphydryl moiety of the cysteine residue leading to the formation of oxidized GSH in form of the disulfide-bonded compound GS-SG. The enzyme glutathione reductase utilizes NADPH to re-reduce one molecule of GS-SG into two molecules of GSH: 
\[
GS-SG + 2NADPH \rightarrow 2GSH + 2 NADP^+ 
\]

Catalases are almost exclusively found in peroxisomes to remove \(H_2O_2\) formed during \(\beta\)-oxidation of long chain fatty acids. Catalases have only been detected in heart mitochondria but not in other mitochondria (Radi et al., 1991).

Mitochondrial uncoupling may provide another line of anti-oxidant defense (Skulachev, 1996). Uncoupling decreases the mitochondrial membrane potential, leading to increased respiration, oxidation of the RC and lowered \(O_2^-\) formation. A threshold membrane potential exists below which no ROS is formed (Korshunov et al., 1997). In rat hepatocytes, the futile cycle of proton pumping and proton leak may account for 20-25% of respiration. In perfused rat muscle, this value is increased to 35% in contracting and 50% in resting muscle. Mitochondrial uncoupling proteins (UCP) have been proposed to regulate \(O_2^-\) formation and there is evidence that \(O_2^-\) directly activates UCP (Brand, 2000).

Damage repair and removal systems have been documented for oxidized proteins (Davies, 2000). Cysteine residues can form a disulfide bond on the same protein or a disulfide crosslink between two different proteins upon oxidation. The re-reduction is performed by disulfide reductases. Oxidized proteins can be also be recognized by proteases and completely degraded into amino acids. In the cytoplasm and the nucleus of eukaryotic cells the oxidized proteins can be recognized and degraded by the proteasome complex. In mammalian mitochondria, there is a separate set of proteases that conduct the degradation of oxidized proteins, for example LON protease (Bota and Davies, 2002).

Membrane phospholipids undergo lipid peroxidation upon oxidative stress (Halliwell and Gutteridge, 1989). Lipid peroxidation is the result of a chain reaction: It is initiated by extraction of a hydrogen atom from an unsaturated fatty acyl, for
example by OH· (O₂· is not reactive enough to induce lipid peroxidation by itself) giving rise to a lipid radical (L·). L· then reacts with oxygen producing a lipid peroxy radical (LOO·). LOO· can further propagate the peroxidation chain reaction by extracting a hydrogen atom from another unsaturated fatty acid. The lipid hydroperoxides (LOOH) can easily decompose into other reactive species. Peroxidized membranes become rigid, loose selective permeability, and can even loose their integrity. Water-soluble lipid peroxidation products, such as dialdehydes, can diffuse from membranes into other subcellular compartments and cause protein aggregation leading to formation of the age pigment lipofuscin. Lipid peroxidation products can also impair enzyme function or may form DNA adducts and induce mutations. Oxidized lipid bilayers become more susceptible to the action of phospholipases, which hydrolyze the phospholipid glycerol backbone to release a free fatty acid hydroperoxide. Fatty acid hydroperoxides are detoxified by glutathione peroxidases reducing them to their corresponding hydroxy fatty acid (Davies, 2000).

Oxidative damage of DNA interferes with DNA replication and transcription. The extent of oxidative DNA damage has been estimated to be as high as 1 base modification per 130,000 bases in nuclear DNA. Damage to mtDNA is estimated to be even higher at 1 per 8,000 bases. Oxidants can give rise to strand breaks (single and double) by damaging the phosphodiester backbone, sister chromatid exchange, DNA-DNA and DNA-protein crosslinks, and base modifications. The base modifications can result in dissociation of the base from the DNA double strand, creating so called apurinic/apyrimidinic sites. Several DNA repair enzyme systems exist that can repair oxidatively damaged DNA (Davies, 2000).

Adaptive responses of cells to oxidative stress involves transient growth-arrest to enable DNA damage repair and expression of factors involved in damage removal/repair and antioxidant defenses, for example catalase, GPx and SOD2, mediated by several transcription factors (Shull et al., 1991) (Davies, 2000).

Cells exposed to oxidative stress may ultimately undergo apoptosis or necrosis. The mechanism involves activation of the mitochondrial permeability transition pore and cytochrome c release (see below).
Mitochondrial uncoupling proteins and their role in energy expenditure and control of ROS homeostasis

The mitochondrial uncoupling proteins (UCP) belong to the large family of anion carriers of the mitochondrial inner membrane containing six transmembrane domains (Ricquier and Bouillaud, 2000). The first identified uncoupling protein, UCP1, is specifically expressed in brown adipocytes. Based on cDNA sequence similarity, two homologues of UCP1 were identified and named UCP2 (Fleury et al., 1997) and UCP3 (Boss et al., 1997; Gong et al., 1997; Vidal-Puig et al., 1997). UCP2 and UCP3 share, respectively, 72 and 57% amino acid identity with UCP1. Of note, UCP2 and UCP3 are adjacent genes on human chromosome 11 and mouse chromosome 7. UCP2 mRNA is found almost ubiquitously, but the protein has been identified only in some organs and cell types including spleen, lung, stomach, brain, kidney, thymocytes and pancreatic β-cells (Pecqueur et al., 2001; Zhang et al., 2001). UCP3 is mainly expressed in skeletal muscle. UCP1 KO mice are cold sensitive but not obese and an extension of these studies demonstrated that UCP1 is the only effector of adaptive non-shivering thermogenesis to cold exposure (Enerback et al., 1997; Golozoubova et al., 2001). UCP1 creates a proton leak across the inner membrane of brown fat mitochondria to dissipate the electrochemical proton gradient into heat. Brown adipose tissue (BAT) is a particular form of adipose tissue required for thermogenesis in infants at birth or rodents exposed to cold. Brown adipocytes differ from white adipocytes by direct sympathetic innervation, a central nucleus, and numerous mitochondria. Sympathetic innervation of brown adipocytes induces lipolysis, respiration and thermogenesis, accompanied by a marked increase in blood flow and heat conductance to other organs by the blood stream. UCP1 is abundantly expressed in brown adipocytes accounting for up to 4% of the total and 8% of the mitochondrial protein content upon cold acclimation. UCP1 expression is induced by the Norepinephrine/cAMP-pathway leading to the activation of cAMP response elements (CRE) in the UCP1 promoter. Various transcription factors, i.e. PPARα, PPARγ, and thyroid hormone T3 bind to a complex enhancer region upstream of the UCP1 promoter and can stimulate UCP1 expression by co-activation with PGC-1α, but the precise roles of these factors in the regulation of UCP1 expression have not been clarified yet (Cannon and Nedergaard, 2004). Reconstituted UCP1 exhibits fatty-acid dependent proton translocating activity (Klingenberg et al., 2001). The proton
translocation activity of native UCP1 is stimulated by fatty acids and inhibited by purine nucleotides, such as GDP, in a competitive manner (Shabalina et al., 2004). Of note, fatty acids are obligatory for uncoupling. There are currently two competing models explaining the proton location mechanism of UCP. In the first model, UCP1 is a pure proton carrier (Klingenberg and Echtay, 2001). In the second model, UCP1 is an anionic fatty acid exporter: protonated fatty acids move from the external to the internal lipid layer of the mitochondrial inner membrane by a flip-flop mechanism, release a proton into the matrix and then return as an anionic fatty acid to the cytosol by UCP-mediated export (Garlid et al., 1998).

UCP2 and UCP3 were proposed to work as uncouplers similar to UCP1. Polymorphic markers encompassing the UCP2-UCP3 locus show genetic linkage to the resting metabolic rate (Bouchard et al., 1997). Similarly, polymorphisms in the coding region of the UCP2 gene are linked to the metabolic rate at sleep (Walder et al., 1998). However, homozygous UCP2 and UCP3 KO mice have normal metabolic rates (Arsenijevic et al., 2000; Vidal-Puig et al., 2000) but compound homozygous UCP2/UCP3 KO mice have not been analyzed yet due to linkage of the UCP2 and UCP3 genes. UCP2 and UCP3 KO mice show signs indicative of increased ROS production (Arsenijevic et al., 2000; Vidal-Puig et al., 2000) supporting the hypothesis that UCP2 and UCP3 function as uncouplers to lower mitochondrial ROS formation. Consistent with this hypothesis, skeletal muscle mitochondria from UCP3 KO mice show improved respiratory control ratios (Vidal-Puig et al., 2000) and enhanced in vivo ATP production rates (Cline et al., 2001) indicative of improved coupling. UCP2 and UCP3 have been reconstituted into liposomes and both exhibit proton translocation activity that requires the presence of fatty acids and is inhibited by GDP (Jaburek et al., 1999; Jezek et al., 2004). A recent report demonstrated superoxide–mediated activation of UCP1, UCP2 and UCP3. This mechanism was proposed as a feedback control to lower excessive \( O_2^- \) production by uncoupling (Echtay et al., 2002b). Superoxide–mediated activation of UCP is fatty-acid dependent and inhibited by purine nucleotides (Echtay et al., 2002b). The superoxide-activation of UCP2 occurs from the matrix side (Echtay et al., 2002a). This finding has potentially important consequences for energy metabolism due to the widespread tissue distribution of UCPs and their suggested role as uncouplers. The hypothesis that \( O_2^- \) activates UCPs however is not universally accepted (Couplan et al., 2002).
The experiments leading to the conclusion that superoxide activates UCP were conducted on isolated mitochondria exposed to supraphysiological levels of exogenous superoxide (Echtay et al., 2002a; Echtay et al., 2002b) and only one study that used forced, adenovirus-mediated SOD2 overexpression in islet cells has indicated that superoxide-stimulated UCP-mediated uncoupling may also occur in vivo (Krauss et al., 2003).

High fat diet of human subjects does not change mitochondrial coupling despite clear upregulation of UCP3 protein content in muscle (Hesselink et al., 2003a). It has been proposed that UCP3 functions as an anionic fatty acid exporter to prevent accumulation of free fatty acids inside the mitochondria that cannot enter the mitochondrial fatty acid β-oxidation cycle. Accumulation of anionic free fatty acids causes mitochondrial dysfunction, a phenomenon called lipotoxicity. This role of UCP3 is consistent with: (i) upregulation of UCP3 expression by conditions increasing circulating FFA (fasting, high fat diet, diabetes, and obesity); (ii) an inverse relationship between mitochondrial fat oxidation capacity and UCP3 protein levels (low fat oxidation capacity in untrained individuals correlates with high UCP3 protein content; high fat oxidation capacity in trained individuals correlates with low UCP3 protein levels) (Hesselink et al., 2003b).

