Mitochondrial and cellular effects of mitochondrial DNA damage

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CONTENTS

CONTENTS 1
ACKNOWLEDGEMENT 3
LIST OF PAPERS 4
ABBREVIATIONS 5
INTRODUCTION 7
Mitochondria: Origin and function 7
Electron transport chain and ATP production 8
The mitochondrial DNA 9
Replication of mtDNA 10
Transcription of mtDNA 12
Vulnerability of the mitochondrial DNA 13
Reactive oxygen species and mutagenic potential 14
The mitochondrial theory of aging and disease 15
mtDNA mutagenesis: contributors 16
Mitochondrial DNA repair 17
Base excision repair in mitochondria 17
Other repair systems in mitochondria 19
Cockayne Syndrome group B protein in BER 20
DNA maintenance and metabolic response 21
Damage: discard or repair? 22
AIMS 23
SUMMARY OF PAPERS 24
DISCUSSION 28
mtDNA damage 28
A function of CSB in mitochondria 28
CSB in mtDNA repair 28
CSB involvement in mitochondrial transcription 29
CSB in ETC organization 30
OGG1 in mitochondria 31

Impact of OGG1 in the repair of 8-oxoG 31
Ogg1 in replication and transcription 31
OGG1 and metabolism 32
mtDNA mutations in aging and disease 32
CONCLUSION 33
FUTURE PERSPECTIVES 34
REFERENCE LIST 35

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Oslo, juli. 2011

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LIST OF PAPERS

PAPER I

Accumulation of mitochondrial DNA damage and bioenergetic dysfunction in *CSB* defective cells

Pia Ø. Osenbroch, Pia Auk-Emble, Ruth Halsne, Rune J. Forstrøm, Ingrid van der Pluijm and Lars Eide

The FEBS Journal, 2009, March2811-2821

PAPER II

Cellular sensitivity of Cockayne Syndrome B deficient cells to oxidative stress is mediated by 8-oxoguanine DNA glycosylase and is associated with inefficient mitochondrial transcription

Ruth Halsne, Janne Strand and Lars Eide

In progress

PAPER III

Novel regulation of citric acid cycle involves mitochondrial DNA repair

Janne Strand, Ruth Halsne, Pia Osenbroch, Ragnhild Skinnes, Wei Wang and Lars Eide

To be submitted

PAPER IV

Lack of the DNA glycosylases MYH and OGG1 in the cancer prone double mutant mouse does not increase mitochondrial DNA mutagenesis

Ruth Halsne, Ying Esbensen, Wei Wang, Katja Scheffler, Rajikula Sugantham, Magnar Bjørås and Lars Eide

To be submitted

ABBREVIATIONS

8-oxoG	-8-oxoguanine
ADP	-adenosine diphosphate
APE1	-AP endonuclease
AP sites	-apurinic/apyrimidinic sites
ATP	-adenosine triphosphate
BER	-base excision repair
bp	-base pair
CS	-Cockayne Syndrome
CSA	-Cockayne Syndrome complementation group A
CSB	-Cockayne Syndrome complementation group B
dNTP	-deoxynucleotide triphosphate
DSB	-double strand break
E.coli	-*Escherichia coli*
ETC	-electron transport chain
faPy-A	-formamidopyrimidine
faPy-G	-formamidopurine
FEN1	-flap endonuclease 1
H chain	-heavy chain
HD	-Huntington's disease
HR	-homologous recombination
IMM	-inner mitochondrial membrane
L chain	-light chain
LP-BER	-long-patch BER
LSP	-light strand promoter
MMR	-mismatch repair
mtDNA	-mitochondrial DNA

mtSSB	-mitochondrial single stranded binding protein
NEIL1	-endonuclease VIII like 1
NHEJ	-non-homologous end-joning
O_2^-	-superoxide anion radical
OGG1	-8-oxoguanine DNA glycosylase
OH·	-hydroxyl radical
OOM	-outer mitochondrial membrane
PCNA	-proliferating cell nuclear antigen
PD	-Parkinson's disease
PGC-1α	-proliferator-activated reseptor γ coactivator-1 alpha
POLRMT	-mtRNA polymerase
POLγ	-mtDNA polymerase γ
ROS	-reactive oxygen species
S.cerevisiae	- *Saccharomyces cerevisiae*
SP-BER	-short-patch BER
TFAM	-mitochondrial transcription factor A
TFB1M	-mitochondrial transcription factor 1
TFB2M	-mitochondrial transcription factor 2
UNG1	-uracil DNA glycosylase
UV	-ultra violet

INTRODUCTION

Mitochondria: Origin and function

The mitochondrion is a double-membraned organelle found in most eukaryotic cells, including plants, fungi, protists and animals. The two membranes comprise all components required for cellular mitochondrial function and have distinct structural properties. While the outer mitochondrial membrane (OMM) is rather permeable, allowing the free diffusion of small proteins and contains specialized channels for import of larger proteins, the inner mitochondrial membrane (IMM) is highly impermeable. Additionally, the IMM has a characteristic folding morphology which forms cristae. Between the two membranes is the intermembrane space, and enclosed by the IMM the mitochondrial matrix is formed (Zorov et al. 2009). The mitochondria contain their own mitochondrial DNA (mtDNA) which encodes a subset of proteins essential for the oxidative phosphorylation system, being the pathway to use redox energy via membrane potential for adenosine triphosphate (ATP) production. The electron transport chain (ETC), embedded in the IMM, utilizie the process of oxidative phosphorylation to produce ATP. The generation of cellular energy, in form of ATP is the main function of the organelle (Burger et al. 2003; Liu and O'Rourke 2009; Mitchell and Moyle 1979). However, the mitochondrion also participates in calcium homeostasis, heat production, cell-signaling and initiation of apoptosis.

According to the endosymbiotic theory, the formation of the mitochondrion is believed to be a key event in the origination of the eukaryotic cell. mtDNA share common features with DNA from prokaryotes, thus a fusion event between an anaerobic host organism and a prokaryote is considered to be a crucial step in the evolution of eukaryotic cells (Gray et al. 1999).

All proteins required for mtDNA replication, transcription and translation are encoded in genes in the nucleus. During the course of time, a number of mitochondrial genes have been relocated to the nuclear genome. However, some genetic material is kept within the mitochondrion and several reasons are proposed for preserving a separate mitochondrial genome. Hydrophobic proteins are difficult to import across mitochondrial membranes and are therefore produced within the mitochondrion (Falkenberg et al. 2007). In addition, the code-usage in mtDNA differs from the universal code in the nucleus. Finally, the assembly of mtDNA encoded subunits in the ETC is rate-limiting and by preserving a mitochondrial genome the subunits are transcribed when needed and occur

independently of the total mitochondrial population (Scheffler 2001; Falkenberg et al. 2007).

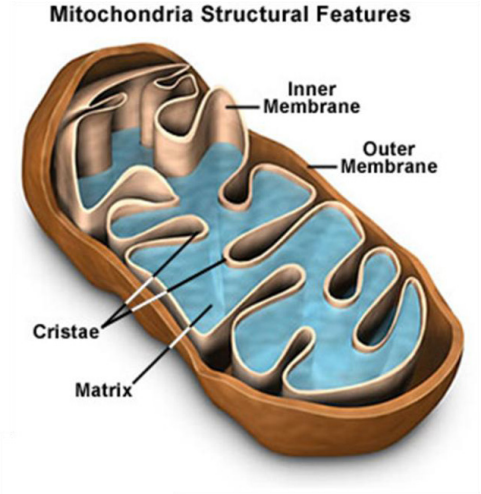

Figure 1: An overview graphic of the mitochondrial organelle divided into distinct compartments as defined by the outer and inner membranes. (Adapted with permission from Florida State University, http://micro.magnet.fsu.edu/cells/mitochondria/mitochondria.html)

Electron transport chain and ATP production

In the cell, carbon nutrients (like glucose) are utilized by catalytic processes (e.g. glycolysis and the citric acid cycle) to produce energy-rich conjugates like NADH and FADH. The oxygen-independent glycolysis is located in the cytosol and catabolize glucose into pyruvate. Pyruvate is then converted to Acetyl-CoA in an oxidative decarboxylation reaction, and the formed NADH is further oxidized in the stepwise catabolizing citric acid cycle inside the mitochondrion. The cycle is allosterically regulated by NADH and other reaction intermediates like pyruvate and oxaloacetate. Lack of cellular oxygen will increase NADH concentrations which will inhibit citric acid cycle. From the citric acid cycle the net energy gain is 3 NADH and 1 FADH, which are subsequently used as electron donors in the ETC. Four enzyme complexes that reside in the IMM constitute the ETC. By means of serial redox reactions in the ETC, oxygen is reduced to water and an electrochemical gradient is established across the IMM. The energy provided by this gradient is utilized by ATP synthase to generate ATP via

phosphorylation of adenosine diphosphate (ADP) (CHANCE and WILLIAMS 1956; Mitchell et al. 1979).

The four ETC enzyme complexes are: NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:cytochrome C oxireductase (complex III) and cytochrome C: O2 oxidoreductase (complex IV). NADH and $FADH_2$ are oxidized by complex I and complex II, respectively. Electrons from NADH and $FADH_2$ are transferred to complex III via ubiquinol: one out of two mobile electron carriers. The other electron carrier, cytochrome C (cyt C), is reduced by complex III, re-oxidized by complex IV and oxygen is reduced into water. As a result of the redox-reactions in complex I, III and IV, protons are translocated across the membrane, creating an electrochemical gradient. The flow of protons back from the intermembrane space into the matrix is coupled to the synthesis of ATP via the ATP synthase (Mitchell and Moyle 1979; Mitchell et al. 1979)

The individual respiratory complexes physically interact with each other to form supercomplexes. Different compositions of supercomplexes exist in the membrane and the ratio of components is proposed to facilitate the stability of the supercomplexes (Vonck and Schafer 2009).

The mitochondrial DNA

The mitochondrial genome is double-stranded, circular and consist of approximately 16 600 base pairs (bp) in human cells and 16 300 bp in mouse cells. The mtDNA encodes two ribosomal RNAs, 22 transfer RNAs and 13 of the approximately 90 proteins present in the respiratory chain of the mammalian mitochondria (Falkenberg et al. 2007). The two mtDNA strands are termed heavy (H) and light (L) strand according to their guanine content, leading to a distinct separation on a cesium chloride density gradient. In contrast to nuclear genes, the mtDNA is compact with no or little intergenic regions. The only noncoding sequence is the noncoding region, which harbors gene elements required for regulation of transcription and replication that are recognized by specific transcription factors.

The number of mtDNA copies in one mitochondrion is in the range of two to ten (Smeitink et al. 2001). mtDNA molecules are organized in structural units termed nucleoides, which are connected to the IMM (Garrido et al. 2003). Proteins active in a variety of dynamic processes, like replication and transcription, are associated with the

nucleoid structure. The mitochondrial transcription factor (TFAM), single stranded DNA binding protein (mtSSB), the mitochondrial helicase TWINKLE, mtRNA polymerase (POLMRT) and mtDNA polymerase γ (POLγ) are some of the proteins associated to the nucleoid structure (Wang and Bogenhagen 2006; Bogenhagen et al. 2003).

mtDNA is maternally inherited, while mitochondria from the sperm are degraded upon fertilization (Sutovsky et al. 1999)**.** However, the exclusion of paternal components is not absolute.

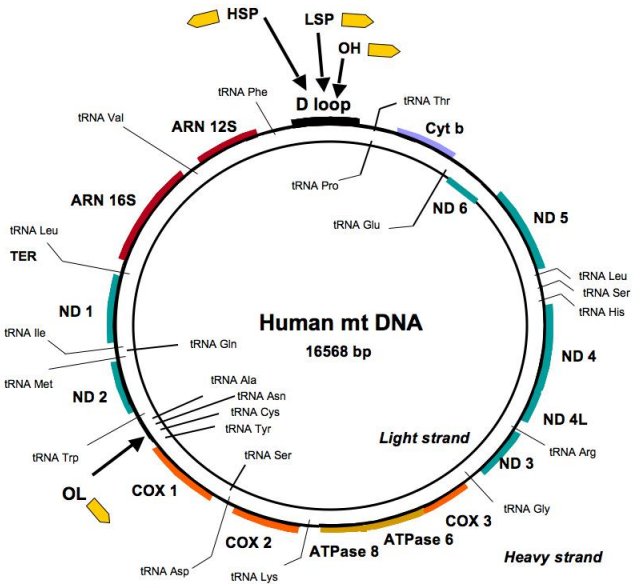

Figure 2: The human mitochondrial DNA molecule. (Adapted with permission from (Bellance et al. 2009)).

