ANJA ROVIO

DNA Polymerase Gamma Mutations in Male Infertility and Ageing

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ACADEMIC DISSERTATION
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To life-long learning and education,

Finnish SISU

and

My Marvellous Daughter Minna

1. ABSTRACT

The nuclear encoded catalytic subunit of polymerase gamma (POLG) is the only polymerase in humans that is known to be located in mitochondria and has been proposed as the replicase of mitochondrial DNA. In its coding region lies a short polyglutamine tract, which is absent in *POLG* of mouse or *Drosophila*. Microsatellite repeats have been shown to evolve in length during the course of primate evolution and show considerable variation between individuals. Polyglutamine repeat expansions are a frequent cause of disease, in many cases involving also infertility. Mitochondrial involvement in infertility has been proposed, based on the high energy demand of sperm cells for motility, and mtDNA deletions and point-mutations described in sperm cells. POLG harbours a polymerase domain and an exonuclease domain, both needed for faithful replication of mtDNA, and mutations along the gene have been reported in connection with diseases associated with mitochondrial DNA deletions or depletion. Cell lines expressing POLG with an exonuclease domain mutation accumulate high loads of mtDNA point mutations compared to control cell lines. Higher mtDNA point mutation loads have been documented in tissues of ageing subjects than of young individuals.

These findings prompted a number of questions: How variable is the *POLG* microsatellite in different populations? Do our primate relatives have the repeat and does the polyglutamine in humans have a role in common mitochondrial disease? Does *POLG* sequence variation have an effect on cells that are highly dependent on biological energy during cell maturation, differentiation and function, like sperm?

To address these questions I analysed POLG genotype in different human populations, in various types of disease, and in non-human primates. The most common allele of *POLG* with 10 CAGs was shown to be maintained at a frequency of 0.88 in different human populations, suggesting maintenance by selection. Expansions of the repeat were ruled out as a common cause of mitochondrial disease. A homozygous lack of the common $(CAG)_{10}$ allele of *POLG* was seen in 9 % of males in whom no other reason for infertility was known, compared to 1.2 % amongst unselected controls or none of 98 males known to be fertile. The semen of men affected by the mutation showed low number of sperm with poor motility and/or morphological defects, but not azoospermia, severe oligozoospermia or isolated asthenozoospermia. Later studies by others have shown that couples with male *POLG* CAG mutation can be helped to reproduce with the help of ICSI treatment. I therefore propose that in cases of idiopathic infertility the males should thus be analysed for POLG

CAG repeat variation and if found to lack the common allele, the couples should be offered ICSI as the first and effective treatment.

Infertile males had more mutations in their mtDNA control region than fertile controls and a positive correlation between the mutation load and the absence of the common POLG length variant was seen. An appreciable proportion of infertile males heterozygous for *POLG* CAG repeat length harbour also, in addition to the length variant, a second mutation somewhere else in their *POLG* gene.

The length variants of the POLG polyglutamine tract were almost exclusively due to the elongation of the CAG repeat. Nucleotide level variation was seen only in 4 out of 1210 control alleles sequenced, all of whom had the same variant in which one of the CAG codons was changed to a CGG (arginine). The polyglutamine tract appears to have evolved during primate evolution from a short arginine-glutamine-leucine-proline region in monkeys to the ´clean` and extended polyglutamine in humans. Each primate species was shown to have its own specific repeat length with high frequency, similar to humans.

Homozygous knock-in mice having a point mutation in the second exonuclease domain of *Polg* were shown to have a three- to five-fold increase in the amount of mtDNA point mutations, and they also had increased amounts of aberrant mtDNA molecules. The mtDNA point mutations accumulated linearly during lifetime of the mice but affected different mtDNA sequence segments differently. The mice showed a premature ageing phenotype with weight loss, reduced subcutaneous fat, alopecia, osteoporosis, kyphosis, anaemia, heart enlargement and reduced fertility, the males being practically infertile. The ageing related phenotypes start to manifest at ~24 weeks of age and the maximum lifespan of the ´mutator` mice was 61 weeks, which is less than half of the normal life span of laboratory mice.