Mitochondrial regulation of apoptosis

Apoptosis is a distinct genetic and biochemical pathway required for normal embryonic development and maintenance of normal tissue homeostasis. Apoptosis was initially defined as a morphological entity. The morphological hallmarks of apoptosis are cell shrinkage, condensation and fragmentation of the cell at later stages followed by phagocytosis of the cell remnants by neighbouring cells. Apoptosis is an active process that does not cause inflammation. In contrast to apoptosis, necrosis is a passive process caused by massive cellular injury. The cell swells because it is unable to maintain ion homeostasis, leading to rupture and release of intracellular contents causing inflammation (Kerr et al., 1972). Mitochondria are key regulators of apoptosis because many factors activating apoptosis are kept in the mitochondrial intermembrane space (IMS) and become active when released into the cytosol. These pro-apoptotic factors include cytochrome c, apoptosis-inducing factor (AIF), SMAC/DIABLO, and Endonuclease G (Endo G) among others. Release of pro-
apoptotic factors from the mitochondrial IMS is regulated to prevent accidental activation of apoptosis. The pro-apoptotic factors are impermeable to the mitochondrial outer membrane. At least three distinct release mechanisms have been proposed:

(i) The existence of a proteinaceous permeability transition pore (PTP) has been postulated at contact sites between the inner and the outer mitochondrial membrane (Figure 10) (Susin et al., 1998). The PTP is proposingly formed by the adenine nucleotide translocator (ANT) and cyclophyllin D at the inner membrane associated with VDAC and the peripheral benzodiazepine receptor in the MOM. Sustained opening of the PTP results in osmotic equilibration of ions between matrix and cytosol, dissipation of the mitochondrial inner membrane potential and mitochondrial swelling, a biophysical state called permeability transition (PT). The MOM ruptures because the MOM cannot expand as much as the highly invaginated MIM. Several triggers of PT have been described, notably lowered mitochondrial membrane potential, Ca$^{2+}$, fatty acids, ROS hyperproduction, NAD(P)H depletion, Nitric oxide (NO) and ATP depletion. Cyclosporin blocks opening of the PTP, while atractyloside activates PTP opening. Interactions between BCL-2 protein family members, BAX and BCL-2 (see below), modulate the opening of the postulated PTP pore. However, gene knockout studies do not support a requirement for ANT, one of the postulated components of the PTP, for cells to undergo PT (Kokoszka et al., 2004). Furthermore, the importance of the PTP has been questioned in the majority

![Figure 10](image)

*Figure 10* A putative model of the mitochondrial permeability transition pore (Green and Reed, 1998).
of cell deaths because protein release can often occur in the absence of any of the hallmarksof PT (Bossy-Wetzel et al., 1998; Newmeyer and Ferguson-Miller, 2003).

(ii) Members of the BCL2-family form channels in the MOM that allow passage of large macromolecules (Danial and Korsmeyer, 2004). The proteins of the BCL-2 family are structurally classified according to the presence of four conserved regions called the BCL-2 homology (BH) 1 - 4 domains. Anti-apoptotic BCL-2 family proteins (BCL-2, BCL-xL, A1 and BCL-W) contain all BH1-4 domains. Pro-apoptotic BCL-2 family proteins can be subdivided into members containing the BH1 - 3 domains (BAX, BAK) or the BH3 domain only (BID, BAD, BIM, Noxa, Puma). Cell line studies showed that the pro-apoptotic BCL-2 family members, BAX and BAK, present as inactive monomers in unstimulated cells homo-oligomerize to form channels that allow passage of cytochrome c (Desagher et al., 1999; Wei et al., 2000). Homo-oligomerization of recombinant monomeric BAX requires recombinant protease-activated BID (tBID) in the absence of any other protein to permeabilize membrane vesicles, allowing passage of extremely large macromolecules (Kuwana et al., 2002). This model accounts for the fact that most of the cytochrome c release clearly occurs prior to swelling of the mitochondria, and that BAX/BAK compound homozygous KO cells are resistant to most stimuli that activate mitochondrial induction of apoptosis (Lindsten et al., 2000; Wei et al., 2001a). Anti-apoptotic BCL-2 family members, BCL-2 and BCL-xL, bind and sequester pro-apoptotic BCL-2 family members by interaction via their BH3 domain (Cheng et al., 2001; Oltvai et al., 1993). Accordingly, the ratio of anti- to pro-apoptotic molecules is one determinant of susceptibility to apoptosis induction (Oltvai et al., 1993).

(iii) BCL-2 family proteins interact with VDAC in the mitochondrial outer membrane to release pro-apoptotic factors from the IMS. VDAC reconstituted into liposomes mediates passage of cytochrome c. In this model, BAX and BAK stimulate opening of the VDAC while BCL-xL stimulates VDAC closure (Shimizu et al., 1999). However, genetic proof for this model is lacking and genetic studies indicate that one of the three VDAC isoforms, VDAC-2, binds BAK and inhibits BAK oligomerization and BAK-mediated apoptosis (Cheng et al., 2003).

The release of cytochrome c is rapid and the extent almost complete (Goldstein et al., 2000). The minor fraction of the total cytochrome c, about 20%, is located in the very narrow IMS adjunct to the MOM, whereas most of the cytochrome
cytochrome c (about 80%) is stored within involutions of the MIM, so called tubular cristae which is a highly sequestered compartment separated from the more narrow IMS through narrow cristae junction. In one EM study, tBID induced striking remodeling of the tubular cristae, mobilizing the stores of cytochrome c (Scorrano et al., 2002). The redistribution was accompanied by a transient opening of the PTP but not by swelling of the mitochondria. In the opening state of the PTP solutes of up to 1500Da are permeable (Bernardi et al., 1999). Transient PTP opening thus remodells tubular cristae and redistributes the cytochrome c pool.

Upon release from the IMS, cytochrome c binds to Apaf-1 and this complex recruits pro-caspase 9 in the presence of ATP to form the apoptosome (Li et al., 1997; Liu et al., 1996). The binding of cytochrome c and ATP to Apaf-1 results in a conformational change of Apaf-1 that enables Apaf-1 to bind six pro-caspase 9 molecules (Acehan et al., 2002). Caspases are specific proteases that activate effectors of the apoptotic programme (Shi, 2002). Caspases are usually present as inactive zymogens (pro-caspase) possessing a large and small subunit. They cleave specific motifs possessing an aspartate. The cleavage depends on a cysteine and occurs at the aspartate (Asp) site, therefore the name “caspase”. Cleavage of internal specific Asp residues releases one large and one small subunit that dimerize to form the active site of the enzyme. Caspases can be divided into two groups, initiator and effector caspases. Initiator caspases are usually upstream caspases that possess the ability of autocatalytic activation. Downstream effector caspases need initiator caspases for their activation. The formation of the apoptosome enables autolytic activation of pro-caspase 9 into active caspase 9. The binding of six pro-caspase 9 molecules to one Apaf-1 molecule allows for an amplification of the initial apoptotic signal. Activated caspase 9 in turn activates the effector caspases 3 and 7. Effector caspases cleave structural components, for example actin and nuclear lamin, and DNA fragmentation factor 45 (DFF45). Cleavage of DFF45 by caspase 3 releases the DFF45-DFF40 dimer. DFF40 subsequently degrades chromosomes into nucleosomal fragments (Enari et al., 1998; Liu et al., 1997). Mitochondria also release AIF (Susin et al., 1999) and EndoG (Li et al., 2001). AIF and Endo G translocate to the nucleus and induce caspase-independent large-scale DNA fragmentation. Mitochondria also release SMAC/DIABLO, an inhibitor of the inhibitor of apoptosis proteins (IAP) that block effector caspases (Chai et al., 2000; Verhagen et al., 2000).
Apoptosis can be triggered by the activation of cell death receptors on the plasma membrane and can involve mitochondrial release of cytochrome c. The binding of Fas ligand to Fas (receptor for Fas ligand) or of TNFα to the TNFα receptor, results in homo-oligomerization of the receptor, binding of adaptor proteins to the oligomerized receptors and recruitment of pro-caspase 8, an initiator caspase, to the receptor complex (Baud and Karin, 2001) leading to autocatalytic activation of caspase 8. Activated caspase 8 can directly cleave caspase 3 in so-called type-I cells, for example in immune cells. In so called type-II cells, for example in hepatocytes, a mitochondrial amplification step is required for efficient activation of caspase 3 by cell death receptors (Scaffidi et al., 1998). In type-II cells, activated caspase 8 cleaves BID yielding tBID that induces cytochrome c release (Li et al., 1998; Luo et al., 1998) by mediating oligomerization of BAK (Wei et al., 2001a) or BAX (Desagher et al., 1999).

Ca²⁺ can trigger induction of mitochondrial PT and promote apoptosis or necrosis. IP₃-mediated Ca²⁺ release from the endoplasmic reticulum (ER) can cause PT and apoptosis (Szalai et al., 1999). The Ca²⁺ flow between the ER and mitochondria is facilitated by close contact sites (Rizzuto et al., 1998). BCL-2 reduces the amount of Ca²⁺ releasable from the ER and thus prevents mitochondrial Ca²⁺ overload (Vanden Abeele et al., 2002). Massive cytosolic Ca²⁺ overflow can cause necrosis following a wide variety of cellular injuries, for example following excitotoxic stimulation of neurons. It has been demonstrated that glutamate-induced neuronal cell death by stimulation of NMDA receptors requires mitochondrial Ca²⁺ uptake (Stout et al., 1998). Glutamate induces necrosis or apoptosis of neurons in culture dependent on mitochondrial function as reflected by the mitochondrial membrane potential and cellular ATP content (Ankarcrona et al., 1995). The intracellular ATP level determines the cell death fate by apoptosis or necrosis (Eguchi et al., 1997; Leist et al., 1997). Inhibition of oxidative phosphorylation with oligomycin reduces staurosporine (STP) and FAS-stimulated apoptosis of T-lymphocytes and leads to enhanced necrosis (Eguchi et al., 1997; Leist et al., 1997). An ATP depletion of more than 50-70% is required to shift the cell death modus towards necrosis (Leist et al., 1997). Addition of glucose to cells pretreated with oligomycin changes the mode of cell death from necrosis to apoptosis indicating that
glycolytic ATP production may allow execution of apoptosis in cells devoid of oxidative phosphorylation (Leist et al., 1997).

**Free radical theory of aging**

The free radical theory of ageing proposes that mitochondrial ROS generation determines the rate of ageing (Sohal and Weindruch, 1996). This is supposed to result from an imbalance between prooxidants and antioxidants leading to an age-dependent accumulation of oxidative cell-damage that exceeds the capacity of cellular repair systems. This theory is based on the following observations: (i) Variations in longevity among different species inversely correlate with the rates of mitochondrial generation of superoxide and hydrogen peroxide; (ii) Restriction of caloric intake lowers steady-state levels of oxidative stress and damage, retards age-associated changes, and extends the maximum life-span in mammals (Sohal and Weindruch, 1996). ROS may damage subunits of the RC, membrane lipids causing proton leak, and enzymes of the TCA cycle leading to lowered energetic efficiency, cell dysfunction and cell death through activation of the PTP (Figure 11) (Wallace, 1992; Wallace, 2001). It is assumed that chronic ROS formation increases the mutant mtDNA load over time leading to synthesis of defective RC subunits, which in turn increase ROS formation and the mutant mtDNA load further (Figure 11). Correlative