Replication of mtDNA

Replication of the mitochondrial genome, in contrast to the replication of nuclear DNA, occurs independently of the cell cycle. Maintenance of an adequate mtDNA copy number is crucial in order to preserve functional mitochondrial activity and cell growth. Both high and low mtDNA copy numbers are associated with negative alterations in mitochondrial function and/or mtDNA. Several actors, like TWINKLE, Peroxisome

Proliferator-activated Reseptor γ Coactivator-1 alpha (PGC-1α) and TFAM, have been shown to function as control mechanisms for mtDNA number in the mitochondrion (Ylikallio et al. 2010; Scarpulla 2002; Jeng et al. 2008). Polymerase γ (POLγ) is encoded in the nucleus and is the only mtDNA polymerase identified (Spelbrink et al. 2000). Together with POLγ, mtSSB and TWINKLE represent the minimal components required for replication at the leading strand in mtDNA (Falkenberg et al. 2007). Based on the presence of ribonucleotides in closed circular mtDNA and analysis of single stranded mtDNA replicative intermediates, a strand-displacement model was hypothesized, as reviewed in Clayton, 1982 (Clayton 1982). In this model, H and L strand replication starts from two distinct origins. H strand replication starts from the H-strand DNA replication origin (O_H) and proceeds in the leading direction until two third of the H strand is synthesized. At this point, the origin for the L strand, L-strand DNA replication origin (O_L), is exposed and synthesis of the L strand starts and continues in the opposite direction until both strands are replicated. However, new data obtained from two-dimensional agarose gel electrophoreses studies of replication intermediates suggest another model, in which conventional leading and lagging strand replication with multiple bidirectional replication forks takes place (Bowmaker et al. 2003).

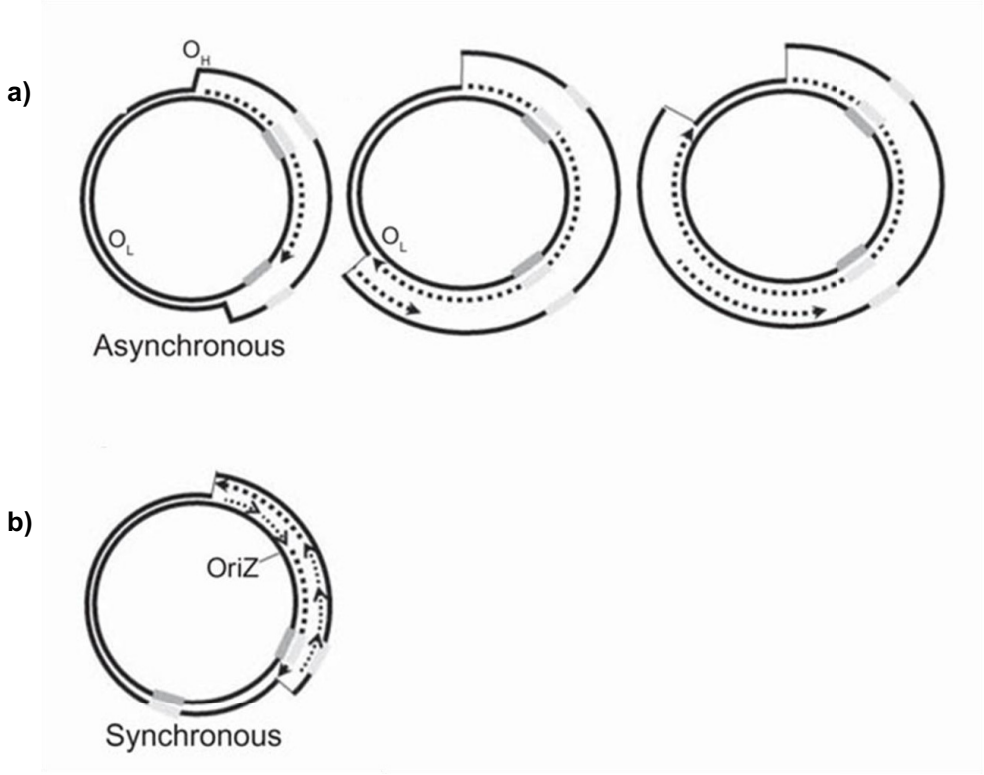

Figure 3: Asynchronous method of replication. **a**) Replication of mtDNA begins in the D loop at the O_H, displacing the light strand from the heavy strand. The light strand is single stranded until synthesis of the nascent heavy strand exposes O_L. At this point, replication of the light strand begins in the opposite direction until both strands have been fully replicated. **b)** Synchronous or coupled replication. In the suggested coupled replication model, replication begins from a zone of replication (OriZ) on the genome and replicates initially bidirectionally via conventional coupled leading- and lagging-strand synthesis. (Adapted with permission from Nature Publishing Group (Copeland and Longley 2008).

Transcription of mtDNA

There are two main transcription promoters in the mtDNA. Transcription from the light strand promoter (LSP) produces short mRNA molecules, which serves as primers for initiation of DNA synthesis at the origin of the H strand (Chang and Clayton 1985;

Falkenberg et al. 2007). Transcription from the H strand is initiated at two different promoter sites, H1 and H2. H1 produces a short rRNA transcript, and from H2 a polycistronic molecule is synthesized and further processed to yield mRNA-, tRNA- and rRNA-molecules. A mitochondrion specific RNA polymerase, POLRMT, is responsible for transcription of the mitochondrial genes (Yaginuma et al 1982). POLRMT requires assistance from the TFAM and one of the two mitochondrial transcription factors, TFB1M or TFB2M, to interact with the promoter and initiate transcription(Falkenberg et al. 2007). A family of mitochondrial transcription termination factors (mTERF 1-4) is suggested to be involved in termination of transcription, with mTERF3 as a negative regulator of mammalian mtDNA transcription (Park et al. 2007).

TFAM is an important component in both transcription and replication. TFAM is associated with mtDNA and present in such large amounts in the mitochondria that the protein is able to cover the entire mtDNA (Kanki et al. 2004). The protein has also been shown to be important to maintain a stable mtDNA number with copy number correlating to the levels of TFAM (Ekstrand et al. 2004). Furthermore, TFAM may play a role in suppression of damaged mtDNA by binding preferably DNA with 8-oxoguanine (8-oxoG) damage (Yoshida et al. 2002; Canugovi et al. 2010). Additionally, TFAM is present in nucleoids together with BER proteins and is believed to play an active role in the response to mtDNA damage (Kanki et al. 2004).

Vulnerability of the mitochondrial DNA

Environmental agents such as UV light, ionizing radiation and genotoxic chemical compounds might alter the chemical properties of the DNA molecule. The other main source of DNA damage is byproducts from normal cellular metabolism, especially reactive oxygen species (ROS). It is demonstrated that mtDNA is more susceptible to oxidative stress compared to nuclear DNA (de Souza-Pinto et al. 2001; Richter et al. 1988). This vulnerability is suggested to be caused by its binding to the inner mitochondrial membrane, where oxidants are formed, and because of the lack of protective histones. A recent study demonstrate that histones are present in the mitochondrial membrane but not directly bound to the mtDNA (Choi et al. 2011). However, some degree of protection might be provided by TFAM which binds to mtDNA (Alam et al. 2003; Kanki et al. 2004).

Reactive oxygen species and mutagenic potential

ROS production is a byproduct of aerobic respiration in the mitochondria. Oxidative damage caused by ROS is associated with disease and is a hallmark of aging. Oxygen reacts with electrons from energy rich reactions such as the ETC and can produce superoxide anion radical (O_2^-). Superoxide anion is unstable, with a short half-life and is normally detoxified by conversion to H_2O_2 by dismutases. Hydrogen peroxide is more stable than superoxide and is able to diffuse through the mitochondrial membrane. The toxic effect of peroxide stems from its ability to form the highly reactive hydroxyl radical ($OH\cdot$) in the presence of divalent metal ions (Fe, Cu, Co or Ni). This radical is suspected to cause the most detrimental effects in mtDNA (Adam-Vizi and Chinopoulos 2006; Andreyev et al. 2005). In addition, ROS can cause many types of oxidative damage to the DNA including DNA strand breaks, base modifications, DNA-protein cross linking and apuridine/apyrimidine sites (AP sites) (Bjelland and Seeberg 2003). Oxidative base modifications include formamidopyrimidines (faPy-A), which is a ring-opened adenine, and faPy-G, which is a ring-opened guanine lesion. Other well known oxidative modifications are bases with retained aromaticity which have potential mispairing properties. Among the four DNA bases guanine has the lowest oxidation potential and is therefore the most easily oxidized base (Kovacic and Wakelin 2001). 8-oxoG is the most commonly used marker for DNA damage (Bjelland and Seeberg 2003).

The mutagenic potential of oxidative lesions is illustrated in repair defective strains of *Escherichia coli* (*E.coli*), *Saccharomyces cereveisia* (*S.cerevisiae*) and mouse (Michaels and Miller 1992; Thomas et al. 1997; Asagoshi et al. 2000; Xie et al. 2004). In *E.coli* the repair system of oxidative damage is called the GO system and consists of MUTT, MUTM and MUTY, corresponding to the mammalian homologues MTH, OGG1 and MYH respectively. The power of the GO system to prevent mutagenesis is shown with the double deficiency of MUTM and MUTY demonstrating a synergistic effect in transversion mutations (Michaels and Miller 1992). For a lesion two prerequisites are necessary to cause mutations. First, the repair enzymes must fail to remove the damaged base before replication and, second, it must have different coding properties than the correct base. 8-oxoG will pair with adenine as frequently as with cytosine (Grollman and Moriya 1993) and if adenine is not removed prior to replication, adenine will be the template for thymidine in replication and lead to GC to TA transversions (Wood et al. 1992; Moriya et al. 1991; Cheng et al. 1992). MUTT hydrolyses 8-oxoG from the nucleotide pool in *E.coli*. Mitochondria have their own pool of deoxynucleotide triphosphate (dNTPs), distinct from the larger nuclear dNTP pool (Marcelino and Thilly 1999). The number of dNTPs in the mitochondrial pool is regulated separately from the

nuclear pool, probably because of the difference in replication control mechanisms of mitochondrial replication compared to nuclear replication (Clayton 2000). Oxidation of free nucleotides, like dUTP and 8-oxo2'-deoxyguanisin triphospate (8-oxo-dGTP) followed by incorporation into DNA contributes to mutations. The mammalian MYH is the major mechanism for the removal of premutagenic 8-oxo-dGTP from the dNTP pool (Nakabeppu 2001).

The mitochondrial theory of aging and disease

The mitochondrial theory of aging postulates that damage caused to mtDNA, proteins and lipids during the lifespan, leads to impaired mitochondrial function and aging of the organism (Harman 1981). An impaired electron transport chain can result in a negative feedback loop generating additional ROS. This additional ROS may erode mtDNA even further, giving rise to a secondary impairment of mitochondrial function. mtDNA damage is considered especially important in aging (Barja and Herrero 2000). Normal aging is associated with declined mitochondrial functions, and a variety of diseases and pathological conditions like neurodegenerative disorders, diabetes and cancer are linked to mitochondrial dysfunction.

The mitochondria participate in many different functions in the cell, and changes in mitochondrial function are detrimental to the cell. Alzheimer's, Parkinson's disease (PD) and Huntington's disease (HD) are neurodegenerative diseases characterized by loss of neurons in the brain due to apoptotic cell death. Mitochondrial abnormalities have been found in neurodegenerative diseases and studies show accumulation of mtDNA damage in these diseases reflecting the importance of mtDNA maintenance (Jeppesen et al. 2011; Yang et al. 2008). The brain is dependent on glucose and has a high energy demand. Maintenance of the mitochondrial integrity for efficient ATP production is important to the brain, and loss of this function is strongly associated with neurodegeneration. It has been shown that neurons are highly sensitive to mitochondrial toxins, and disruption of the mitochondrial respiratory chain by such toxins leads to neuronal death and phenotypes that resembles PD and HD (Yang et al. 2008).

Studies have shown that reduced food intake (caloric restriction) extend the lifespan in organisms such as yeast and rodents. In addition to extended lifespan, caloric restriction has shown to protect against diabetes, cancer and cardiovascular disease. Genetic alterations leading to decreased activity of nutrient-signaling pathways show the same protection against diseases and prolonging effect on lifespan (Fontana et al. 2010).

Several studies show that accumulation of oxidative damage in the DNA is reduced after caloric restriction (Heydari et al. 2007).

To gain more insight into the contribution of mutations in mtDNA a mtDNA-mutator mouse expressing a defective mtDNA polymerase (*POLγ*) has been generated (Trifunovic et al. 2004). The proofreading activity of this mouse is reduced and this leads to an increase in somatic mtDNA point mutations. In line with the theory of aging, increasing amounts of mtDNA mutations cause a progressive respiratory chain deficiency, showing reduced life span and a premature aging phenotype of the mouse. No cancer is associated with the phenotype of this mouse.

mtDNA mutagenesis: contributors

Mutations in mtDNA are underlying factors in many mitochondrial diseases. Additionally, mtDNA mutations are found to be associated with neurodegenerative diseases, diabetes, cancer and aging. Mutations in mtDNA can arise from exposure to environmental mutagens, from unrepaired DNA damage, replication error in DNA synthesis and/or from defects in the mtDNA degrading system (Krokan et al. 1997).

Since polγ is the only DNA polymerase found present in the mitochondria, polγ is most likely to be the responsible polymerase for replication errors leading to mutations. The POLγ has a catalytic subunit that has DNA polymerase, 3'-5' exonuclease and 5' dRP lyase activities (Graziewicz et al. 2006). The fidelity of POLγ is high for nucleotide selectivity, exonucleolytic proofreading and for base incorporation in short, repetitive sequences. However, the frameshift fidelity of POLγ when copying homopolymeric sequences longer than four nucleotides is lower (Longley et al. 2001). A study claim that 85 % of mutations detected *in vivo* are probably due to misincorporation by the mitochondrial polymerase (Zheng et al. 2006). There is no study that estimates how much DNA damage contributes to mutations in the mtDNA.

Whether mutations arise mainly from replication errors inserted by POLγ or by unrepaired damage remain unclear.

Mitochondrial DNA repair

Mitochondria have their own distinct repair systems which resemble the nuclear systems but are less complex. The mitochondrial genome does not encode repair proteins and thus, they are encoded by nuclear genes, and imported into the mitochondria. Mitochondrial proteins might differ from the nuclear versions by splice variation, post-translational modification or alternative translation-initiation (de Souza-Pinto et al. 2008).

Base excision repair in mitochondria

The mitochondrial BER pathway is a multistep process for removal and replacing chemically altered bases, such as oxidized base lesions, in addition to repair of apurinic/apyrimidinic (AP) sites and single strand breaks. The repair pathway is evolutionally conserved and is initiated by different DNA glycosylases that recognize and excise specific base lesions. The remaining AP-site is processed by an AP endonuclease (APE1) prior to the action of POLγ filling the single nucleotide gap. The remaining nick is sealed by DNA ligase IIIa (Bogenhagen et al. 2001; Pinz and Bogenhagen 1998; de Souza-Pinto et al. 2008). These four steps constitute the short-patch BER (SP-BER). In addition, activity of Long-patch BER (LP-BER) has been detected in mitochondrial extracts (Liu et al. 2008a). From the gap-filling step the BER pathway can be sub-divided into SP- and LP-BER. While in SP the polymerase fills the single nucleotide gap, the LP performs a repair synthesis of several nucleotides (2-13). The replaced nucleotides are removed by the flap endonuclease 1, FEN1, whose activity is stimulated by proliferating cell nuclear antigen (PCNA) (Szczesny et al. 2008; Liu et al. 2008b). Both FEN1 and DNA2, a mitochondrial nuclease/helicase needed for LP, are found in the mitochondria (Szczesny et al. 2008; Zheng et al. 2008). The discovery of LP-BER in mitochondria is recent and additional studies are needed to determine the exact mechanism of this sub-pathway.

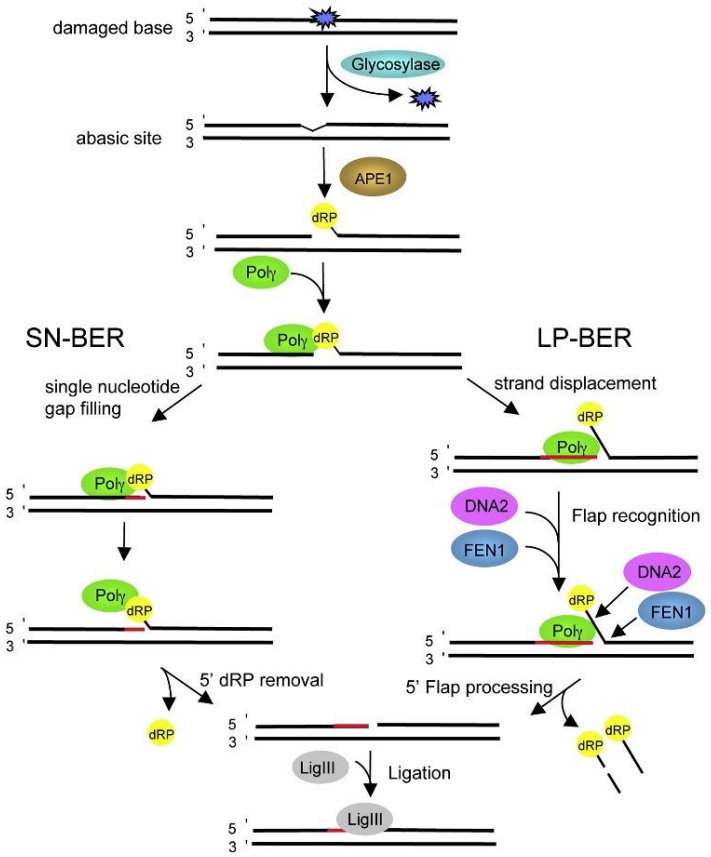

Figure 4: mtDNA base excision repair: An oxidized or damaged base is excised by a specific glycosylase, leaving an abasic site, after which APE1 generates a strand break 5′ to the lesion and a 5′deoxyribose phosphate group on the downstream DNA. Repair can proceed via the single nucleotide BER (SN-BER) pathway (left) or the LP-BER pathway (right). In SN-BER, the single-nucleotide gap is filled by POLγ and the 5′dRP moiety is removed by POLγ's dRP lyase activity to make a ligatable substrate. In LP-BER, DNA synthesis by POLγ displaces the downstream strand to produce a 5′flap structure, which can be processed by DNA2 and FEN1. (Adapted with permission(Krishnan et al. 2008)).

DNA glycosylases are encoded in the nucleus and are actively transported to the mitochondria. Damaged bases recognized by DNA glycosylases are released from the sugar by breaking the N-glycosylic bond. The 8-oxoguanine DNA glycosylase (OGG1) and the endonuclease III homologues 1 (NTH1) are the main DNA glycosylases for

removal of the oxidized bases lesions from mtDNA like 8-oxoG and Fapy, respectively. The human OGG1 (hOGG1) exist in two spliceforms, the α- and β- hOGG1, with the β-hOGG1 containing a mitochondrial localization signal. However, the β-HOGG1 exhibits no glycosylase activity and αhOGG1 is most likely participating in 8-oxoG incision in mitochondria (Hashiguchi et al. 2004). In addition to OGG1, endonuclease VIII like (NEIL1) removes faPy and is found in mitochondria (Hu et al. 2005). OGG1 is, together with NEIL1 and NTH1, bi-functional DNA glycoslyases with 3'AP lyase activity to perform single strand breaks in addition to the glycosylase activity. In mouse, NTH1 is mainly transported to mitochondria while the human NTH1 is mostly localized to the nucleus (Karahalil et al. 2003; Ikeda et al. 2002). Uracil DNA Glycosylase 1 (UNG1) removes uracil from DNA, a result either from a deaminated cytosine (Lindahl 1993) or a misincorporated dUMP instead of dTMP during replication (Krokan et al. 1997). Misincorporated adenine opposite 8-oxoG is recognized and removed by MYH, which is found to localize to the mitochondrial matrix (Takao et al. 1998).

Other repair systems in mitochondria

While BER is a multi-step repair pathway, direct repair is a single step process and does not involve breaking of the phosphodiester bond (Hakem 2008). The main direct repair protein, methyl guanine methyl transferase (MGMT), has not been found in mitochondria. However, some methyl-groups are repaired in mtDNA whereas for the more complex alkylation damage no repair has been detected (LeDoux et al. 1992). A photolyase protein capable of direct repair of ultra violet (UV) mtDNA damage is directed to mitochondria in *S.cerevisiae* (Yasui et al. 1992), while no photolyase activity is detected in mammalian cells.

Mismatch repair (MMR) is a conserved DNA repair pathway for recognition and repair of misincorporated bases, insertions and deletions arising during DNA replication and recombination (Jiricny 2006) and thus, improving the fidelity of replication. Proteins participating in MMR have been detected in mitochondria from *S.cerevisiae* (Chi and Kolodner 1994), but not for mammalian mitochondria. However, low but significant MMR activity has been detected in mitochondrial extracts from rats. It is debated whether MSH2, a key MMR protein, is present in mitochondria and the findings that Y-box binding protein (YB-1), a multifunctional protein, participates in mitochondrial MMR, indicate a novel pathway distinct from the nuclear one (Mason et al. 2003; de Souza-Pinto et al. 2009).

Double strand break (DSB) are induced by exogenous sources, like ionization radiation and intracellular sources, like ROS, during failure from replication and repair events (Larsen et al. 2005). DSBs are repaired by two distinct repair pathways in the nucleus: homologous recombination (HR) and non-homologous end-joining (NHEJ). *In vitro* studies indicate recombination activities of both pathways in mammalian mitochondrial extracts (Bacman et al. 2009; Fukui and Moraes 2009). Identification of proteins that binds double-stranded DNA ends in mammalian mitochondrial extracts support findings of recombination activity. However, the end-joining activity was independent of Ku70, a protein known to be involved in nuclear double-stranded DNA repair, suggesting that the mechanism is different from that of mitochondria (Coffey et al. 1999).

Cockayne Syndrome group B protein in BER

Cockayne Syndrome (CS) is a severe inherited disease characterized by premature aging and neurological impairment. The patients suffer from hearing loss, retarded growth and other symptoms that resemble accelerated aging (Nance and Berry 1992; Weidenheim et al. 2009; Licht et al. 2003). CS patients have a mutation in the CS complement group A (CSA) or B (CSB) protein, most commonly in CSB. These proteins are known to participate in DNA maintenance through transcription coupled repair (TC-NER) (Licht et al. 2003). In CS patients, TC NER is defective for the repair of UV induced cyclobutane pyrimidine dimers.

The CSB gene encodes a 168 kDa protein (Troelstra et al. 1992) with homology to the SWI/SNF family of proteins. The CSB include an acidic region, a glycine rich region, two putative nuclear localization signal sequences, and an ATPase domain. The ATPase domain includes seven conserved ATPase motifs found in all SWI/SNF family proteins and supports DNA helicase activity.

csb$^{m/m}$ cells accumulate oxidative mtDNA damage (Osenbroch et al. 2009; Stevnsner et al. 2008) and are defective in repair of 8-oxoG (Stevnsner et al. 2002). When *ogg1* is additionally mutated a strong increase of oxidative damage accumulation is apparent (Osterod et al. 2002). Together, these results are strongly implying that CSB is involved in BER of oxidative lesions in genomic DNA and maybe mitochondrial DNA.