![Figure 11 Free radical theory of ageing. Chronic mitochondrial ROS formation damages the RC and increases the mtDNA mutant load leading to progressive decline of oxidative phosphorylation, and further increases in ROS formation and mtDNA mutations with age. The final outcome is cell dysfunction and cell death.](image-url)
evidence suggests a causative link between ROS and accumulation of mtDNA mutations over time. Prolonged exposure to oxidative stress causes more extensive and persistent damage to mtDNA than to nuclear DNA (Yakes and Van Houten, 1997). The levels of an oxidative DNA modification marker were 10-fold increased in mtDNA compared to nuclear DNA (Bowling et al., 1993). Several reasons have been proposed to contribute to this selective vulnerability: (i) the lack of histones that protect from oxidative DNA damage; (ii) the lack of an efficient repair system in mitochondria; (iii) the compact gene organization of mtDNA increasing the likelihood that a gene is mutated by ROS; (iv) the nearby location of mtDNA to the mitochondrial inner membrane, the major site of ROS production (Shigenaga et al., 1994). MtDNA point mutations and mtDNA deletions have been reported to increase with age (Larsson et al., 1990; Michikawa et al., 1999; Wang et al., 2001b). Complex I-IV deficiency, isolated or in combination was reported along with oxidative damage to mtDNA in the brains of patients with age-associated neurodegeneration, i.e. Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis, and Huntington disease (Kirkinezos and Moraes, 2001). However, a causative relationship between ROS and the accumulation of mtDNA mutations remains to be demonstrated. A proof reading deficient mtDNA polymerase γ knockin mouse that accumulates somatic mtDNA mutations over time develops a premature ageing phenotype (Trifunovic et al., 2004). In C. elegans, mutations have been documented that confer increased resistance against ROS and significant prolongation of lifespan: (i) clock-1 mutants that cannot synthesize ubiquinone and accumulate the biosynthetic precursor demethoxyubiquinone, a more potent anti-oxidant, that can replace Q functionally, at least in part (Lakowski and Hekimi, 1996); (ii) Isp-1 mutants with a mutation in the Rieske iron sulphur protein of Complex III leading to low oxygen consumption (Feng et al., 2001). EUK-8 and EUK-134, antioxidant compounds that act simultaneously as SOD and Catalase mimetic increased lifespan in C. elegans (Melov et al., 2000). Simultaneous overexpression of SOD1 and Catalase prolonged lifespan in Drosophila melanogaster (Orr and Sohal, 1994).
Respiratory chain diseases

Diseases of the respiratory chain (RC) have an incidence of at least 1 in 10’000 live births (Chinnery and Turnbull, 2001). RC diseases are caused by mutations in the nuclear or the mitochondrial genome. MtDNA mutations include point mutations in protein coding genes, tRNA genes, rRNA genes or deletions that usually encompass at least one tRNA gene (Smeitink et al., 2001). Nuclear mutations involve protein subunits of the RC, factors required for the assembly of respiratory complexes and for maintenance of mtDNA. Furthermore there are several nuclear mutations affecting proteins that are important to preserve mitochondrial homoestasis and indirectly affect the RC, for example components of the mitochondrial protein import machinery.

The largest group of patients with a respiratory chain disease display only an enzymatically verified deficiency but no identified mutation (Graff et al., 2002). The same clinical features can be caused by various mutations in mtDNA or nuclear DNA. Conversely, the same genetic defect in mtDNA can lead to different clinical manifestations. The segregation of mutant mtDNA is one key determinant of this variability. It has been demonstrated the percentage of heteroplasmy varies between different individuals, tissues and cells within a patient (Chinnery et al., 2000; Chinnery et al., 1999). However, heteroplasmy is not always sufficient to explain this clinical variability: Firstly, in numerous cases the percentage of mutant mtDNA does not correlate with the clinical phenotype (Zhou et al., 1997). Secondly, symptoms may develop only in specific tissues even when the mtDNA mutation is homoplasmic in all tissues. Thirdly, nuclear mutations affecting one oxidative phosphorylation complex can also cause variable phenotypes or involve selective tissues (DiMauro and Schon, 2003) (Rossignol et al., 2003).

The level of heteroplasmy that causes disease is called the phenotypic threshold (Rossignol et al., 2003). The phenotypic threshold depends on the type of the mutation and can be approximated to >90% for most mutations in protein coding genes, 65%-90% for tRNA gene mutations and 60% for deletions (Hayashi et al., 1991). The phenotypic threshold effect is based on an excess of wild-type mtDNA, mRNA, tRNA, and active RC complexes compared to what is needed for normal respiration. This reserve provides a safety margin against deleterious mutations. The
phenotypic threshold is the sum total of thresholds at the level of transcription, translation, enzyme activity and respiration (Rossignol et al., 2003).

The steady-state transcript level of mtDNA encoded subunits appears to correlate with the mtDNA copy number (D'Aurelio et al., 2001; Tang et al., 2000). Heteroplasmic point mutations of protein coding genes give rise to proportionate amounts of mutated mRNAs (Bai et al., 2000; D'Aurelio et al., 2001), while heteroplasmic mtDNA deletions result in proportionate reductions in mRNA levels of the deleted genes (Hayashi et al., 1991). The mutant tRNAs do not necessarily rise proportionately to the level of heteroplasmy (Chomyn et al., 2000), for example the A3243G tRNA^Leu^ MELAS mutation may affect transcription or be subject to degradation (Jacobs, 2003). A selective upregulation of transcription of wild-type mtDNA over mutant mtDNA has not been observed in heteroplasmic cells (Bai et al., 2000; D'Aurelio et al., 2001) (Hayashi et al., 1991). This implies that the transcriptional threshold is determined by (i) the ratio of mutant to wild-type mtDNA or (ii) by the number of wild-type mtDNA copies. The relationship between mtDNA copy number and steady state transcript levels may however not be straightforward as there are indications for posttranscriptional regulation in mitochondria (Gagliardi et al., 2004). Pathogenic mtDNA mutations or deletions impair translation of wild-type subunits, when the fraction of mutated mRNA (Bai et al., 2000), tRNA (Boulet et al., 1992) or mtDNA deletions (Hayashi et al., 1991) exceeds 40-50%. Again the translational threshold may be influenced by posttranscriptional regulation (Gagliardi et al., 2004). Pathogenic mutations in both mtDNA and nuclear DNA can impair the activity of the respective respiratory complex by (i) decreasing the total amount of assembled active enzyme complexes and/or (ii) by changing the intrinsic kinetic properties of the complex. Significant reductions in the protein levels of different subunits may not result in lowered enzyme activity (Spelbrink et al., 1994; Triepels et al., 2001). In cell lines, a decrease in respiration has been reported following an 85% reduction in Complex I activity (Barrientos and Moraes, 1999), or a 40-80% reduction in Complex IV activity (Davey et al., 1998; Villani and Attardi, 1997), dependent on the cell lines and experimental conditions used. The respiratory threshold can reflect an excess of active respiratory chain complexes, activation of inactive complexes (Grivennikova et al., 2001), recruitment and integration of inactive complexes into respiratory super-complexes (Schagger and Pfeiffer, 2000), or biochemical regulation.
of the kinetic properties of the complex, for example cAMP-mediated phosphorylation of Complex I (Rossignol et al., 2003). The respiratory threshold for a given complex may also relate to inhibition of the metabolic flux upstream of the enzymatic deficiency leading to variations in the concentrations of intermediary metabolites (Rossignol et al., 1999). The transcriptional, translational and respiratory thresholds are clearly tissue-specific (Rossignol et al., 1999) and may explain some of the phenotypic differences caused by mtDNA mutations. Treatment of mitochondrial disease may therefore aim at modulating these threshold (Taylor et al., 2001).

MtDNA mutations in protein coding genes and tRNA genes cause multisystemic disorders, for example MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes) and MERFF (myoclonic epilepsy and ragged red fibres), or present as organ-specific disease, for example mitochondrial diabetes, mitochondrial cardiomyopathy, mitochondrial myopathy, mitochondrial deafness, Leigh syndrome and LHON (Lebers hereditary optic neuropathy).

LHON is usually caused by mutations of the ND1, ND4 and ND6 subunits of Complex I. The mutations are usually homoplasmic. The penetrance is low and an additional X-linked factor has been suggested. Affected young adult patients display a bilateral optic neuropathy with degenerating retinal ganglion cells.

Leigh syndrome is a histopathological entity characterized by bilateral necrosis of the striatum that usually affects infants. Leigh syndrome is caused by mitochondrial or nuclear mutations. The most common mitochondrial mutation involves the ATPase 6 subunit (T8993C). Another mutation at the same site of the ATPase 6 subunit (T8993G) causes a milder phenotype known as the NARP syndrome (neurogenic weakness, ataxia and retinitis pigmentosa) with adult onset. The threshold level to develop symptoms has been found at 80-90% in blood for the T8993C mutation and 60-70% in blood for the T8993G mutation (White et al., 1999).

The most documented mutation leading to MELAS is the A3243G mutation of the tRNA$^{Leu}$ gene, while MERFF most commonly associates with the A8344G tRNA$^{Lys}$ gene mutation. The MELAS and MERFF mutations are usually heteroplasmic. In MERFF, symptoms develop above a mutant load of 90% in muscle (Chinnery et al., 1997). Mutations of tRNA genes usually produce a variety of structural and functional defects that result in impaired aminoacylation, impaired pre-tRNA processing, loss of modifications in the anticodon leading to mistranslations
and lowered translational activity (Yasukawa et al., 2001; Yasukawa et al., 2000) (Jacobs, 2003).

MtDNA deletions are most commonly associated with multi-systemic disorders. MtDNA deletions arise spontaneously, probably somewhere between oogenesis and early embryogenesis, and are rarely transmitted. They are always heteroplasmic and include at least one tRNA gene. The most common deletion (called “common deletion”) is found in one third of all patients carrying mtDNA deletions. Large-scale mtDNA deletions encompassing several tRNA genes cause Pearson or Kearns-Sayre syndrome. Pearson syndrome has an onset early in childhood and is characterized by severe bone marrow deficiency and exocrine pancreatic dysfunction. Children can recover from Pearson syndrome but patients are at risk to develop Kearns-Sayre Syndrome later in life, characterized by ophthalmoplegia, ptosis, retinal degeneration, ataxia, and heart block. An increased level of mutated mtDNA has been found over time in muscle of patients with Kearns-Sayre Syndrome probably representing a mechanism for disease progression (Larsson et al., 1990).

Nuclear mutations cause at most 10% of all known respiratory chain disorders, but they probably represent the majority of all cases with deficient respiratory chain function (Graff et al., 2002). Nuclear gene mutations affect: (i) subunits of the RC, so far identified only in Complex I and II, that can result in Leigh-syndrome or leukodystrophy. Complex II mutations are also associated with hereditary paragangliomas and pheochromocytomas (DiMauro and Schon, 2003; Smeitink et al., 2001); (ii) assembly factors of respiratory chain complexes, so far identified for complex III and IV, and associated with Leigh syndrome (de Lonlay et al., 2001) (Tiranti et al., 1998; Zhu et al., 1998); (iii) proteins regulating the stability of mtDNA leading to mtDNA depletion or mtDNA deletions, for example mitochondrial thymidine kinase 2 (Saada et al., 2001), thymidine phosphorylase (Nishino et al., 1999), adenine nucleotide translocator 1 (Kaukonen et al., 2000), DNA pol γ (Lamantea et al., 2002) and Twinkle (Spelbrink et al., 2001). The affected proteins are required to maintain mitochondrial nucleotide pools or to replicate mtDNA. The clinical hallmark of most of these diseases is ophthalmoplegia.
Mitochondrial diabetes