In 2010, two distinct publications confirmed the presence of the CSB protein in mitochondria (Aamann et al. 2010; Kamenisch et al. 2010). CSB was demonstrated to localize to mitochondria, with increased mitochondrial distribution following oxidative stress induced by menadione (Aamann et al. 2010). In addition to mitochondrial

localization, CSB (and CSA) was found to form a complex with mtDNA, OGG1 and mtSSBP upon oxidative stress. The exact function of CSB in mitochondria and the connection to OGG1 is still unclear. It is hypothesized that CSB influences the 8-oxoG repair activity by regulating OGG1 expression and/or activity. This hypothesis is supported by the work of Dianov et al 1999 who report reduced OGG1 expression in *csb*$^{m/m}$ cells (Dianov et al. 1999) and by increased expression of OGG1 in CSB depleted cells retransfected with CSB (Stevnsner et al. 2002). Elevated levels of mtDNA mutations were found in reduced subcutaneous fat tissue of aged mice with CSA or CSB deficiency. Reduced fat in subcutaneous tissue is a hallmark of aging and this correlates with a role of CSB (and maybe CSA?) in protecting DNA (Kamenisch et al. 2010)

DNA maintenance and metabolic response

In *S.cerevisiae*, mitochondrial metabolism via the protein Aconitase is involved in mtDNA maintenance (Chen et al. 2005). Mitochondrial Aconitase catalyses the isomerization of citrate to isocitrate in the citric acid cycle. This protein is sensitive to oxidative stress and it is readily used as a marker of oxidative stress (Bulteau et al. 2003). Aconitase has been found to interact with OGG1 independently of mtDNA repair, and a role of human OGG1 as a mitochondrial Aconitase chaperone has been postulated (Panduri et al. 2009). This action might be important in the defense against toxicity induced by oxidative damage. The yeast study demonstrates a new role of mitochondrial Aconitase (Chen et al. 2005). They found mitochondrial Aconitase to bind mtDNA and be crucial for DNA maintenance, thereby linking cellular metabolism to mtDNA maintenance (Chen et al. 2005). Furthermore, mitochondrial Aconitase is found to interact with the β-hOGG1 in lung epithelial cells (Panduri et al. 2009). By overexpressing a mutated α-hOGG1, with no glycosylase activity and with a mitochondrial translocation signal, mitochondrial dysfunction and apoptosis was prevented. The lack of glycosylase activity suggests a novel mechanism of OGG1 as a mitochondrial Aconitase chaperone protein that prevents mitochondrial dysfunction and apoptosis induced by oxidative damage.

A mouse deficient in the NEIL1 DNA glycosylase show increased levels of mtDNA damage and deletions when compared to wild-type (Vartanian et al. 2006). In response to deficient DNA maintenance, the mouse develops severe obesity, dyslipidemia and fatty liver disease. Whether mtDNA damage is the cause of this metabolic syndrome or the consequence of nuclear DNA damage and disease are discussed by the authors (Vartanian et al. 2006). For further examination of the possible metabolic response to mtDNA

damage, the NEIL1 deficient mice were exposed to oxidative stress in the form of a high-fat diet (Sampath et al. 2011). This greatly enhanced the development of obesity in NEIL1 deficient mice, in addition to a significant reduction in mtDNA and protein content. The authors propose that animals lacking NEIL1 are less tolerant of oxidative DNA damage and that deficient mtDNA maintenance leads to disease.

The metabolic responses to oxidative mtDNA damage are complex and involve many processes. The nuclear transcription factor p53, a tumor suppressor gene involved in apoptosis in mitochondria, has been shown to induce cell arrest in response to DNA damage (Lakin and Jackson 1999). The Sirtuin family of deacetylases consists of seven proteins, some of them located in the mitochondria. Especially the SIRT1 is activated by the redox status in the mitochondria, more specifically by NADH (Alcain and Villalba 2009). A study shows that the fate of neuronal progenitors is related to SIRT1 as a mediator of the redox status (Prozorovski et al. 2008). The expression of both p53 and SIRT1 is upregulated after oxidative stress (Lakin and Jackson 1999; Alcain and Villalba 2009).

Damage: discard or repair?

Considering the multicopy nature of mtDNA it was originally believed that repair of mtDNA was unnecessary and the theory of mtDNA degradation in response to damage was therefore accepted. With time DNA repair systems have been demonstrated in mitochondria.

Selective degradation of damaged DNA is shown in mitochondria. A study by Mita and coworkers showed that the mitochondrial genome of HeLa cells accumulated few mutations in response to chemical carcinogens, suggesting that mtDNA contained large amounts of damaged DNA that was not replicated (Mita et al. 1988). Several studies have targeted site-specific restriction endonucleases to mitochondria as a mean to demonstrate that large amounts of double-strand breaks cause mtDNA degradation (Kukat et al. 2008; Bacman et al. 2009; Fukui and Moraes 2009), while low levels of DSBs lead to recombination (Bacman et al. 2009). Shokolenko and coworkers showed that extensive oxidative damage lead to degradation of mtDNA. Additionally, when APE1 activity is inhibited, the degradation of mtDNA is further enhanced (Shokolenko et al. 2009). It is speculated that DSB triggers mtDNA degradation, probably by forming a stalled complex with DNA or RNA polymerases on the damaged mtDNA template (Liu and Demple 2010). Both strand breaks and AP sites lack coding information and it is important to repair or eliminate such damage to prevent mutagenesis. The process of degradation of damaged DNA is unique to the mitochondrial DNA.

AIMS

Mitochondrial dysfunction is believed to be the underlying cause in many life-style diseases like diabetes, neurodegenerative diseases, cancer and DNA damage in general. Damage to the mitochondrial genome is thought to be the main factors leading to mitochondrial dysfunction. In this study, we aimed to use cellular models with apparent signs of mitochondrial dysfunction or, alternatively, deficiency in the mitochondrial DNA maintenance capacity, to study how damage to the mitochondrial genome manifest into mitochondrial or cellular dysfunction. The purpose was to evaluate the impact of mtDNA damage on the processes resulting in mitochondrial dysfunction.

In addition to mtDNA damage, nuclear DNA damage, metabolic alterations and environmental toxins can result in mitochondrial dysfunction. In the present study we aimed to investigate how the contribution of mtDNA damage leads to mitochondrial dysfunction.

Four different systems were used to assay this interaction:

1) Mouse cells with a *CSB* truncated protein, mimicking the damage human disease Cockayne syndrome (CS), were used as a model. CS is a neurodegenerative progressive disorder which resembles a mitochondrial disease. The CSB protein is involved in DNA maintenance in the nucleus but has also been shown to participate in regulation of proteins with localization to mitochondria.
2) Cells from genetically modified mice that were either deficient in the base excision repair (BER) (*ogg1*$^{-/-}$*),* the nucleotide excision repair (NER) of UV induced damage (*csb*$^{m/m}$), or both, were used in this study. By looking into mtDNA processes in these mutant cells the relative contribution of the two separate repair-mechanisms in mtDNA could be estimated.
3) Oxidative stress is formed naturally in the cell and is known to damage mtDNA. Aconitase is a protein participating in the citric acid cycle and is readily used as a marker of oxidative stress. Using the cell lines deficient in BER, (*ogg1*$^{-/-}$*)* Aconitase was used to look into how metabolic changes interfere with the mitochondrial function.
4) In order to investigate whether mtDNA damage leads to mutations we used a mouse deficient in two DNA glycosylases that is participating in BER (*myh*$^{-/-}$ */ogg1*$^{-/-}$). OGG1 and MYH are known to localize both to nuclear and mitochondrial compartments.

SUMMARY OF PAPERS

Accumulation of mitochondrial DNA damage and bioenergetic dysfunction in *CSB*-defective cells

Cockayne syndrome (CS) is a severe inherited human disease characterized by premature aging and neurodegeneration. The disease is caused by a mutation in the *CSB* gene, whose protein product is involved in transcription coupled repair of DNA in the nucleus. A $csb^{m/m}$ mouse model was utilized to investigate mitochondrial DNA damage in *CSB*-defective cells.

CS shares many clinical signatures associated with mitochondrial dysfunction, like developmental problems, neurodegeneration, muscular and skeletal defects, deafness, and premature aging as well as accumulation of oxidative DNA damage. In order to evaluate potential bioenergetic defects in $csb^{m/m}$ cells, we analyzed the sensitivity to inhibition of bioenergetic defects in these cells. Finally, mitochondrial function was evaluated by measuring the respiration capacity and electron transport chain (ETC) organization.

We found that $csb^{m/m}$ cells were more sensitive than wt to all bioenergetic inhibitors, which is indicative of an energy-critical phenotype. While the sensitivity to rotenone, 3NP, antimycin, KCN and oligomycin in general was stronger than in wt cells, the relative sensitivity to 2-deoxyglucose, an inhibitor of glycolysis, was especially prominent. This implies that the $csb^{m/m}$ cells are relatively dependent on glycolytic energy conversion. In association with the bioenergetic susceptibility, we identified altered organization of the ETC subunits into supercomplexes. These alterations correlated to some extent with the mitochondrial DNA (mtDNA) damage in that liver mtDNA from $csb^{m/m}$ accumulated more 8-oxoG and were less capable of being amplified by PCR than wt mtDNA. However, we could not directly couple mtDNA damage to the alterations in ETC organization, suggesting that the CSB protein is involved in both mtDNA and mitochondrial function. Our data indicate that mitochondrial dysfunction could be a possible underlying cause of Cockayne Syndrome.

Cellular sensitivity of Cockayne Syndrome B deficient cells to oxidative stress is mediated by 8-oxoguanine DNA glycosylase and is associated with inefficient mitochondrial transcription

The study described above implies that CSB is involved in the maintenance of mitochondrial DNA (mtDNA). In the process of this PhD-project, another group demonstrated the presence of CSB in mitochondria and involvement in the base excision repair of 8-oxoG from mtDNA. In order to evaluate the impact of CSB in the maintenance of mtDNA, we used cells from genetically modified mice that were either deficient in base excision repair (*ogg1*$^{-/-}$), nuclear transcription-coupled repair of UV-induced DNA damage (*csb*$^{m/m}$), or both, to study the relative contribution of OGG and/or CSB on mtDNA repair, as well as possible overlap. Since our work has demonstrated that CSB was additionally implicated in cellular function beyond mtDNA damage (paper I), we further investigated the impact on mtDNA damage on both cellular replication and transcription. Our results demonstrated that the mutant cells were sensitive to the mitochondrial oxidant menadione, in line with a function of OGG1 and CSB in repair of mtDNA. Interestingly, the phenotype of the cells indicates that CSB belongs to the same complementing group as OGG1 in the repair of oxidative damage, but not for the removal of UV-induced damage. Our data suggest that CSB functions downstream of OGG1 and facilitates efficient progression of repair intermediates. Interestingly, mtDNA damage was not specifically inhibiting mitochondrial replication but rather mitochondrial transcription. This indicates that oxidative stress-induced mtDNA damage is particularly detrimental for mitochondrial transcription. Surprisingly, the mitochondrial transcription rate in mtDNA repair mutants is less than 10 % of that in wt cells, suggesting that spontaneously formed mtDNA damage have the potential to inhibit transcription if not repaired efficiently.

Novel regulation of citric acid cycle involves mitochondrial DNA repair

Oxidative mtDNA damage is formed under conditions of oxidative stress. Such oxidative stress is also expected to modify cellular processes like intramitochondrial metabolic pathways. The function of mitochondrial Aconitase is to catalyze the isomerization of citrate to isocitrate in the citric acid cycle. It is one of the most sensitive enzymes to oxidative stress in the mitochondria, and it is readily used as a marker of oxidative stress. Interestingly, it has been found to interact with OGG1 independently of mtDNA repair. Here, we sought to understand the interplay between metabolism and mtDNA repair during and after oxidative stress. Our results suggest that the OGG1-Aconitase interplay represents a molecular link for regulating aerobic metabolism in a mtDNA-dependent manner. Hence, these results also demonstrate that mtDNA damage modifies mitochondrial function by regulating metabolic pathways inside the organelle.