MtDNA mutations cause approximately 0.5-1% of all types of diabetes mellitus. At least 42 different mtDNA mutations have been associated with diabetes (Mathews and Berdanier, 1998), for example mtDNA rearrangements such as deletions associated with partial mtDNA duplications (Ballinger et al., 1992; Rotig et al., 1992), or mtDNA triplications (Dunbar et al., 1993). The most common mutation reported in patients with mitochondrial diabetes is the mitochondrial A3243G tRNA<sub>Leu</sub> gene mutation (Maassen et al., 2004). The majority of these patients become clinically manifest at the age of 35-40 years. Hearing impairment precedes in most cases the onset of clinically manifest diabetes by several years. The A3243G tRNA<sub>Leu</sub> mutation is generally heteroplasmic and present in all the tissues but the heteroplasmy levels seem to be highest in tissues with low mitogenic activity, for example in muscle. However, additional studies did not identify insulin resistance as a common pathogenic factor in most carriers of the A3243G tRNA<sub>Leu</sub> mutation, although insulin resistance has been reported in some cases. Increased hepatic glucose production may represent another mechanism leading to hyperglycemia but no data are at present available regarding hepatic glucose production in carriers of the A3243G tRNA<sub>Leu</sub> mutation. Diabetic patients with the A3243G tRNA<sub>Leu</sub> mutation exhibit impaired pancreatic insulin secretion in response to glucose stimulation (Velho et al., 1996). Pancreatic mtDNA deficient β-cell lines show lowered glucose-stimulated insulin release (Kennedy et al., 1998; Soejima et al., 1996; Tsuruzoe et al., 1998). Loss of mtDNA gene expression by the A3243G tRNA<sub>Leu</sub> mutation is expected to impair oxidative phosphorylation. Lowered ATP levels are thought to block the stimulus-secretion coupling, whereby blood glucose stimulates insulin secretion by the pancreatic β-cell (Maechler and Wollheim, 2001). The stimulus-secretion coupling is initiated by glucose uptake through the Glucose-2 transporter. Glucose enters the glycolytic pathway and is metabolized to pyruvate yielding ATP. Pyruvate enters the mitochondria and stimulates oxidative phosphorylation leading to further ATP synthesis. The increase in cytosolic ATP originating from both sources, glycolysis and oxidative phosphorylation, results in closure of ATP dependent K<sup>+</sup> channels, depolarization of the plasma membrane, opening of voltage dependent L-type Ca<sup>2+</sup> channels and Ca<sup>2+</sup> influx. Cytosolic Ca<sup>2+</sup> triggers exocytosis of insulin containing
granules located near the plasma membrane. Impaired oxidative phosphorylation may thus inhibit closure of ATP dependent K⁺ channels and pancreatic insulin-secretion.

Some studies indicate that other molecular processes contribute to mitochondrial dysfunction. Firstly, it has been reported that high levels of the A3243G tRNA^{Leu} mutation can cause severe mitochondrial dysfunction without significant reduction in mitochondrial protein synthesis (Janssen et al., 1999). Secondly, studies of transmитochondrial cell lines (cells repopulated with donor-derived wild-type or mutant mitochondria) containing the A3243G tRNA^{Leu} mutation indicate a high threshold level for a reduction in oxygen consumption and oxidative phosphorylation at 80-90% mutant mtDNA versus wild type mtDNA (Chomyn et al., 1992). These high levels of heteroplasmy are not frequently found in diabetic patients with the A3243G tRNA^{Leu} mutation.

In situ characterization of islets of diabetic patients with the A3243G tRNA^{Leu} mutation revealed severely deficient cytochrome c oxidase (COX) activity and a decreased number of pancreatic β–cells. The heteroplasmy levels of micropunched pancreatic islets were quantified by laser densitometry and last cycle hot PCR labeling and revealed that they were 63 ± 5% (Kobayashi et al., 1997). Similarly, immunohistochemical studies of pancreas from patients with MELAS syndrome revealed a reduction in the number of pancreatic β- and α-cells and in total islet mass. The level of heteroplasmy for the A3243G tRNA^{Leu} mutation found in this study was 43% (Otabe et al., 1999). Interestingly, none of these studies revealed apoptosis markers in pancreatic islets.

**ROS formation in respiratory chain deficient cells**

Fibroblasts of patients with Complex I deficiency and different mitochondriopathies showed a significant increment in ROS formation (Luo et al., 1997; Pitkanen and Robinson, 1996) along with upregulation of SOD2 (Pitkanen and Robinson, 1996). Correlations between the levels of Complex I deficiency, ROS production, lipid peroxidation and apoptosis were observed in studies of human xenomitochondrial cybrids harboring a 40% Complex I deficiency. Cell death was in this case quantitatively associated with ROS production rather than with Complex I deficiency (Barrientos and Moraes, 1999). Complex II mutations causing hereditary paraganglioma likely result in increased O₂⁻· formation as a consequence of impaired
ubiquinone binding (Yankovskaya et al., 2003). In trans mitochondrial cybrids containing a mitochondrial cytochrome b gene mutation of a patient with MELAS and parkinsonism, high levels of the mutation were associated with Complex III deficiency and increased intracellular hydrogen peroxide levels (Rana et al., 2000). Fibroblasts of patients with the NARP mutation of the mitochondrial ATP synthase 6 gene show massive superoxide generation along with upregulated SOD activity (Geromel et al., 2001). Fibroblasts of patients with MELAS or MERFF display increased intracellular H$_2$O$_2$ levels and oxidative damage to lipids and DNA (Wei et al., 2001b). In whole blood cells of patients with MELAS-related mitochondriopathy, ROS-associated telomere shortening was observed (Oexle and Zwirner, 1997). In a mouse model of sarcopenia, the age-related decline of muscle mass and function, mtDNA deletions were found to colocalize with sites of oxidative damage to nucleic acids (Wanagat et al., 2001). Finally, mtDNA depleted (rho-0) cells showed an induction of SOD2 and other antioxidant defenses conferring enhanced resistance against oxidative stress-induced cell death (Park et al., 2004). The molecular mechanisms whereby mtDNA mutations induce ROS formation are not well defined. Mutations of respiratory subunits may block electron transfer within or between the respiratory complexes leading to electron leakage and superoxide formation. The mutations can also impair complex assembly as demonstrated for complex III (Rana et al., 2000), IV (D'Aurelio et al., 2001; Hanson et al., 2001), and V (Nijtmans et al., 2001), leading to loss of the respective complex or to a misassembled dysfunctional complex (Nijtmans et al., 2001), block in electron transfer and/or electron leakage and superoxide formation (Rana et al., 2000). Finally the mutations may inhibit ubiquinone binding to the complex causing electron leakage (Yankovskaya et al., 2003). Impaired mtDNA expression, caused by tRNA gene mutations, mtDNA deletions or mtDNA depletion may result in misassembled complexes and ROS formation. However, there are 2 tendencies that potentially counteract ROS formation in this situation: (i) the lack of mtDNA-encoded subunits could block assembly of several complexes and only deplete the number of fully active complexes without causing any block or leak in electron transfer; (ii) the combined deficiency of several respiratory complexes lowers oxygen consumption dramatically.
Metabolic alterations and cell death in respiratory chain deficient cells

Mitochondria have a central role in energy metabolism. Firstly they provide the vast amount of cellular ATP though oxidative phosphorylation. Secondly, glycolysis and mitochondrial fatty acid β-oxidation are linked to respiration (Stryer, 1995). Severe RC deficiency increases the [NADH]/[NAD⁺] ratio and slows down the activities of the TCA cycle and fatty acid β-oxidation that are both primarily regulated by the [NADH]/[NAD⁺] ratio (Erecinska and Wilson, 1982). Deficient oxidative phosphorylation lowers the [ATP]/[ADP] ratio that serves as the primary cytosolic sensor for altered energy metabolism and controls glycolytic rates (Erecinska and Wilson, 1982). Lowered mitochondrial ATP production is compensated for by up-regulation of glycolysis (Hansson et al., 2004; Wang et al., 2001a) and increased mitochondrial biogenesis (Hansson et al., 2004; Heddi et al., 1999; Wredenberg et al., 2002). This is also reflected by the formation of ragged red fibres in muscle (DiMauro and Schon, 2003). The increased mitochondrial biogenesis increases the overall mitochondrial ATP production rate in respiratory chain-deficient skeletal muscle (Wredenberg et al., 2002) but not in respiratory chain-deficient heart muscle (Hansson et al., 2004). Respiratory chain-deficient heart muscle displays a switch from fatty acid metabolism to glycolysis early in the progression of cardiac mitochondrial dysfunction prior to the increase in mitochondrial mass (Hansson et al., 2004). The observed switch from fatty acid metabolism to glycolysis is unlikely to benefit energy homeostasis in the respiratory chain-deficient hearts because fatty acid oxidation yields more ATP than glycolysis and is the chief myocardial energy source (Barger and Kelly, 1999). In most cancer lines with deficient oxidative phosphorylation, the energy for growth is almost exclusively derived from glycolysis. One reason for this is that glycolysis provides ATP at a low yield but high rate whereas oxidative phosphorylation produces ATP at a lower rate but higher yield (Pfeiffer et al., 2001). Upregulation of glycolysis results in lactic acidosis due to lowered pyruvate utilisation by the TCA cycle. Severe RC deficiency is thus expected to result in lowered mitochondrial ATP production, lowered TCA cycle activity, impaired amino acid and heme biosyntheses, impaired fatty acid degradation, up-regulation of glycolysis and increased mitochondrial biogenesis. MtDNA depleted (rho-0) cells can grow in glucose medium supplemented with uridine and pyruvate. The growth rate is lowered but there is no apparent increased cell death rate under these conditions (Dey...
and Moraes, 2000; Jiang et al., 1999; Wang et al., 2001a). Oxygen consumption of rho-0 cells is almost abolished but rho-0 cells still preserve the mitochondrial membrane potential to some extent (Jiang et al., 1999). This occurs through the reverse action of the F$_1$-ATP synthase that acts as a soluble ATP hydrolase in the absence of the mtDNA-encoded subunits of the F$_0$ part. The electrogenic exchange of mitochondrial ADP$^+$ for cytosolic ATP$^+$ maintains the membrane potential in rho-0 cells and upregulation of glycolysis provides ATP$^+$. Preservation of the mitochondrial membrane potential is likely required for protein import and mitochondrial biogenesis in order to maintain some important metabolic functions, for example biosynthetic pathways (Stryer, 1995) or buffering of apoptotic factors. Pharmacological inhibition of the reverse action of the soluble F$_1$ ATPase in rho-0 cells has detrimental effects (Buchet and Godinot, 1998). It is therefore not clear to what extent RC function is indeed required for cell maintenance in vivo. RC deficiency lowers the mitochondrial membrane potential and may increase ROS formation rendering the cell more susceptible to induction of PT (Susin et al., 1998). RC deficiency may significantly impair Ca$^{2+}$ homeostasis by decreasing the mitochondrial Ca$^{2+}$ buffering capacity, the activity and expression of ATP dependent endoplasmic and plasma membrane-bound Ca$^{2+}$ pumps (Arai et al., 1994; Carafoli, 2004; Wang et al., 2001a; Wankerl and Schwartz, 1995) leading to cytosolic Ca$^{2+}$ overflow, mitochondrial Ca$^{2+}$ uptake and either necrosis or apoptosis. Apoptosis is an energy dependent process, for example the formation of the apoptosome requires ATP (Liu et al., 1996). Furthermore, the intracellular ATP level determine the cell death fate, either by necrosis or apoptosis (Ankarcrona et al., 1995; Eguchi et al., 1997; Leist et al., 1997). Enhanced glycolytic ATP production in the absence of oxidative phosphorylation can allow execution of apoptosis (Leist et al., 1997). Nevertheless, mtDNA depleted cells are more resistant against induction of apoptosis in culture (Dey and Moraes, 2000; Park et al., 2004). Reports regarding apoptosis/cell death in respiratory chain disease are very scarce. It is very difficult to monitor cell death in vivo and the availability of patient material is rather limited. Cell death is a well-documented pathological hallmark of Leigh syndrome and LHON. Cell loss has also been documented in diabetic carriers of the A3243G tRNA$^{Leu}$ mutation exhibiting a reduction in the number of pancreatic insulin-producing $\beta$-cells in post-mortem pancreas sections (Kobayashi et al., 1997; Otabe et al., 1999). Apoptosis has also been observed in COX-negative muscle fibres of
patients carrying > 40% mtDNA deletions and > 70% tRNA point mutations (Mirabella et al., 2000). Cultured fibroblasts of patients with NARP harboring the 8993 mutation of the mitochondrial ATP synthase 6 gene display massive superoxide-induced apoptosis (Geromel et al., 2001). Thus, it is possible that increased cell death is a general pathogenic mechanism of RC disorders.