Impact of mitochondrial DNA mutations in a DNA repair deficient mouse model with elevated carcinogenesis and shortened lifespan

Reactive oxygen species (ROS) are formed as natural byproducts during aerobic metabolism. The 8-oxoG DNA glycosylase 1 (OGG1) and MutY homologue 1 (MYH1) are proteins participating in Base Excision Repair (BER) of DNA. Studies from *E.coli* have shown that deletions of both enzymes lead to a synergetic increase in G to T transversions in DNA. A mouse model deficient in *OGG1* and *MYH* suffers from increased tumorigenesis and shortened lifespan. Mitochondrial DNA (mtDNA) mutations have been linked to many forms of cancer.

It has been shown that damage accumulates in nuclear DNA in the OGG/MYH double deficient animals, and our laboratory has shown that OGG1 is especially important for the removal of 8-oxoG from mtDNA. Since both OGG1 and MYH have shown localization to mitochondria, we hypothesized that accumulation of mtDNA damage could lead to mutations with age and, thus be manifested into mitochondrial dysfunction. Examination of tissue from both young and adult mice may indicate to what extent possible mitochondrial dysfunction was responsible for the increased carcinogenesis (and shortened lifespan) of these animals. Tissue (brain, lung and liver) from young and adult mice were analyzed for mtDNA damage and mutations. The function of the mitochondria from the different tissues was analyzed for comparison, as measured by the ability to generate large PCR products. Despite the absence of OGG1, we could not detect reduced mtDNA integrity in the repair double knockout mice except for in liver. Possibly, this is due to the fact that 8-oxoG, that is expected to accumulate, is not detected by this method. Surprisingly, we found no differences in mutation levels between knockout mice or wt and no accumulation of mutations with age. In line with this data, mitochondrial function was normal in all tissues at both ages, except in brain, where we discovered a significant 25 % reduction in complex I activity and corresponding respiration capacity. We suspect that efficient backup mechanisms for removal of premutagenic 8-oxoG is responsible for the innocuous phenotype.

DISCUSSION

Our aim was to look closer into the connection between mtDNA damage, mitochondrial dysfunction and aging/disease.

mtDNA damage

The influence of mtDNA damage was evaluated in cells that lack either OGG1 or CSB proteins. Thus, unrepaired mtDNA damage will alter processes that require these protein functions. By comparing phenotypes during different situations, we have compared impact of mtDNA damage on transcription, replication, mutagenesis and mitochondrial metabolism. Our data indicate that both OGG1 and CSB function in the base excision repair of mtDNA damage, independently of the nuclear role of the same proteins. Moreover, important differences in cells deficient in one or both of these functions indicate what processes that are associated with mtDNA maintenance.

A function of CSB in mitochondria

CSB in mtDNA repair

The role of CSB in the repair of UV adducts via the nucleotide excision repair pathway has been known for some time (Balajee et al. 2000), and hypersensitivity to UV irradiation is a hallmark of the CS phenotype (Stevnsner et al. 2008). More recently, it was shown that extracts from *csb*$^{m/m}$ cells are deficient in repair of 8-oxoG (Dianov et al. 1999). Additionally, Stevnsner and coworkers demonstrated a role of CSB in repair of 8-oxoG in mitochondrial DNA (Stevnsner et al. 2002). The stimulation of repair was exclusive for 8-oxoG which is the substrate of OGG1 in the BER pathway. However, previous studies have shown that repair of 8-oxoG in the mitochondria involves only BER and not NER, in which CSB is known to participate (Anson et al. 1998). Furthermore, CSB has been found in the nucleus exclusively. By characterization of the *csb*$^{m/m}$ mice cells we found that they were sensitive to all inhibitors of mitochondrial complexes and especially to the inhibitor of glycolysis (Paper I). In addition, we show that *csb*$^{m/m}$ cells accumulate mtDNA damage, supporting a role of CSB in mtDNA

maintenance (Stevnsner et al. 2002). Furthermore, the *csb*$^{m/m}$ cells are sensitive to induced mitochondrial oxidative damage (PAPER II) in an OGG1-dependent manner. In sum these results imply a role of CSB in response to oxidative damage in mtDNA, which is independent of the nuclear function in nucleotide excision repair. For CSA, the other complement group protein causing Cockayne Syndrome, separate roles in response to UV and oxidative damage was found (Nardo et al. 2009). In 2010 two studies report of CSB found in mitochondria (Kamenisch et al. 2010; Aamann et al. 2010). Both CSA and CSB were found to relocalize to mitochondria after exposure to oxidative stress. This is consistent with our and others result suggesting a role of CSB in processing mtDNA damage. Finally, the CS proteins were found in connection with OGG1 (Kamenisch et al. 2010), demonstrating the CSB sensitivity in an OGG1-dependent manner (PAPER II). Our result, showing csbm/m sensitivity against menadione, coincides with the result of CSB to translocate to mitochondria in response to menadione (Aamann et al. 2010).

We believe that CSB acts downstream of OGG1 in the BER pathway, a role independent of its function in the nucleotide excision repair. The exact mechanism of CSB in mtDNA repair is still unclear. It is suggested that CSB may act as a scaffold or organizing protein to connect the BER process to the mtDNA in the inner mitochondrial membrane (Aamann et al. 2010). It is also suggested that CSB is important to reduce the toxic effect of repair intermediates (PAPER II). CSB has been shown to stimulate the catalytic activity of apurinic endonuclease 1 (APE1), and protect against toxic effects from APE1 substrates (Wong et al. 2007). The exact mechanism for import of CSA and CSB into the mitochondria is not clear. However, since the CS proteins are relocalized in response to mtDNA damage, a functional role in processing these damages is clearly supported.

CSB involvement in mitochondrial transcription

Looking into cellular mechanisms we found that oxidative stress inhibits mtDNA replication similarly as nuclear DNA replication. For the mitochondrial transcription we found it to be inefficient in mutant cells compared to wt cells, indicating that repair of mtDNA is more important to facilitate efficient transcription rather than efficient mtDNA replication (PAPER II). CSB is also involved in controlling the levels of mitochondrial transcripts after oxidative stress (PAPER II). The influence of mtDNA repair on mitochondria transcription efficiency is a relatively unexplored field, and for CSB a connection to transcription is mainly due to findings in the nucleus, where it has been shown to bind to a fraction of the RNA polymerase II (RNA pol II) which catalyzes the transcription of DNA to synthesize precursors of mRNA, snRNA and micro RNA (van

Gool et al. 1997). Several models have been proposed regarding how CSB plays a role in the rescue of RNA pol II complexes that are stalled at oxidative DNA lesions, but the details remain unclear (Stevnsner et al. 2008; de et al. 2004). However, RNA pol II has not been found in mitochondria.

The coexistence of TFAM in the nucleoid (Bogenhagen et al. 2003) might provide a molecular mechanism for how the mtDNA damage facilitates transcription arrest. TFAM is present at levels sufficient to cover mtDNA entirely and binds preferentially to branched DNA and mtDNA damage (Kanki et al 2004). Recently, TFAM was found to colocalize with CSB in response to oxidative stress in mitochondria (Aamann et al. 2010) supporting a role of CSB in transcription.

CSB in ETC organization

According to the mitochondrial theory of aging accumulated mtDNA damage could lead to impaired mitochondrial function. The respiration in $csb^{m/m}$ cells is normal, but the arrangement of respiration complexes into supercomplexes in the inner mitochondrial membrane is altered in $csb^{m/m}$ cells (PAPER I). The rearrangement of supercomplexes was not due to inhibition of transcription/replication, and might be related to a yet unidentified role of the CSB protein. In summary, these data implies a mitochondrial dysfunction as an underlying cause of Cockayne Syndrome. Reports of the presence of CSB protein in mitochondria strengthen the theory of mitochondrial influence in the pathology of Cockayne Syndrome (Aamann et al. 2010; Kamenisch et al. 2010).

OGG1 in mitochondria

Impact of OGG1 in the repair of 8-oxoG

An increase in oxidative damage in mitochondria does not give rise to increased mutation rates, at least not by the action of OGG1 (PAPER IV). Possible back-up systems for 8-oxoG lesions in mitochondrial DNA could be the DNA glycosylase NEIL1. A weak, but not significant excision activity towards 8-oxoG was detected (Jaruga et al. 2000). In HeLa cell a second OGG (OGG2) has been identified and partially characterized (Hazra et al. 1998). While OGG1 preferentially excise 8-oxoG opposite a guanine, the OGG2 shows activity for 8-oxoG opposite guanine or adenine. In addition, the same group found a DNA glycosylase with sequence homologue to MUTM (OGG1) and NEI, Nei homolog (NEH1) (Hazra et al. 2002). NEH1 show a weak activity for 8-oxoG: C and stronger activity for 8-oxoG: A activity, but the excision activity is stronger for FaPy lesion than 8-oxoG. None of these proteins have been detected in mitochondria. Finally, mismatch repair activity of single G-T and G-G mismatches has been detected in mitochondria and shown to contribute to remove oxidative lesions from mtDNA (Mason et al. 2003). If the G-T mismatch is not recognized by BER, MMR could function as a back-up mechanism.

Ogg1 in replication and transcription

The transcription rate was as ineffective for OGG1 deficient cells as for CSB deficient cells, while the replication rate in the mutants did not differ compared to wild-type. The blocking potential of 8-oxoG is debated but the lesions is generally believed not to cause a block for the polymerase (Tornaletti 2005). A plasmid model demonstrate a transcription blocking potential of 8-oxoG in cells with functional BER (Kitsera et al. 2011). The excision of 8-oxoG by OGG1 leads to the generation of a single-strand break which in turn blocks the RNA polymerase II. CSB is known to improve gene expression in the presence of single-strand breaks (Khobta et al. 2010). According to our hypothesis of CSB working downstream of OGG1 in BER, the repair intermediates from oxidative lesions probably have a higher blocking potential than 8-oxoG.

OGG1 and metabolism

According to the aging theory, oxidative damage manifest into mitochondrial dysfunction and disease. We describe a novel ROS mediated regulation of the TCA cycle by the OGG1 mtDNA repair enzyme. The reversible inactivation of Aconitase connotes the importance of blocking the TCA cycle under conditions of superoxide anion stress. Reactivation of Aconitase is dependent on the mtDNA repair enzyme OGG1, and correlates with its repair function. We find that inactivated Aconitase is depleted in the absence of OGG1, which supports a role of OGG1 as a scaffold protein to facilitate enzymatic recovery and protection against degradation, as has been suggested (Panduri et al. 2009).

mtDNA damage repair of OGG1 is additionally shown to be important for the differentiation potential of neuronal stem cells. Accumulation of mtDNA damage in OGG1 deficient stem cells contributes to a shift towards astrocytic lineage which may be related to redox condition and SIRT1 activation in neural stem cells (Prozorovski et al. 2008) (Wei et al. 2011 in press). The redox ratio, which is a key regulator of SIRT1, is influenced by mitochondrial activity thus, the underlying mechanism for how mtDNA damage influence the differentiation in stem cells might be influenced by how mitochondrial alterations in NADH/NAD ratio regulate SIRT1 (Wei et al. 2011 in press).

mtDNA mutations in aging and disease

mtDNA mutations are associated with aging and disease. The POLγ mouse with an elevated level of mutations show reduced lifespan and -related phenotypes, supporting the mitochondrial theory of aging. However, in the mouse deficient in MYH and OGG1 no elevated levels of mutations were found (PAPER IV). This mouse also shows reduced lifespan in addition to cancer development (Xie et al. 2004). The oxidative damage load is high in tissue from *myh*$^{-/-}$/*ogg1*$^{-/-}$ (Russo et al. 2004) and *E.coli* with deficiency in the same DNA repair genes demonstrate a strong mutation frequency (Michaels and Miller 1992). As we could not find mutations in the mouse deficient in oxidative mtDNA repair our results (PAPER IV) support the theory of mutations arising primarily from replication errors (Zheng et al. 2006) rather than from unrepaired damage. More research is required in this field to elucidate how mutations emerge and how the mutation level in mtDNA is related to aging and disease.