**Mouse models for respiratory chain disease**

It is often assumed that ATP deficiency is the main cause of RC disease but there is evidence that additional processes influence the phenotype (Hansson et al., 2004; Wredenberg et al., 2002). The limited availability of human tissue makes it necessary to perform studies of molecular pathogenesis in model organisms. Mice and humans display quite similar gene content, comparable types of internal organs and physiological processes. The generation of transgenic mice with mutant mtDNA molecules is a significant technical problem because attempts to transf ect mitochondria with whole mtDNA have been unsuccessful. This problem has been solved by two alternative approaches: (i) cybrid-mediated transfer of mutant mtDNA into ES cells or pro-nucleus stage embryos followed by propagation of the mutation through the mouse female germline; (ii) inactivation of mtDNA gene expression through disruption of the gene encoding Tfam. In addition to mouse models that directly affect the expression of subunits of the RC, mouse models have been created that alter the homeostasis of the mitochondria and thereby indirectly affect RC function, for example gene ablation of SOD2, Gpx1, ANT1.

Cybrid-mediated transfer of mtDNA has been used to create: (i) Heteroplasmic mice harboring mtDNA haplotypes from two different mouse strains by fusion of enucleated zygotes from one mouse strain to pronucleus-stage embryos from another mouse strain (Jenuth et al., 1996; Jenuth et al., 1997); (ii) Mice carrying deleted mtDNA (ΔmtDNA) (Inoue et al., 2000). Mouse cell lines heteroplasmic for mtDNA with a 4969bp-deletion were propagated in a mouse rho-0 cell line. These cybrids were enucleated and fused to pro-nucleus-stage embros. Heteroplasmic mice transmitted the ΔmtDNA to several generations, displayed high levels of rearranged mtDNA in most tissues and RC deficiency in heart, skeletal muscle and kidney leading to myopathy and kidney failure. Surprisingly, a partially duplicated mtDNA
molecule was identified in postmitotic tissues despite being undetectable in the cybrids used to create these mice and it is therefore unclear whether ΔmtDNA was transmitted intact through the female germline or generated later by intramolecular rearrangements. ΔmtDNA syndromes in humans have in most instances an adult-onset, are not transmitted, exhibit high levels of ΔmtDNA in postmitotic tissues, such as brain, heart and skeletal muscle and cause no kidney failure. Thus the limitation of this mouse model is that it does not reproduce well the pathology found in adult patients with ΔmtDNA (Larsson and Rustin, 2001). (iii) Mice carrying a mutation in the 16S rRNA gene (chloramphenicol resistance, CAP$^R$ mtDNA) (Sligh et al., 2000). CAP$^R$ mtDNA was propagated in a rho-0 cell line. The enucleated cybrids were fused to a female ES cell line. The cybrid ES cells were injected into C57/B16 blastocysts and high degree chimeras were obtained. Mice heteroplasmic for CAP$^R$ mtDNA transferred the mutant mtDNA through the female germline for several generations and exhibited a severe phenotype including myopathy and cardiomyopathy but the respiratory defects in these mice have not been demonstrated yet.

Disruption of the T\textit{fam} gene causes depletion of mtDNA, mitochondrial transcripts, loss of mtDNA encoded polypeptides and severe RC deficiency (Larsson et al., 1998; Wang et al., 1999). T\textit{fam} KO mice therefore mimic human disease caused by impaired mtDNA gene expression, for example ΔmtDNA syndromes, mtDNA mutations of tRNA genes, and mtDNA depletion syndromes. Homozygous T\textit{fam} KO mice show severe developmental delay and die between embryonic day 8.5 and 10.5. They exhibit complete absence of Tfam protein, complete absence of mtDNA and abolished oxidative phosphorylation. Heterozygous T\textit{fam} KO mice have a 50% reduction in T\textit{fam} transcripts and proteins, a 34% reduction in mtDNA copy number, a 22% reduction in mitochondrial transcripts and a mild respiratory chain deficiency in heart (Larsson et al., 1998). Tissue-specific inactivation of T\textit{fam} in heart results in postnatal death due to dilated cardiomyopathy. These animals develop conduction defects with prolongation of the PQ interval and intermittent atrioventricular blocks under anesthesia. Heart-specific T\textit{fam} KO mice exhibit a reduction in Tfam protein, mtDNA levels and mtRNA levels in heart and muscle, and reduced activities of Complex I and IV. Histochemical analyses of the T\textit{fam} KO hearts revealed a mosaic-staining pattern with COX-negative and SDH-hypereactive cardiomyocytes. The
heart–specific Tfam KO mice thus reproduce typical morphological and physiological features of human mitochondrial cardiomyopathy (Wang et al., 1999).

ANT1−/− mice develop a myopathy with ragged red fibres and a hypertrophic cardiomyopathy associated with a marked proliferation of mitochondria (Graham et al., 1997). The inhibition of ADP/ATP exchange deprives the ATP synthase of substrate leading to hyperpolarization of the mitochondrial membrane potential, inhibition of electron transport and increased superoxide production. Consistently, ANT1−/− mice exhibit six- to eight fold increased H₂O₂ production in skeletal muscle and heart mitochondria and upregulated SOD2 and GPx1 enzyme activities (Esposito et al., 1999). MtDNA rearrangements were observed in hearts and skeletal muscle of aged ANT1−/− mice and may be explained by the enhanced ROS formation acting as a mutagen or alternatively by alterations of the mitochondrial nucleotide pool impairing mtDNA replication (Esposito et al., 1999).

SOD2 and GPx1 KO mice were created to assess the consequences of enhanced superoxide and hydrogen peroxide formation, respectively, on mitochondrial function. On the CD1 background, SOD2−/− mice die of dilated cardiomyopathy at the age of 8 days (Li et al., 1995). On the B6 background, SOD2−/− mice die at the age of 18 days due to neurodegeneration (Lebovitz et al., 1996). While inactivation of SOD2 proves to be lethal early in life, the inactivation of either the cytosolic Cu/Zn-SOD (SOD1) (Reaume et al., 1996) or the extracellular SOD (SOD3) (Carlsson et al., 1995) have little effect on the viability of the mice. This suggests that mitochondrial production and toxicity of O₂⋅− is far more deleterious than the cytosolic and extracellular formation of O₂⋅−. SOD2−/− mice develop massive lipid droplets in the liver, deficient activities of Complex I and II, citrate synthase and aconitase in heart. Increased O₂⋅− concentrations thus inactivate enzymes containing mitochondrial iron-sulphur centers blocking the TCA cycle and electron transport (Melov et al., 1999). Respiratory measurements demonstrated signs indicative of increased mitochondrial basal proton leak. Mitochondria from SOD2−/− mice are more susceptible to activation of the PTP indicating that O₂⋅− sensitizes the PTP (Kokoszka et al., 2001). Similar effects on respiration and accumulation of ROS-induced protein and lipid damage are observed in middle-aged SOD2+/− mice and in wild-type mice at a later age. Furthermore SOD2+/− mice display a three-fourfold increased apoptosis-rate in liver (Kokoszka et al., 2001).
Prior to generating GPx1 KO mice, a reporter cassette (β-galactosidase) was inserted into the GPx1 mouse gene and revealed that the GPx1 gene locus is mainly active in liver, brain, kidney and weakly in heart and skeletal muscle. GPx1 protein is found in the cytosol and the mitochondria of liver and kidney. GPx1−/− mice are viable but display a 20% weight reduction. H₂O₂ levels are increased fourfold in liver mitochondria. Respiratory measurements indicated an increased mitochondrial uncoupling in liver. However augmented mitochondrial H₂O₂ production in liver, brain and kidney appears to cause a rather mild phenotype in the living animal compared to inactivation of SOD2 (Esposito et al., 2000).
Aims of this study

RC deficiency causes specific mitochondrial disorders. In addition, various types of evidence links RC deficiency with age-associated common disorders and ageing. RC deficiency is expected to lower mitochondrial ATP generation. However, there are potentially other factors contributing to the pathophysiology of RC dysfunction. The specific aims of this proposal are to investigate whether:

1) RC deficiency in pancreatic β-cells impairs glucose-stimulated insulin release and β-cell survival.
2) RC deficiency affects cell survival.
3) RC deficiency causes ROS formation.
4) Mitochondrial superoxide regulates uncoupling protein activity, RC function and energy expenditure.
Comments on methods

All methods used in this study have been described in the original publications that are included in the end of the text. Central methods that have not been described or are only referred to in the original publications will be described in this section.

Tfam knockout strategy

The mouse Tfam gene consists of 7 exons. LoxP sites were introduced into the mouse Tfam gene by homologous recombination of ES cells (Larsson et al., 1998). The loxP sites flank exon 6 and 7 of the mouse Tfam gene including the polyadenylation signal. Mice homozygous for the floxed Tfam allele (TfamloxP/TfamloxP) were mated to different transgenic strains expressing cre-recombinase from cell-type specific promoters: (i) mice expressing a β-actin-cre transgene (Lewandoski et al., 1997) ubiquitously in the preimplantation embryo to generate germline Tfam KO mice (paper II); (ii) mice expressing cre-recombinase from the rat insulin 2 promoter (RIP2-cre) (Postic and Magnuson, 1999) to generate pancreatic β-cell specific Tfam KO mice (paper I); (iii) mice expressing cre-recombinase from the muscle creatine kinase promoter (Ckmm-cre) (Bruning et al., 1998) to disrupt Tfam in cardiomyocytes (paper II); (iv) mice expressing cre-recombinase from the calcium dependent calmodulin kinase II promoter (CaMKII-cre) (Xu et al., 2000) to disrupt Tfam specifically in forebrain neurons (Paper III). Cre-mediated excision of exon 6 and 7 of the Tfam gene results in loss of the polyadenylation signal and Tfam transcript instability.