CONCLUSION

In conclusion, the use of CSB and OGG1deficient cells enable us to evaluate the impact of mtDNA damage on mitochondrial function and cellular homeostasis. Since these proteins are involved in mtDNA repair, it is expected that at least some of the phenotypes of the *csb*$^{m/m}$ and *ogg1*$^{-/-}$ mutants would be representative under conditions that are associated with increased mtDNA damage, like during disease and aging.

Our observations contribute to unravel apparent DNA-unrelated factors that may be involved in metabolizing mtDNA. The facts that *csb*$^{m/m}$ cells display altered organization of the respiratory complexes in the inner mitochondrial membrane (PAPER I), is one example of such unexpected correlation between ETC and mtDNA maintenance. Furthermore, the regulation of citric acid cycle by OGG1, via mtDNA integrity dependent recovery of Aconitase (PAPER II), is another example for how mtDNA damage alters mitochondrial function.

Surprisingly, we do not find experimental evidence to support that mtDNA damage can generate mutations, even in the double mutant *myh*$^{-/-}$/*ogg1*$^{-/-}$ (PAPER IV). We believe that the existence of back-up mechanisms prevent unrepaired damages from being fixed into mutations. Further experiments with exposure to exogenous agents will unravel whether these back-up mechanisms are sufficient to prevent oxidant induced mutagenesis.

We believe the cellular effects of mtDNA damage are best visualized by its ability to inhibit mitochondrial transcription (PAPER II). Upon oxidative stress, mitochondria undergo temporary inhibition of transcription and replication. However, the cell sensitivity correlates best with the selective suppression of transcription. Moreover, mtDNA damage mediates global regulation of replication and transcription processes. Further work is required to identify how these processes are controlled by mtDNA integrity.

It is interesting to note that regardless of oxidants, mtDNA damage by itself is less toxic to the cell than repair intermediates that accumulate in mutants or maybe under excessive mtDNA repair initiation.

FUTURE PERSPECTIVES

Future work will include biological characterization of CSB's role in mtDNA repair and transcription. In this respect, recombinant proteins are available with mutations in the ATpase and helicase domains in CSB. These domains have been shown to be essential for specific cellular function of CSB.

Efforts to distinguish transcribed mtDNA from replicated mtDNA could potentially be used to evaluate specific roles of CSB in either process. It should be mentioned in this respect that CSB has now been shown to interact with the mitochondrial RNA polymerase (Vilhelm Bohr, personal communication), in support of our data.

Does mtDAN base damage cause mutation or arrest? Data collected and reported so far suggest that misincorporation by POLγ appears to be more important for mtDNA mutagenesis than premutagenic base lesions. CSB is involved in preserving mitochondrial function, and may play a role in preserving mtDNA integrity during replication and/or transcription. Perhaps future studies will identify a role of CSB in transcription coupled repair of mtDNA?

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I

II

III

IV

Title page

To Cancer Research Research article

Lack of the DNA glycosylases MYH and OGG1 in the cancer prone double mutant mouse does not increase mitochondrial DNA mutagenesis

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Abstract

Reactive oxygen species (ROS) are formed as natural byproducts during aerobic metabolism and readily form premutagenic base lesions in the DNA. The 8-oxoguanine DNA glycosylase (OGG1) and MutY homologue 1 (MYH1) synergistically prevent mutagenesis and cancer formation in mice. The mitochondrial localization of the OGG1 and MYH1 DNA glycosylases suggests that mutations in mitochondrial DNA (mtDNA) could play a significant role in the pathology of the *myh*$^{-/-}$*/ogg1*$^{-/-}$ double knockout mouse. As mtDNA mutations are frequently detected in various cancer cells, we hypothesized mtDNA mutations could result in mitochondrial dysfunction that is responsible for the carcinogenesis in these mice.

In order to test this hypothesis, we analyzed mtDNA mutagenesis and mitochondrial function in young (1 month) and adult (6 months) wt and *myh*$^{-/-}$*/ogg1*$^{-/-}$ mice. To our surprise, the absence of OGG1 and MYH did not increase mtDNA mutations, even at the onset of cancer. This shows that mtDNA mutagenesis is not responsible for the carcinogenesis of these mice. In line with these results, age-matched mitochondria from the cancerous tissues liver and lung displayed similar respiration characteristics in wt and *myh*$^{-/-}$*/ogg1*$^{-/-}$ mice, whereas a significant reduction was observed in brain mitochondria from the adult *myh*$^{-/-}$*/ogg1*$^{-/-}$ mouse. Biochemical analyses confirmed these results as there was no difference between wt and *myh*$^{-/-}$*/ogg1*$^{-/-}$ mitochondria with respect to complex I and V activities in liver and lung mitochondria, but complex I activity in brain mitochondria was reduced by 25 % in the adult *myh*$^{-/-}$*/ogg1*$^{-/-}$ double knockout mice.

Our results demonstrate that mtDNA mutations are not responsible for the pathology of *myh*$^{-/-}$*/ogg1*$^{-/-}$ mice, and the reduced mitochondrial function in brain mitochondria is probably due to nuclear DNA mutations in these mice. Furthermore, our results show that OGG1 and MYH are not important for protecting mtDNA against spontaneously induced mutations.

Introduction

DNA mutations, either induced by exogenous agents or spontaneously formed, are responsible for cancer. Mutations in nuclear tumor suppressors or protooncogenes predispose to cancer development. The impact of mitochondrial (dys)function on tumor biology is illustrated by the dependence of aerobic glycolysis, first observed by Warburg (1). Tumor-promoting suppression of citric acid cycle and as well as tumordependent utilization of anabolic pathways can be supported by mutations in the protooncogene succinate dehydrogenase and increased expression of specific isoforms of pyruvate kinase and hexokinase (2;3). The oxidative degradation of metabolites generates reducing equivalents that drives the electron transport chain (ETC) in the inner mitochondrial membrane, which is important for cell signaling, apoptosis control, membrane potential and ATP production; all central processes in cancer cell biology. The ETC are composed of five different complexes where complex I is the largest and consists of 45 subunits. In addition to the catalytic subunits of complexes, assembly factors, chaperone proteins and lipids are involved in the stabilization and organization of ETC complexes in the inner mitochondrial membrane (4;5). The mtDNA encodes 13 essential subunits of the ETC, and mutations in mtDNA are associated with disease, neurodegeneration, aging and cancer (6-8). The effect of mtDNA mutations has shown to be mediated by increased ROS production (9) and it has been shown that the mitochondria pool determines the cancer phenotype (9; 10).

Mutations arise from premutagenic lesions, such as 7, 8-dihydro-8-oxoguanine (8-oxoG) as this lesion readily mispairs with adenine during DNA replication. The cellular defense to combat 8-oxoG-induced mutagenesis lesions includes the following evolutionary conserved functions: 1) the Fpg/OGG1 DNA glycosylase, which removes premutagenic 8-oxoG from DNA opposite cytosine (11), 2) MutY DNA glycosylases which removes misincorporated adenine opposite 8-oxoG or G (12), and 3) MutT Nudix hydrolases which hydrolyze 8-oxoGTP to prevent incorporation into the DNA (13). The combined action of Fpg and MutY type of proteins reduce the mutagenic potential of 8-oxoG in *E. coli* by more than 100 times (14). While the cancer prone phenotypes of the single knockouts $ogg1^{-/-}$ and $myh^{-/-}$ are mild, the $myh^{-/-}/ogg1^{-/-}$ double knockout mouse display strong tumorigenesis with severe effect on lifespan (15). More specifically, the double knockout mice have a lifespan expectancy of LD50 about six months. The frequencies of lung and liver carcinomas are increased in these mice, and correlates with

age-dependent accumulation of 8-oxoG in the nuclear DNA from liver and lung, but not in brain (16). No tumors were identified in the brain from these animals, an observation that correlates with stable levels of the 8-oxoG with age. The tumorigenic capacity of the 8-oxoG:adenine mispairing event was supported by the fact that the majority of the tumors carry G-T transversion mutations in the *k-RAS* oncogene (16).

Besides being important for nuclear DNA stability, the OGG1 and MYH proteins are additionally located in mitochondria. Mitochondrial 8-oxoG accumulates readily in liver mtDNA from *ogg1*$^{-/-}$ single knockout mice (17). Thus, it is expected that the *myh*$^{-/-}$*/ogg1*$^{-/-}$ double deficient mouse would suffer from increased mtDNA mutagenesis, which may contribute to and even be responsible for the cancer phenotype of the *myh*$^{-/-}$*/ogg1*$^{-/-}$ double knockout mice. Mutations in the mtDNA are associated with premature aging, and the lifespan of the *myh*$^{-/-}$*/ogg1*$^{-/-}$ mouse is similar to that of the mitochondrial mutator mouse generated by Trifunovic and coworkers (16;18), although the phenotypes are different.

Here, we evaluated the mtDNA integrity in different tissues from the OGG1/MYH double deficient mouse and correlated these findings with mitochondrial function, assessed by respiration characterization and biochemical analyses of ETC complexes. Our results unexpectedly show that spontaneous mtDNA mutagenesis is independent OGG1 and MYH and that mitochondrial alteration does not contribute to the cancer phenotype of these mice. Furthermore, the absence of mtDNA mutagenesis suggests that nuclear DNA mutations are responsible for the mitochondrial dysfunction in brain from the *myh*$^{-/-}$*/ogg1*$^{-/-}$ mouse.

Materials and Methods

Mice strains, mitochondria and materials

The c57 Bl/6:*myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mouse has been generated previously (15) and was kindly provided by Dr.T.Lindahl (Clare Hall Laboratories, Cancer Research UK London Research Institute, South Mimms, Herts, UK). Wt c57 Bl/6 was bred inhouse in the same room. Animals were bred in accordance with European regulations FELASA category C. The genotypes of the mice were routinely tested by a PCR-based protocol upon tissue collection; Fig. S1. Liver DNA from 35 weeks old mice expressing proof-reading deficient DNA polymerase γ in one (*polg*$^{mut/+}$) or both alleles (*polg* $^{mut/mut}$) was obtained from Nils-Göran Larsson, Max-Planck Institute, Germany.

Liver, lung and brain tissue were harvested from young (1 month) and adult (6 months) wt and *ogg1*$^{-/-}$/*myh*$^{-/-}$ double knockout mice and homogenized in MSHE buffer (19) using potter-elvehjem homogenizer. Samples were collected for DNA isolation and stored at -20°C. The remaining homogenate was used for preparation of mitochondria, using the method of Croteau et al (19), except that the gradient purification step was omitted.

All chemicals and enzymes were obtained from SIGMA unless otherwise specified.

Quantification of mtDNA damage

Total DNA was isolated from tissue homogenates using a DNA purification kit ("DNeasy Blood & Tissue Kit" cat. # 69506; Qiagen). mtDNA damage was quantified by the ability of selectively inhibit amplification of a large PCR product over a small PCR product (20) and modified slightly as described in Osenbroch et al. (21).

Quantification of mtDNA mutations

Random Mutation Capture assay (RMC)

Mutations were quantified using the RMC method developed by Vermulst et al (22) with the following modifications: Total DNA was digested both with S1 nuclease (10 U per DNA sample in 50 µl containing S1 buffer, 15 min 60°C) to remove single stranded DNA that otherwise escapes Taq1 digestion and consequentially results in artificially high level of mutation rate

unless excessive amounts of TaqI is used. Taq1 digestion mixture contained 100 U Taq1 (New England Biolabs) in 50 µl with 1xBSA and was incubated for 10 hours at 60°C and subsequently inactivated at 95ºC. Serial dilutions of each DNA preparation were made to estimate mtDNA copy number. The RMC was then applied on 10 000 copies of mtDNA in 96 well plates. To eliminate possible contamination and ensure complete digestion, 1 U Taq1 was added to the qPCR mix. Real-time qPCR was used to detect positive mutants in a reaction mixture (20 µl) containing DNA, SYBRGreen buffer, 1 U Taq1, 0.5 pmol sense and antisense primers spanning the TaqI site at position 634 (22). To distinguish positive PCR products from artificial qPCR signals (e.g. primer-dimer), any signal with C_T <34 was regarded as positive.