Enzyme histochemistry

Cryostat sections were prepared and stored at -20° C. To detect COX enzyme activities, cryostat sections were incubated 30-60 min at room temperature with a reaction mix containing 1mg/ml cytochrome c (Sigma-Aldrich Sweden AB), 2mg/ml catalase (Sigma-Aldrich Sweden AB), 75mg/ml sucrose (BDH AnalaR, England), 0.5mg/ml 3,3’- diaminobenzidine tetrahydrochloride dihydrate (DAB, Fluka Chemicals, Switzerland) in 0.05M phosphate buffer (0.01M NaH₂PO₄, 0.04M Na₂HPO₄, pH 7.4). To stain for succinate dehydrogenase (SDH) enzyme activity,
cryostat sections were incubated 30-90 min at 37 °C with a reaction mix containing 54mg/ml succinate acid Na-salt (Sigma-Aldrich Sweden AB), and 1mg/ml 4-nitroblue tetrazolium chloride (NBT) (Boehringer Mannheim Scandinavia, Sweden) in 0.2M phosphate buffer (0.16M Na₂HPO₄, pH 7.2-7.6). COX/SDH double staining was performed by first staining for COX and then for SDH.
Results and Discussion

Pathogenesis studies in a mouse model of mitochondrial diabetes (Paper I)

Mutations of mtDNA cause approximately 0.5-1% of all types of diabetes mellitus and studies of mtDNA depleted pancreatic β-cell lines suggest that RC function is critical for insulin release. To study the pathogenesis of mitochondrial diabetes, we inactivated the *Tfam* gene specifically in pancreatic β-cells by *cre*-mediated excision of a loxP-flanked *Tfam* allele. To this end, transgenic mice expressing *cre*-recombinase from the rat insulin-2 promoter (*RIP2-cre*) (Postic and Magnuson, 1999) were bred to knockin mice harboring a loxP flanked *Tfam* allele (Larsson et al., 1998). The *RIP2-cre* strain expresses *cre*-recombinase specifically in pancreatic β-cells and in certain hypothalamic regions as evidenced by crosses between the *RIP2-cre* strain and a mouse strain harboring a conditional lacZ gene (Soriano, 1999) (Gannon et al., 2000) and immunohistological analyses of *cre*-recombinase expression (Gannon et al., 2000). As expected, PCR analyses demonstrated the presence of the *Tfam* KO allele only in pancreas and brain of young (7-week old) homozygous *Tfam* KO mice (*RIP2-cre/+, *Tfam*loxP/TfamloxP*).

At the age of 7 weeks there was a highly efficient inactivation of mtDNA gene expression in homozygous *Tfam* KO mice as revealed by: (i) *in situ* hybridization studies of pancreas sections showing severe mtDNA depletion in islets of mutant mice; (ii) enzyme histochemical double staining for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activities showing a severe COX deficiency and normal SDH activities in islets of mutant mice. This result was expected because COX contains mtDNA encoded catalytic subunits whereas SDH is completely nucleus-encoded; (iii) EM pictures of insulin producing β-cells demonstrating abundant, abnormally appearing mitochondria in mutant islets.

The homozygous *Tfam* KO mice developed diabetes with decreased blood insulin concentrations from the age of about 5 weeks onwards. Insulin tolerance tests showed normal insulin sensitivity in younger (12-week old) mutant mice and enhanced insulin sensitivity in older (27-33 week) mutant mice. Histochemical analyses of islet cell composition showed a normal cell-type composition and point counting morphometry demonstrated normal β-cell mass in young (7-week old)
mutant mice. Older (33-39 week old) mutant mice showed a marked change in the cell-type composition of the islets and a reduction in β-cell mass. There was a patchy loss of β-cells and a relative increase in glucagon- and somatostatin-producing α- and δ-cells, respectively. These data conclusively demonstrate that mutant β-cells had been lost over time. However, we found no evidence for increased apoptosis (<1% of TUNEL reactive islet cells) or islet inflammation as a consequence of necrosis in young 7-week old mutant mice.

Next, we analyzed the β-cell stimulus-secretion coupling at the age of 7 weeks, when β-cell mass was still normal in mutant mice. The β-cell stimulus-secretion coupling is the signal transduction pathway whereby glucose stimulation of the β-cell results in insulin secretion. We found a decreased hyperpolarization of the mitochondrial membrane potential in isolated mutant islets in response to glucose stimulation consistent with a RC deficiency. We then monitored intracellular Ca\(^{2+}\) after glucose stimulation of isolated islets. We found a decreased rise in intracellular Ca\(^{2+}\) and an almost complete absence of the fast Ca\(^{2+}\) oscillations in mutant islets. When both, mutant and control islets, were exposed to extracellular K\(^{+}\) which depolarizes the plasma membrane potential, both had normal intracellular Ca\(^{2+}\) rise indicating that the stimulus-secretion pathway downstream of ATP-dependent K\(^{+}\) channels worked normally in mutant islets but also demonstrating that the lack of a Ca\(^{2+}\) rise in response to glucose stimulation was not due to cytosolic Ca\(^{2+}\) overflow. Next, we studied the kinetics of glucose-induced insulin release by perfusing islets isolated at the age of 7 weeks. Insulin release was significantly reduced in mutant islets. We also determined insulin content and found no significant differences between mutant and control islets. The homozygous Tfam KO mice thus displayed impaired stimulus-secretion coupling at the age of 7 weeks.

Blood glucose levels dropped in older mutant mice but remained elevated. Older (14- and 27-39-week old) mutant mice showed presence of the Tfam allele only in brain but not in pancreas. Consistently, islets of older mutant mice showed normal COX activity and normally appearing mitochondria on EM pictures. Immunofluorescence analyses showed that cre-recombinase was abundantly expressed in islets of 7-week old mutant mice while cre-recombinase was not expressed at all or only in very few islet cells in older (22-25 week old) mutant mice. Thus, the recombination event inactivating the Tfam allele and the RC deficiency
ceased when the mutant mice became older. The diabetic phenotype in older mutant mice is thus caused by the loss in β-cell mass and the improvement in glucose homeostasis observed in the older mutant mice is likely attributable to expansion of β-cell mass over time and enhanced insulin sensitivity.

This study provides the first genetic in vivo evidence that loss of mitochondrial DNA gene expression in pancreatic β-cells causes diabetes. This study identified two phases in the progression of mitochondrial diabetes: mutant β-cells first display impaired stimulus-secretion coupling. This is later followed by β-cell loss. This mouse model reproduces the clinical features of patients with mitochondrial diabetes, i.e. impaired pancreatic insulin secretion and β-cell loss.

**Apoptosis studies of cells and tissues lacking mtDNA gene expression (Paper II)**

Apoptosis may contribute to the pathology of RC disease. Mitochondria are key regulators of apoptosis and a RC deficiency may directly activate cytochrome c release because of lowered mitochondrial membrane potential and ROS formation (Susin et al., 1998). MtDNA depleted (rho-0) cells do not exhibit increased cell death in culture and are reportedly more resistant against apoptosis induction suggesting that intact RC function is required for apoptosis execution (Dey and Moraes, 2000).

In order to clarify whether respiratory chain-deficient cells are able to undergo apoptosis and are more apoptosis-prone in vivo we investigated heart-specific Tfam KO mice, Tfam KO embryos and an mtDNA depleted osteosarcoma rho-0 cell line. Mice with heart-specific inactivation of the Tfam gene exhibit postnatal onset of severe mitochondrial cardiomyopathy (Wang et al., 1999). We found increased apoptosis in homozygous heart-specific Tfam KO hearts as reflected by an increase in TUNEL reactive cells, activation of caspase 3 and caspase 7, and apoptotic DNA fragmentation. Trichrome staining of heart sections did not reveal fibrosis in mutant hearts as a consequence of necrosis. Northern blot analysis revealed increased expression of genes regulating apoptosis, namely BAX and BCL-xL, and massive up-regulation of glycerldehyde-3-phosphate dehydrogenase (Gapdh) indicative of increased glycolysis. We next analyzed homozygous Tfam KO embryos. Homozygous Tfam KO embryos die between E8.5 and 10.5 and have abolished oxidative phosphorylation (Larsson et al., 1998). We found massive induction of
apoptosis in homozygous Tfam KO embryos at E9.5 as reflected by massive TUNEL reactive cells and activation of caspase 3, followed by resorption of the embryo at E10.5. We next reexamined wether mtDNA depleted (rho-0) human osteosarcoma cells were able to undergo apoptosis. We stimulated apoptosis using Staurosporine (STP), TNFα and anti-Fas antibodies (anti-Fas) that stimulate Fas, the receptor of Fas ligand. STP stimulates mitochondrial cytochrome c release while TNFα and anti-Fas antibodies (Anti-Fas) activate caspase 8 which activates the effector caspase 3 or the pro-apoptotic BID protein (tBID) that in turn induces mitochondrial cytochrome c release. Human osteosarcoma cells were scored for apoptosis by simultaneous labeling of the cells with Annexin V and Propidium iodide followed by flow cytometry to determine the percentage of apoptotic cells (Annexin positive and Propidium iodide negative). Rho-0 cells were able to undergo apoptosis in response to all apoptotic stimuli and they were less sensitive to induction of apoptosis by STP and more sensitive to induction of apoptosis by TNFα and Anti-Fas. Apoptosis was further confirmed by detection of DNA fragmentation.

Thus, the human rho-0 osteosarcoma cells exhibited an increased threshold for induction of the cytochrome c release pathway as previously reported (Dey and Moraes, 2000) and were more sensitive to induction of the death receptor pathway as previously observed in cytochrome c KO cells (Li et al., 2000). The significance of these differences however remainns unclear because cancer-cell lines carry chromosomal rearrangements, and thus likely consist of a mixed genetic background.

We explored whether ROS formation was associated with the observed in vivo apoptosis of respiratory chain-deficient cardiomyocytes. We found significantly increased transcript levels of SOD2 and GPx. Next we determined total SOD and GPx activities and found a significant increment in GPx activity, only. Furthermore, the activities of Complex II and of aconitase that are readily impaired by ROS due to inactivation of iron-sulphur clusters proved normal.

This study shows that RC deficient cells can undergo apoptosis and that RC deficient cardiomyocytes and embryos are more prone to undergo apoptosis in vivo. Apoptosis may thus contribute to RC disease. There is evidence for increased ROS formation in RC deficient hearts but the normal activities of enzymes readily impaired by ROS suggest that the enhanced ROS formation is compensated for by upregulation of antioxidant defenses.
Analysis of the relationship between prolonged respiratory chain deficiency, ROS formation and cell death in late-onset neurodegeneration mice (Paper III)

Correlative data suggest a connection between deficient RC function, ROS production and apoptosis induction in age-associated neurodegeneration (Wallace, 1992). We performed a genetic experiment to test whether these processes are linked. To this end, we inactivated the *Tfam* gene in neurons of the frontal neocortex and the hippocampus. Mice expressing *cre*-recombinase from the calcium dependent calmodulin kinase II promoter were crossed to mice with a loxP flanked *Tfam* allele to obtain homozygous tissue-specific *Tfam* KO mice (*Tfam*loxP/loxP, +/-*CaMKII-cre*) called MILON mice (Mitochondrial late onset neurodegeneration mice). In the *CaMKII-cre* transgenic line, *cre*-recombinase is expressed from postnatal day 14 (P14) and maximal recombination of loxP flanked alleles is observed at P29 causing recombination in about 50% of the neurons in neocortex and hippocampus (Xu et al., 2000). Consistent with these earlier observations we found identical levels of the mutant *Tfam* allele at 1 month (relative level, 24 ± 3%) and 5 months of age (relative level, 25 ± 2%) in pre-symptomatic MILON mice confirming that maximal recombination was obtained by 1 month of age. Tfam protein levels in neocortex of MILON mice were reduced from the age of 2 months onwards. MtDNA copy number and mtRNA levels were reduced by 40% from the age of 2 and 4 months, respectively. *In situ* hybridization showed a dramatic reduction of mtRNA in necortex and hippocampus. Biochemical measurements demonstrated reduced activities of Complex I and IV in 4- and 5-month old MILON mice. Enzyme histochemical stainings revealed severe COX deficiency in individual neurons of hippocampus and neocortex of MILON mice at the age of 4 months. The reduction in RC function in tissue-extracts of neocortex is thus the result of profound COX deficiency of individual *Tfam* KO neurons.