Real-time analysis of mutation frequency (RAMF)

We developed a faster, real-time based method for quantification of mtDNA mutation frequency. A similar procedure was recently reported by others (23), except that we included use of S1 DNA nuclease and performed qPCR analysis as described above. In brief, total DNA (6 ng) was either untreated, or digested with S1/Taq1 as illustrated above, and the resulting Δc_t (c_t value for digested DNA minus c_t for nondigested DNA) estimated. Mutation frequency per bp is given by the formula: $(1/ (2\exp (\Delta c_t))/4$.

Biochemical mitochondrial complex activity

Complex I activity measurements was performed as described by Brooks and Krähenbühl (24) in a 96 well plate reader (Wallac) in the absorbance range 355 (+/-25) nm. Complex V activity was measured as described in Barrientos et al and Yu et al (26;27). In brief, the mitochondrial proteins were diluted to 40 µg in 20 µl dH_2O. 150 µl complex V buffer (50 mM Tris HCl pH 8.0, 2mM ATP, 0,3 mM NADH, 2 mM $MgCl_2$, 3 mM Phosphoenolpyruvate, 5 U/ml lactate dehydrogenase, 2,5 U/ml Pyruvate kinase and 2 µg/ml Antimycin A) was added to each well and the plate was measured immediately at 340 nm using the Wallac for 5 min every 30 sec (kinetic program). Oligomycin (50 µg/ml) was added instead of Antimycin A as control. The ATPase activity was measured by the steady decrease in NADH absorbance (355 (+/-25) nm).

Mitochondrial respiration analyses

Respiration characterization of isolated mitochondria was performed using an oxygraph-2K with the integrated software DATLAB 4.2 (Oroboros Instruments). The stirrer speed was set to 750 r.p.m.. Approximately 50-100 µg mitochondria were used in Mir05 buffer (27). Complex I-supported respiration was initiated by pyruvate (5 mM) and malate (2 mM). Rate III respiration was induced by adding ADP (2 mM). Complex II-based respiration was determined after inactivating complex I by rotenone (0.5 µM), followed by succinate (10 mM) supplementation.

Statistics

Each group consists of 5 mice. Unless otherwise stated, average values with SD are shown. Statistical significance is calculated using students t-test.

Results

We used a PCR based method to evaluate mtDNA damage in brain, liver and lung tissues from the *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mouse. Quantification of mtDNA damage is based on the ability to inhibit amplification of a large DNA fragment (20). 8-oxoG has been shown to accumulate markedly in nuclear DNA after 4 months in liver and lung from the *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mouse (16). In order to evaluate the timing of potential damage accumulation in mtDNA, we compared mtDNA from wt and double knockout mice at the age of1 and 6 months. The latter age corresponds to the onset of tumor development in the mutant mice (15). In young animals (1 months), there was no significant effect of OGG1 and MYH deficiency on the mtDNA integrity in liver, lung or brain tissue, as measured by this method (Fig. 1). Since this mtDNA damage detection method scores for the ability of a lesion to block DNA polymerase in a PCR reaction, the results either imply that there is negligible mtDNA damage accumulation in *myh*$^{-/-}$/*ogg1*$^{-/-}$ mice, or alternatively that the accumulated lesions, such as 8-oxoG, are readily bypassed by the PCR DNA polymerase. In adult mice, the integrity of mtDNA from lung and brain was similar (Fig. 1*B* and *C*), while liver mtDNA from the *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice surprisingly scored for less mtDNA damage compared to the age-matched wt mice (Fig. 1*A*). We conclude that the combined deficiency of OGG1 and MYH do not result in accumulation of blocking lesions or strand breaks in mtDNA.

The apparent reduction in mtDNA integrity in wt mtDNA (Fig. 1*A*) could be indicative of repair intermediates, such as single strand breaks, which are readily detected by the mtDNA damage assay. 8-oxoG satisfies the criteria for a lesion that is readily bypassed by DNA polymerases in the *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice. To evaluate the consequence on mtDNA mutagenesis, we employed two distinct methods where both scores for the ability of a mutation to destroy a TaqI restriction enzyme recognition site (TCGA). The restriction enzyme site is located at position 634 in mtDNA, in the 16 S ribosomal gene. First, the two methods were compared by measuring mutation rates in liver mtDNA from wt mouse (4 weeks) and in liver mtDNA from mice expressing proofreading deficient DNA polymerase γ from one and both alleles, respectively. The Random Mutation Capture (RMC) mutation method was developed by Vermulst and coworkers and requires amplification of a single copy of mutated mtDNA (22), and were modified slightly by us to reduce background noise due to single-stranded regions of

mtDNA (see materials and methods). Compared to wt mice, the mtDNA mutation rates in the *polg*$^{mut/+}$ and *polg* $^{mut/mut}$ controls were 10 and 60-fold increased, respectively. The mutation rates for wt liver mtDNA is in the range previously reported for heart and brain mtDNA, using this method (22), but the rates for the mutant mice are lower. We developed another real-time based analysis of mutation frequency (RAMF) method, which is less sensitive to PCR contamination and less cost and time expensive (see Materials and Methods). During the progression of our work, a similar technology was reported (23). The RAMF method yielded 16- and 60-fold increase in mtDNA mutation rates from the *polg*$^{mut/+}$ and *polg* $^{mut/mut}$ mice, respectively, compared to wt (Table 1). Overall, our RAMF method gave slightly elevated mutation rates compared to RMC (approximately 2-fold).

We then quantified the mutation rates in mtDNA from wt and *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice by the two methods. To our surprise, the absence of OGG1 and MYH did not result in increased mtDNA mutation rates in any tissue, either in young or adult animals (Table 2 and 3). There were no differences in mutation rates between the two different age groups, but the mutation rates varied between the tissues. While lung mtDNA was found to suffer from most mutations (5 fold) as judged by the RMC method (Table 2), the RAMF method identified highest mutation rates in liver mtDNA (Table 3). We used the same DNA for both methods hence we believe the reason for the discrepancy is due to tissue-dependent variations in the mtDNA. It is important to note that the difference in absolute frequencies vary similarly in a method-dependent manner between the different tissues and genotypes (Table 2 and 3). In conclusion, using either RMC or RAMF methods, the mutation rates in liver, lung and brain mtDNA from wt and *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mouse are similar, independent of age.

The innocuous effect of deleting *MYH* and *OGG1* on mtDNA mutagenesis is surprising in view of the evolutionary conserved role of these functions for protection against mutagenesis. Since the mutagenesis assay does not score for functionality, it was a possibility that the mutagenesis assay scored for a subpopulation of mtDNA that is specifically protected from mutagenesis or alternatively escapes detection because only one area of mtDNA is investigated. In order to follow up on this point, we evaluated the functionality of isolated mitochondria by respiration characterization. The respiration characteristics were similar for mitochondria from young wt and *myh*$^{-/-}$/*ogg1*$^{-/-}$ mice, regardless of whether complex I or II was supporting respiration (Fig. 2).

In the adult mice, lung and liver mitochondria displayed similar respiration efficiency in wt and *myh*$^{-/-}$/*ogg1*$^{-/-}$ mice. In contrast, we observed a small but significant reduction of respiration efficiency in brain mitochondria during complex I and II-utilization (Fig. 2*C*). This shows that the OGG1 and MYH double deficiency is manifested as a mitochondrial dysfunction in brain at 6 months age.

It was possible that potential ETC dysfunction could be masked by the lower respiration capacity compared to ETC capacity. In order to evaluate the maximal capacity of the ETC, we measured biochemical activity of complex I and V in the different tissues of the two age groups. For complex I, a significant decrease in activity (approximately 25 %) was found in brain mitochondria from 6 months old *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice (Fig. 3*A*), and suggests that complex I insufficiency at least partially contributes to the lower respiration efficiency in Fig. 2. Except for brain mitochondria from adult mice, there were no significant differences between wt and mutant mitochondria from other tissues (Fig 3*A*). When complex V was analyzed, we found no significant difference between wt and *myh*$^{-/-}$/*ogg1*$^{-/-}$ mitochondria, regardless of tissue and age (Fig. 3C). Thus, in conclusion, our functional evaluation of mitochondria from liver and lung tissue fail to demonstrate any correlation between mtDNA repair capacity exerted by OGG1/MYH and mitochondrial function. The reduced mitochondrial activity in brain mitochondria from the adult double knockout mice does not correlate with mtDNA mutagenesis and thus may indicate that nuclear mutagenesis contributes to the mitochondrial dysfunction in these mice.

Discussion

In this study, we have investigated the impact of OGG1 and MYH on mtDNA mutagenesis. To our surprise, the combined deficiency did not result in increased mutagenesis despite that both proteins are localized to the mitochondria. These findings were confirmed by functional assays of mitochondria from lung and liver which displayed similar biochemical and respiration activities as the age-matched wt mice. The cancer prone phenotype of the animals is therefore not due to mitochondrial alterations.

OGG1 has previously been shown to be essential for efficient removal of 8-oxoG from liver mtDNA (17). Thus, the additional removal of MYH was expected to increase the mutagenic potential of accumulated 8-oxoG. We believe the explanation for the innocuous effect of removing OGG1 and MYH in mtDNA is due to efficient backup systems in the mitochondria. Members of the NEIL family of DNA glycosylases have substrate specificities that overlap with that of OGG1. For instance, NEIL1 excises 8-oxoG and has been identified in mitochondria (28). NEIL2 is another 8-oxoG removing enzyme that additionally has been implicated in mtDNA repair (Hazra, personal communication). For the premutagenic 8-oxoG:A basepair, it is possible that this could be handled by the mismatch repair shown to be present in mitochondria (29; 30).

Our findings demonstrate that OGG1 and MYH are indispensible for protection against mtDNA mutations in lung, liver and brain tissue. In Fig. 1, we show that the ability to amplify a 10 kb large fragment from liver mtDNA was 2-fold higher when using template from adult double mutant mice compared to wt mice. If the reason for the decreased PCR efficiency in wt mice is due to ongoing repair of 8-oxoG, it can be calculated that repair frequency is 0.7 per mtDNA, using the poisson distribution model (20). Interestingly, this repair incidence is close to the estimated steady state level of 8-oxoG in liver mtDNA from *ogg1*$^{-/-}$ single knockout mouse (19). Thus, our results suggest that induced 8-oxoG is rapidly recognized and removed by OGG1 in wt. In OGG1 deficient mitochondria, backup mechanisms that either omit strand breaks or alternatively proceed with faster repair rates (to fast for being detected by the mtDNA damage method) prevent progressive accumulation of 8-oxoG and subsequent mutagenesis. During oxidative stress, ring-opened purines (such as formamidopyrimidines; faPy) are formed in addition to 8-oxoG and presumably accumulates in *myh*$^{-/-}$/*ogg1*$^{-/-}$ mice since these lesions are substrates for OGG1 (31). The faPy residues can block DNA synthesis (32). Thus, it is also

possible that apparent lack of mtDNA damage accumulation is due to a combined ability of PCR DNA polymerase to bypass 8-oxoG and being blocked by faPy in the mutant mice, and ability to amplify repaired mtDNA and being blocked by repair intermediates in the wt mice.

The double deficient mice were previously analyzed for nuclear DNA 8-oxoG, where the authors discovered a correlation between age-dependent accumulation of 8-oxoG and cancer incidence in different tissues (16). Interestingly, brains from the *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice were found to be spared for age-dependent accumulation of 8-oxoG, which also correlated with the lack of tumors in this tissue. Since brain has a high metabolic rate, it is nevertheless expected that the frequency of spontaneously formed lesion exerts a detrimental pressure. Thus, backup DNA nuclear repair mechanisms appear to be a plausible mechanism for the relative low damage accumulation in brain from these animals. Our results, however, indicate that mitochondrial dysfunction observed in adult brain from the *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice is due to nuclear mutagenesis, since the mutation rate is similar in wt and mutant mtDNA. One explanation could be that regional accumulation of damage in promoter region of genes in the brain could suppress expression of mitochondrial proteins encoded by nucleus. Such regional localization of damage has been identified previously in brain tissue during aging (33).