MILON mice displayed onset of rapidly progressing, severe neurodegeneration in hippocampus and neocortex at 5-5.5 months and died at the age of 5.5-6 months. Cresyl violet staining showed massive loss of neocortical neurons and of hippocampal neurons of the CA1 and to a lesser extent of the CA3 region of the hippocampus accompanied by a cellular infiltration with macrophages and gitter cells. The S-phase marker PCNA demonstrated numerous dividing glia or inflammatory
response cells in neocortex and hippocampus as judged from their morphological appearance. Immunostainings for neurofilament (NF-10) showed axonal degeneration and immunostainings for GFAP to detect astrocytes showed reactive astrocytosis further confirming the presence of neurodegeneration and an inflammatory response in end-stage MILON mice. End-stage MILON mice, displayed massive induction of TUNEL positive cells in hippocampus and neocortex. In the hippocampus, the first areas displaying TUNEL positive cells were the CA1 and to some extent the CA3 areas, followed by appearance of TUNEL positive cells in the dentate gyrus at a later stage. In order to distinguish between apoptosis and necrosis, we performed additional analyses. Gel electrophoresis of DNA from neocortex and hippocampus revealed no apoptotic DNA fragmentation pattern. However, a more sensitive PCR assay detected DNA fragmentation in endstage MILON mice. Immunostainings did not show presence of activated caspase 3 or 7 in brain sections of end-stage MILON mice. Northern blot analysis revealed no changes in the transcript levels of the proapoptotic BAX and anti-apoptotic BCL-xL. Thus apoptosis markers were mainly negative despite massive neuronal cell death suggesting that a major fraction of dying neurons underwent necrosis rather than apoptosis. Northern blot analyses showed normal levels of the transcripts for glyceroldehyde-3-phosphate dehydrogenase (Gapdh) suggesting that glycolysis is not upregulated in MILON mice.

Transcript levels and enzyme activities of SOD2 and GPx were determined at 2-, 4- and 5-months of age and revealed only an increase in GPx transcripts in 5-month old endstage MILON mice. Western Blot analyses and immunohistochemical stainings showed no enhanced protein nitrosylation, another marker for oxidative stress induced by the conversion of superoxide to peroxynitrite, in 4-6 month old mutant animals. The activity of the nucleus encoded Complex II whose activity is readily impaired by ROS was not changed in mutant mice determined at 2-, 4- and 5-months of age. These results suggest unexpectedly low levels of ROS production in MILON mice at all investigated ages, i.e. at all stages of development of severe RC deficiency. Next we explored whether RC deficient neurons are more susceptible to an excitotoxic challenge with Kainic acid (KA). Excitotoxic insults result in apoptosis or necrosis dependent on the level of ATP present in a neuron (Ankarcrona et al., 1995). We challenged MILON mice with KA at the age of 4 months when MILON mice displayed severe RC deficiency but no signs of neurodegeneration. MILON
mice with level 3-5 seizures (level 5 is the most severe grade) exhibited a much larger number of TUNEL positive cells in the hippocampus (CA3 region) 24 hours after injection of KA. Thus, RC deficient neurons are more susceptible to an excitotoxic challenge.

We conclude that neurons with severe RC deficiency are viable for a prolonged period before they undergo cell death. Surprisingly, there is no apparent upregulation of glycolysis suggesting that glial cells may sustain neuronal energy metabolism. RC deficient neurons likely die by necrosis rather than by apoptosis and are more sensitive to excitotoxicity. Most surprisingly, RC deficient neurons did not exhibit significant induction of ROS defenses or signs of oxidative protein damage during the development of severe RC deficiency.

Regulation of uncoupling protein activity and mitochondrial bioenergetics by superoxide (Paper IV)

Mitochondrial generation of superoxide ($O_2^-$) and other ROS species has been implicated in ageing. However, it is at present not clear whether mitochondrial $O_2^-$ also regulates RC function and energetic efficiency. A recent study reported superoxide-mediated activation of mitochondrial uncoupling proteins (UCP) (Echtay et al., 2002b). Activation of UCPs by $O_2^-$ is expected to increase the proton leak across the mitochondrial inner membrane, to increase respiration and reduce the generation of $O_2^-$ (Korshunov et al., 1997). The mechanism of superoxide-mediated activation of UCPs would thus provide a feedback control to lower further $O_2^-$ production by the RC (Echtay et al., 2002b). The activation of UCP occurs from the mitochondrial matrix side (Echtay et al., 2002a). Superoxide-mediated activation of UCPs is expected to have a significant regulatory effect on whole body metabolism due to the widespread tissue expression of UCPs. For example in BAT, $O_2^-$ may enhance UCP1 function and facilitate nonshivering thermogenesis. In pancreatic islets, $O_2^-$ may activate UCP2 function and lower insulin release. In muscle, $O_2^-$ may activate UCP3 and influence energetic efficiency. The hypothesis that $O_2^-$ activates UCPs is not universally accepted (Couplan et al., 2002). The study reporting superoxide-mediated activation of UCPs was performed using isolated mitochondria exposed to artificial systems generating very high concentration of exogenous $O_2^-$.
(Echtay et al., 2002b) and there is only one study using adenovirus-mediated SOD2 overexpression in islet cells indicating that superoxide-stimulated UCP-mediated uncoupling may also occur in vivo (Krauss et al., 2003).

The aim of this study was to perform a genetic experiment to investigate whether superoxide-activation of UCPs is a regulatory mechanism in vivo. To this end, we generated a genetically modified mouse strain with a regulated increase of SOD2 enzyme activity. We used P1 artificial chromosomes (PAC) harboring the entire human SOD2 gene to create transgenic mice overexpressing SOD2. PAC-mediated overexpression confers physiological regulation of gene expression due to: (i) the presence of sequence elements needed for physiological regulation of gene expression within the large genomic fragment; (ii) minimization of positional effects due to the presence of large amount of flanking sequences (Heintz, 2000); (iii) moderate overexpression of the introduced transgene within a physiologically relevant interval (Ekstrand et al., 2004). The use of the human SOD2 protein will make it easy to establish that the transgene is present and expressed in the transgenic mice by using species-specific DNA and antibody probes for human SOD2 (Ekstrand et al., 2004).

PAC clones from a human genomic library were screened for the presence of the SOD2 gene and ordered in a contig by using Southern blotting with end probes (generated by vectorette PCR) and by size determination with pulse field gel electrophoresis as previously described (Ekstrand et al., 2004). Three PAC clones with overlapping 5’ and 3’ sequences named PAC662, PAC817 and PAC737 were used to generate transgenic lines by pronuclear injections of embryos of the inbred FVB/N mouse strain. Southern blots of tail DNA using a human SOD2 cDNA probe showed integration of the human SOD2 gene into the mouse genome without signs of rearrangements in all founders. We obtained germline transmission from most founder mice. Two independent lines, PAC662D1 (from the founder D1 of clone PAC662) and PAC737D2 (from the founder D2 of clone PAC737), were characterized further. The transgenes of these two lines were constantly transmitted at less than the expected Mendelian ratio of 50% (PAC662D1 34% and PAC737D2 38%; p<0.001 obtained by chi-square test for uneven genotype distribution). Increased expression of SOD2 thus had some effects on fertility, consistent with a previous report (Raineri et al., 2001). Western blot analyses showed expression of the human SOD2 protein in muscle and kidney of the PAC737D2 line and in most tissues
of the PAC662D1 line. SOD enzyme activities were determined in isolated mitochondria of liver, skeletal muscle, brain and BAT and were increased in skeletal muscle of the PAC737D2 line and in all investigated tissues in the PAC662D1 line. The observed SOD enzyme activity pattern was thus consistent with the observed human SOD2 protein expression pattern.

SOD2 overexpression did not affect mitochondrial morphology and mitochondrial biogenesis. EM showed normally appearing mitochondria in heart, skeletal muscle and liver. Mitochondrial mass markers, i.e. mitochondrial volume density by transmission electron microscopy, mtDNA copy number, mtDNA transcript levels and Tfam, cytochrome c and COXII protein levels, revealed no differences in mitochondrial mass between PAC662D1 and wild-type mice in skeletal muscle, BAT and liver. In BAT, following cold acclimation, UCP1 protein and PGC-1α protein levels increased 2-3 fold in both wild-type and PAC662D1 mice, but again no differences were found between the wild-type and the SOD2-overexpressing mice. Thus, while markers of recruitment and mitochondrial biogenesis in BAT responded as expected to cold stimulus, the overexpression of SOD2 was without influence on the process.

Oxidative stress damages cellular membranes (Davies, 1995) and renders mitochondria more prone to induction of mitochondrial permeability transition (Kokoszka et al., 2001). We examined induction of mitochondrial permeability by Ca^{2+} and oleate in liver and muscle mitochondria and found as expected that PAC662D1 mitochondria were more resistant to induction of high amplitude swelling than wild-type mitochondria. The response to alamethicin, a pore forming peptide inducing maximal mitochondrial swelling, was greater in PAC662D1 mitochondria showing that mutant mitochondria were significantly less swollen than wild-type mitochondria prior to the addition of alamethicin. SOD2 overexpression thus conferred resistance to induction of mitochondrial permeability.

We performed respiration measurements of isolated mitochondria to assess UCP activity. If matrix O_{2}^{−} is a significant activator of UCPs, mitochondria overexpressing SOD2 would be expected to show a markedly lower basal respiration because of reduced proton leak. Basal respiration (i.e. state 4, after oligomycin addition) was not changed in liver mitochondria that lack all three UCPs, in brain mitochondria, that have been reported to express UCP2, and in skeletal muscle.
mitochondria, that express UCP3 (see Table I of Paper IV). In brown fat mitochondria, which express UCP1, the equivalent respiration (after GDP addition) was also unchanged (see Table II of Paper IV). Thus in the basal state, mitochondrial superoxide did not have an observable activating effect on proton conductance mediated by UCPs. Similarly, ADP-stimulated state 3 respiration in liver, brain and muscle was unaffected by the SOD2 overexpression thus indicating no change in proton leak (see Table I of the manuscript). However, unexpectedly, in all tissues, SOD2 overexpression increased maximal mitochondrial oxygen consumption (oxidative) capacity upon addition of a chemical unoupler, FCCP (see Tables I and II of Paper IV). The increase in mitochondrial oxidative capacity upon addition of FCCP correlated with the increase in SOD enzyme activity. These observations thus indicate a hitherto unrecognized regulatory effect of endogenous superoxide on mitochondrial oxidative capacity.

It is well-accepted that respiration inhibition by GDP in BAT mitochondria is mediated by effects on UCP1. SOD2 overexpression did not affect the sensitivity for GDP-mediated inhibition of UCP1 in PAC662D1 mitochondria compared to wild-type mitochondria (Km of wild-type = 162 ± 25 µM, n = 3 and of PAC662D1 = 168 ± 28 µM, n = 3). Superoxide-mediated activation of UCPs is fatty-acid dependent (Echtay et al., 2002). SOD2 overexpression did not influence the sensitivity of UCP1 to fatty acids (Km of wild-type = 24 ± 5 nM, n = 5 and of PAC662D1 = 26 ± 8 nM, n = 5). We repeated these measurements with glycerol-3-phosphate which gives rise to reverse electron flow and increased superoxide generation at complex I (Turrens, 2003). We found similar oxygen consumption rates in SOD2-overexpressing and control mitochondria when using glycerol-3-phosphate as a substrate, and GDP titration experiments showed no significant difference between wild-type (Km=169±30, n=5) and PAC662D1 (Km= 146±28, n=5) BAT mitochondria.

Next, we explored UCP3 activities. Purified UCP3 incorporated into liposomes displays a proton translocation capacity that can be stimulated by fatty acids and inhibited by GDP (Jaburek and Garlid, 2003; Jaburek et al., 1999). As UCP3 is found in skeletal muscle mitochondria, we used fatty acid activation and GDP inhibition to estimate UCP3 activity in isolated skeletal muscle mitochondria assuming that fatty acid effects in intact skeletal muscle mitochondria are mediated by UCP3. When respiration was stimulated with pyruvate plus malate, skeletal muscle
mitochondria did not exhibit innate GDP-responsive uncoupling, in accordance with a previous report (Cadenas et al., 2002). However, GDP can inhibit basal proton leak upon induction of reverse electron flow generating high levels of $O_2^-$ (Talbot et al., 2004). We induced reverse electron flow by using succinate in the absence of rotenone and detected a very small GDP-mediated inhibitory effect on basal respiration. However, the degree of inhibition was the same in wild-type and PAC662D1 skeletal muscle mitochondria. Furthermore, we found no significant difference in the sensitivity for fatty-acid mediated activation of UCP3 between wild-type (Km = 2.6 ± 0.6 µM, n=4) and PAC662D1 skeletal muscle mitochondria (Km = 2.5 ± 0.8 µM, n=4). Also, the maximal response to fatty acid was decreased to the same extent by GDP treatment of wild-type and PAC662D1 mitochondria. Thus SOD2 overexpression did not influence the effects of fatty acids or GDP on skeletal muscle mitochondria and, provided that these effects are UCP3-mediated, this demonstrates that UCP3 activity is not influenced by SOD2 overexpression. It has previously been indicated that loss of UCP3 activity through gene ablation enhances ATP production of the skeletal muscle (Cline et al., 2001). We determined mitochondrial ATP production rates (MAPR) in skeletal muscle and found no difference between wild-type and PAC662D1 mice further supporting the conclusion that SOD2 overexpression did not inhibit UCP3 activities.

We also investigated whether signs of a regulatory role for $O_2^-$ could be observed in the intact animal since the above experiments were performed on isolated systems and potentially important regulatory factors may have been absent. No difference in body weight was found when wild-type and PAC662D1 mice of both sexes were compared. The resting metabolic rates determined in mice acclimated to 25°C or 4°C for 4 weeks showed no differences between wild-type and PAC662D1 mice. Thus, SOD2 overexpression does not affect energy expenditure, i.e. the innate UCP activation was apparently not altered. To estimate the importance of superoxide for UCP1 activity in vivo, we induced nonshivering thermogenesis by norepinephrine (NE) injection of mice acclimated to 25°C or 4°C for 4 weeks and measured oxygen consumption. The respiratory response to NE was similar in wild-type and PAC662D1 mice. Thus, SOD2 overexpression did not affect UCP1 activity in vivo.

Lowered activation of UCP1 by superoxide could be compensated for by increased BAT organ mass, mitochondrial mass per BAT cell or UCP1 protein levels.
per cell. We excluded these possibilities by measuring the wet weight and protein concentration of the left lobe of the interscapular BAT, transcript and protein levels of mitochondrial biogenesis markers in BAT (see above) and Western Blot analysis of UCP1 expression in BAT following cold exposure of the mice to 4°C for > 3 weeks. Thus there was no indication that a putative decrease in UCP1 activity was compensated for by augmented recruitment of BAT.

We conclude that SOD2 overexpression did not affect the regulation of UCP1 and 3 activities despite clear effects on other mitochondrial parameters, i.e. oxidative capacity and resistance to inducers of mitochondrial permeability. Also, in a global measure, that of basal metabolic rate in the intact animal, we found no evidence for effects of SOD2 overexpression, i.e. no significant part of basal metabolism appears to derive from superoxide-regulated UCP activity. The absence of a regulatory effect of endogenously generated superoxide on UCP1 and UCP3 activities thus indicates a basic difference between effects of exogenously generated and endogenously occurring superoxide.
Concluding remarks

We have generated a mouse model for mitochondrial diabetes by inactivating the Tfam gene in pancreatic β-cells. We found that severe RC deficiency of pancreatic β-cells causes diabetes. Two phases were identified in the progression of mitochondrial diabetes: first pancreatic β-cells display impaired stimulus-secretion coupling; this is later followed by β-cell loss.

We next explored whether respiratory chain-deficient cells are able to undergo apoptosis and are more apoptosis-prone in vivo. We used the previously characterized germline Tfam KO embryos and heart-specific Tfam KO mice and also reexamined the capacity of mtDNA depleted human osteosarcoma cells to undergo apoptosis. Germline homozygous Tfam KO embryos displayed massive induction of apoptosis at E9.5 while homozygous heart-specific Tfam KO mice showed a moderate induction of apoptosis. MtDNA depleted (rho-0) human osteosarcoma cells were able to undergo apoptosis in response to various stimuli. Respiratory chain-deficient cells are thus able to undergo apoptosis and are more apoptosis-prone in vivo suggesting that apoptosis may contribute to RC disease. We also explored whether apoptosis was associated with increased ROS generation. Respiratory chain-deficient cardiomyocytes showed slight induction of antioxidant defenses but normal activities of iron-sulphur containing enzymes that are readily impaired by ROS. These findings indicate that increased ROS generation is not a major disease mechanism in mtDNA-depleted tissues.

The relationship between RC deficiency, ROS generation and cell death was studied in more detail in a mouse model of mitochondrial neurodegeneration (MILON mice). MILON mice were obtained by inactivation of the Tfam gene in neurons of the neocortex and hippocampus. MILON mice developed late-onset neurodegeneration of neocortex and hippocampus at the age of 5-5.5 months. There was a long lag time between onset of severe RC deficiency at the age of 4 months and onset of neurodegeneration approximately one month later. We observed massive induction of neuronal cell death at the age of 5-5.5 months. Respiratory chain-deficient neurons died rather by necrosis than apoptosis as concluded from absence of apoptosis.
markers and presence of an inflammatory reaction. Most surprisingly, MILON mice displayed almost no upregulation of antioxidant defenses and no signs of oxidative protein damage at different ages, i.e. during the development of severe RC deficiency. Interestingly, RC deficient neurons were more susceptible to apoptosis induction by an excitotoxic challenge with kainic acid.

It can be concluded that cell death is a common feature of respiratory chain-deficient tissues. Embryonal cells, cardiomyocytes, and neurons likely utilize different cell death pathways as indicated by the lag time between onset of severe RC deficiency and cell death, presence of apoptosis markers and inflammatory changes. Glycolysis was significantly upregulated in cardiomyocytes but not in neurons. Upregulation of glycolysis may allow execution of apoptosis (Leist et al., 1997) in cardiomyocytes and its absence may explain why respiratory chain-deficient neurons rather undergo necrosis. ROS formation is not a prominent feature of cardiomyocytes and neurons with a severe RC deficiency induced by mtDNA depletion.

We addressed whether mitochondrial $O_2^-$ regulates UCP activity as indicated by studies of isolated mitochondria that used artificial systems to generate high levels of superoxide. We created PAC transgenic mice with a regulated increase in SOD2 enzyme activity to investigate whether superoxide-mediated activation of UCP is a regulatory mechanism in vivo. SOD2 overexpression improved mitochondrial parameters, i.e. oxidative capacity and resistance against induction of mitochondrial permeability, but did not alter UCP1 or UCP3 activities. The resting metabolic rate and the respiratory response to induction of nonshivering thermogenesis were normal in SOD2 overexpressing mice further confirming that SOD2 overexpression did not affect UCP activities. BAT organ weight, mitochondrial mass and UCP1 protein levels were not upregulated excluding the possibility that the failure to observe alterations in UCP activities were compensated for by increased recruitment of the tissue. We conclude that superoxide does not regulate UCP activities under in vivo physiological conditions, however we observed a hitherto unrecognized effect of superoxide on mitochondrial oxidative capacity. The absence of a regulatory effect of endogenously generated superoxide on UCP1 and UCP3 activities thus indicates a
basic difference between effects of exogenously generated and endogenously occurring superoxide.
Acknowledgements

I wish to express my gratitude to all those who have made this thesis possible, especially:

**Nils-Göran Larsson**, my supervisor. It is a very high privilege to be the student of such a distinguished scientist. You taught me to see the critical issues in science. Thank you for sharing your experience and knowledge with me, for your trust, generosity and support throughout my entire PhD period, for your friendship and for every advice I got from you to take important decisions.

**Claes Gustafsson** for your enthusiasm and optimism, for sharing your wealth of ideas with me, for your help and for all valuable advice I got from you.

I would like to thank **Jan-Åke Gustafsson** for providing excellent scientific resources at the Department of Medical Nutrition.

I would like to thank all the collaborators who have contributed to these studies: **Per-Olof Berggren, Martin Köhler, Pierre Rustin, Lars Olson, Eva Lindqvist, Anders Oldfors, Barbara Cannon, Jan Nedergaard, Irina Shabalina, Natasa Petrovic, Emma Backlund, Rolf Wibom and Kjell Hultenby**. You have all provided important expertise in your fields!

I would like to thank present and former members of the Nils-Göran Larsson’s Group: **Caroline Graff** and **Jianming Wang** for fruitful and pleasant collaborations, **Eric Dufour** for showing me the real altruism, for your constant interest and willingness to help, for valuable inputs and fruitful collaboration, **Mats Ekstrand** for introducing me into new protocols of Molecular Biology and for support whenever I requested it, **Aleksandra Trifunovic** for carefully managing the laboratory and providing a platform to perform scientific work, **Anna Hansson, Nicole Hance, Mina Pellegrini, Muegen Terzioglu** for pleasant company in the laboratory, **Anna Wredenberg** for interesting discussions extending beyond the world of science, **Christoph Freyer** for helping to organize laboratory issues and for organizing the journal club, **Martina Gaspari** for cheerful company, **Chan Bae Park** for support, wisdom and fine sense.
of humour, Ivan Khvorostov for cheerful company and Russian anecdotes at late and lonely laboratory hours, Zekye Cansu for support in practical laboratory matters, Lene Sörensen for sharing with me her IT expertise, Anja Rantanen and Hans Wilhelmsson for teaching me the Finish and Swedish Culture.

Furthermore I would like to thank Maria Falkenberg and all her group members for sharing our scientific activities and for having a great time outside the laboratory.

I wish to acknowledge colleagues and friends in/outside the institute, Maria Eriksson for your friendship and your scientific inputs, Annika Wallberg for extremely valuable advice regarding post-doctoral studies, Margaret Warner and Magnus Nord for critical inputs into my thesis, Gil-Shin Shim for providing me with protocols and technical help and Charlotta Lindvall for your company in my early years in Sweden.

Finally I would like to thank my mother and my father, to whom I dedicate my thesis, for your eternal and unconditional support.
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