Here we used mutation-mediated inhibition of TaqI digestion as tool to quantify mtDNA mutagenesis. By focusing on the TaqI site at position 634, there is a possibility that mutations accumulate outside elsewhere in the mtDNA and escape detection. In particular, the mtDNA region spanning from nt 15.423 to 16.299 region has been found associated with low mutation rates (Jim Steward, personal communication). Since clonal expansions might contribute to increase the impact of mutations outside this area, we cannot exclude the possibility that subregions of mtDNA from the *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockouts suffer from elevated mutagenesis that alter cancer susceptibility. However, the lack of correlation between mitochondrial dysfunction found here and the reported cancer disposition do not favor this possibility. Vermulst et al reported that TaqI sites at positions 7667, 12080 and 15253 displayed similar mutation characteristics with respect to frequency, age-dependence and type of mutations. The RMC method has been reported to be sensitive to artificial background (34). We include S1 nuclease to remove interacting single stranded DNA, which otherwise can induce unspecific PCR priming as well as escaping TaqI digestion and thereby result in false positive signals.

In conclusion, our results show that the OGG1 and MYH DNA glycosylases are dispensable for protecting against mutations in mtDNA. Additional deletion of e.g. NEIL functions may provide more information about the extent that base lesions contribute to mtDNA mutagenesis, in comparison with misincorporation by DNA polymerase γ.

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Figure legends

Figure 1. mtDNA integrity in liver (A), lung (B) and brain (C) from wt and *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice of 1 and 6 months (mo) age, as indicated. Total DNA was isolated and analyzed for mtDNA damage by the ability to generate a large PCR product. Relative mtDNA integrity is presented relative to average of wt (1 mo) values. N=5, *p<0.05.

Figure 2. Respiration characteristics of mitochondria from liver (A), lung (B) and brain (C), from 1 and 6 months (mo) old wt and *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice. Mitochondria were isolated by differential centrifugation and transferred to an oxygraph-2K with the following serial manipulations: Initial complex I-dependent respiration was supported by pyruvate/malate (PM), followed by ADP addition to obtain state III respiration (ADP). Thereafter, rotenone (ROT) was added, to provide complex II-dependent respiration, supported by succinate (SUCC; 10 mM). wt: dark bars, *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice: grey bars. N=5; *p<0.05.

Figure 3. Mitochondrial biochemical complex activities. A, NADH-oxidoreductase (complex I) and B, ATPase (complex V) activities in liver, lung and brain mitochondria were analyzed as described and presented as indicated in the figure. Average values of wt (black bars) and *myh*$^{-/-}$/*ogg1*$^{-/-}$ double knockout mice (grey bars) are provided with SD for mice, age 1 and 6 months (mo), as indicated. N=5, *p<0.05.

Figure 1

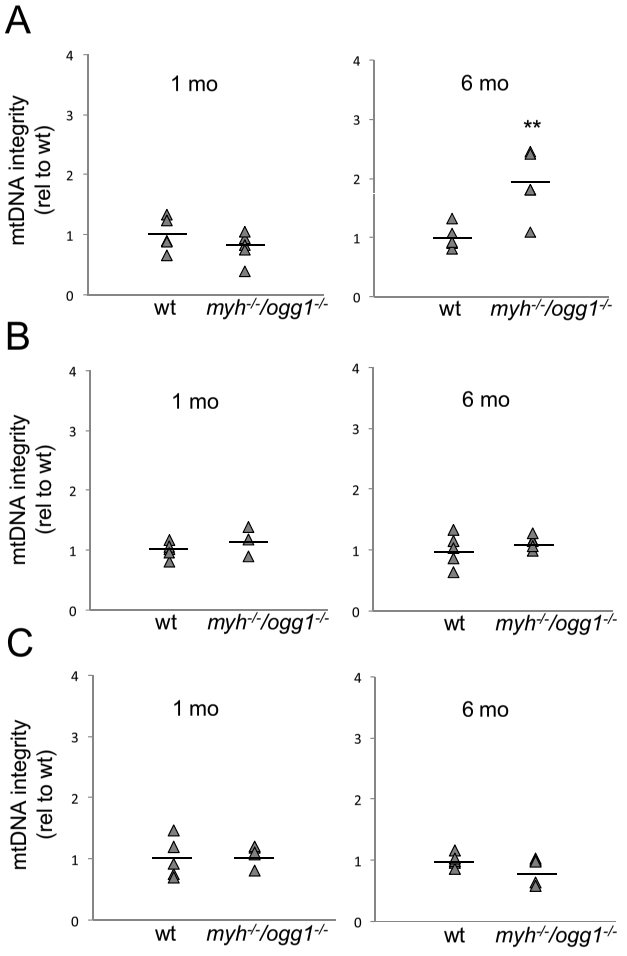
A
mtDNA integrity (rel to wt)
1 mo
6 mo
**
wt
myh-/-/ogg1-/-
B
mtDNA integrity (rel to wt)
1 mo
6 mo
wt
myh-/-/ogg1-/-
C
mtDNA integrity (rel to wt)
1 mo
6 mo
wt
myh-/-/ogg1-/-

Figure 2

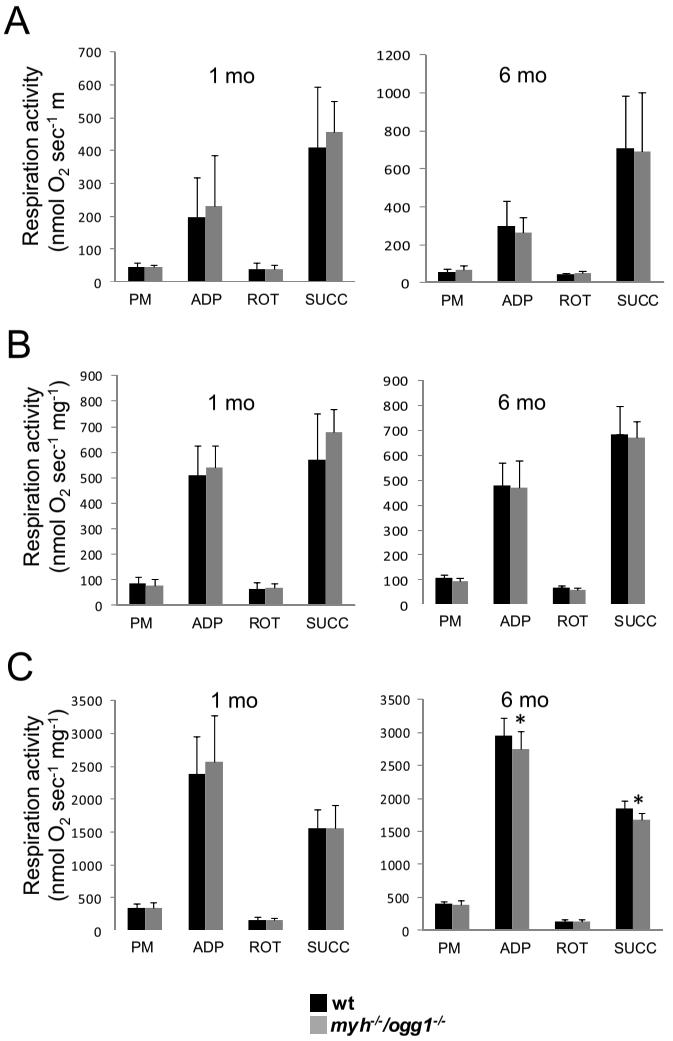
A
1 mo
6 mo
Respiration activity (nmol O2 sec-1 m
PM
ADP
ROT
SUCC
B
1 mo
6 mo
Respiration activity (nmol O2 sec-1 mg-1)
C
1 mo
6 mo
*
wt
myh-/-/ogg1-/-

Figure 3

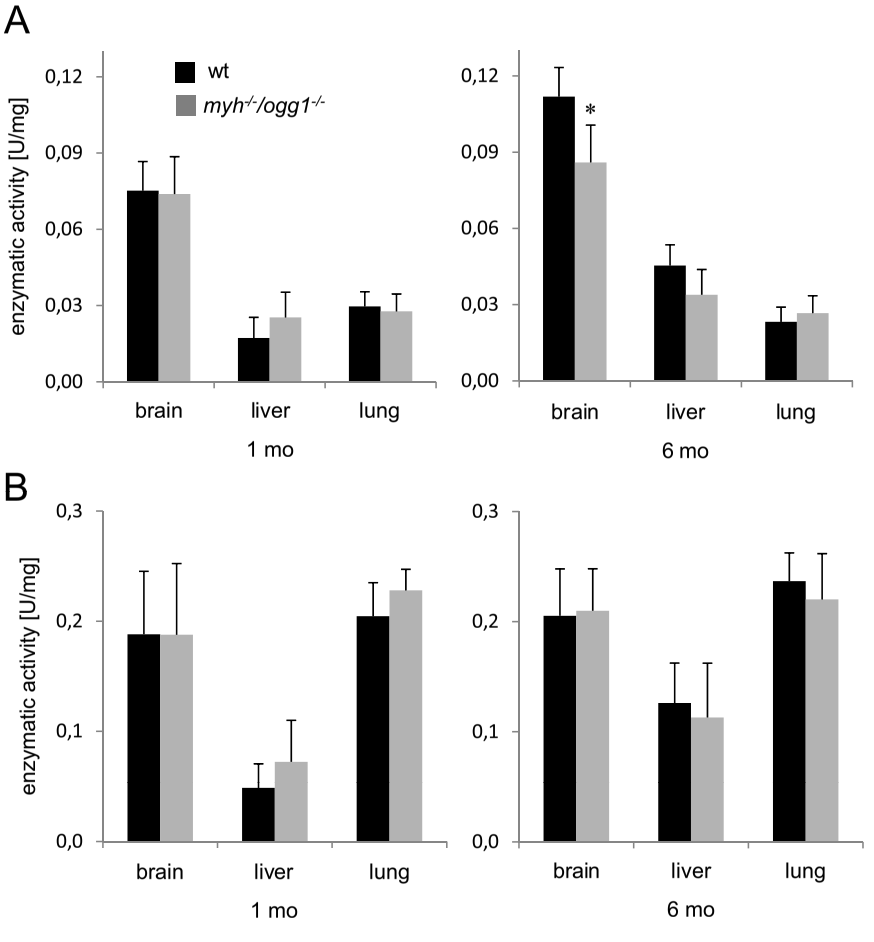
A
wt
myh-/-/ogg1-/-
enzymatic activity [U/mg]
0,12
0,09
0,06
0,03
0,00
*
brain
liver
lung
1 mo
6 mo
B
0,3
0,2
0,1
0,0

Table I. mtDNA mutagenesis analyzed by RMC and RAMF methods

	RMC	RAMF
wt	2,4	4,6
polg$^{mut/+}$	23	75
polg $^{mut/mut}$	150	300

Mouse liver DNA from wt (4 weeks), *polg*$^{mut/+}$ and *polg* $^{mut/mut}$ (35 weeks) were analyzed for TaqI restriction site (nt 635) mutation. Mutation frequencies are presented as pr 10^6 bp.

Table II. mtDNA mutation frequencies, determined by the RMC method

	1 mo		6 mo	
	wt	myh$^{-/-}$/ogg1$^{-/-}$	wt	myh$^{-/-}$/ogg1$^{-/-}$
Liver	2,4 (1.9)	1,8 (1,1)	2,0 (1,3)	1,9 (0,3)
Lung*	16	14	18	16
Brain*	2,6	2,1	1,3	2,6

Mutation frequencies are presented as per 10^6 bp. SD is provided in parenthesis, *DNA from 5 different mice were pooled prior to analysis.

Table III. mtDNA mutation frequencies, determined by the RAMF method

	1 mo		6 mo	
	wt	myh$^{-/-}$/ogg1$^{-/-}$	wt	myh$^{-/-}$/ogg1$^{-/-}$
Liver	4,6	2,8	5,0	3,2
Lung	1,6	1,5	1,3	1,7
Brain	0,53	0,46	0,40	0,33

Mutation frequencies are presented as per 10^6 bp. DNA from 5 different mice were pooled prior to analysis.