This study combined to the phenotypical and biochemical analysis done by collaborators and the data in the literature underline the critical role of DNA polymerase gamma in maintaining optimal function and healthy status of cells and tissues, and thus whole organisms through their whole lifetime.

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2. GLOSSARY and ABBREVIATIONS

AAO; Acute Amino glycoside Ototoxicity
AIS; Androgen Insensitivity Syndrome
Asthenozoospermia (AP); inability of sperm to swim
Azoospermia; no sperm cells in seminal plasma
AR; Androgen Receptor
ARE; Androgen Responsive Element
ART; Assisted Reproduction Technology
ATP; adenosine triphosphate
AZF; azoospermia factor
BER; base excision repair
BMR; basic metabolic rate
bp; base pair
CAG; triplet of deoxynucleotides cytosine, adenine, guanine
cAMP; cyclic adenosine monophosphate
CBAVD; congenital bilateral absence of *vas deferens*
CF; cystic fibrosis
CR; control region
Cryptozoospermia; sperm count <1M/ml
2-D-gel; two-dimensional gel electrophoresis
D-loop; displacement loop
DM; myotonic muscle dystrophy
DNA; deoxyribonucleic acid
ETC; electron transport chain
ETS; electron transport system
F-PCR; fluorescent PCR
FTAX; fragile X-associated tremor/ataxia syndrome
HD; Huntington´s Disease
Globozoospermia; round-headed sperm cells
ICSI; Intra Cytoplasmic Sperm Injection
IM; inner membrane
IMS; inter-membrane space
kDa; kiloDalton, quantity describing mass of a molecule
LBD; ligand-binding domain
MELAS; mitochondrial Myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes
MERRF; Myoclonus Epilepsy associated with Ragged-Red Fibres syndrome
MDS; mitochondrial DNA depletion syndrome

MMP; mitochondrial membrane permeabilization
mtDNA; mitochondrial DNA
mRNA; messenger RNA
NARP; Neuropathy, Ataxia and Retinitis Pigmentosa syndrome
NCR; non-coding region
NO; Nitric Oxide
O_H; origin of heavy strand replication
O_L; origin of light strand replication
Oligozoospermia; low sperm count, <20M/ml
OM; outer membrane
OMIM; Online Mendelian Inheritance in Man
OXPHOS; oxidative phosphorylation
PACC; pre-apoptotic chromatin condensation
PCR; polymerase chain reaction
PEO; Progressive External Ophtalmoplegia
POLG; DNA polymerase gamma
Pu; purine
Py; pyrimidine
RE; responsive element
RMR; resting metabolic rate
RNA; ribonucleic acid
ROL; rate of living
ROS; reactive oxygen species
SAM; sorting and assembly complex
SANDO; Sensory Ataxic Neuropathy, Dysarthria, and Ophthalmoparesis
SBMA; Spinal Bulbar Muscular Atrophy
SNP; single nucleotide polymorphism
Teratozoospermia; morphological defects in spermatozoa
TIM; transporter of the inner membrane
TOM; transporter of the outer membrane
TRED; trinucleotide repeat expansion disease
tRNA; transfer RNA
UTR; untranslated region
WHO; World Health Organization

3. LIST OF ORIGINAL COMMUNICATIONS

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V. Trifunovic A, Hansson A, Wredenberg A, **Rovio AT**, Dufour E, Khvorostov I, Spelbrink JN, Wibom R, Jacobs HT and Larsson N-G. 2005. Accumulation of somatic mtDNA mutations is not accompanied by elevated ROS production. PNAS. 102:17993-17998.

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4. INTRODUCTION

I think we all agree that ageing itself is not a disease and thus should be taken as the natural final phase of life. It is anyhow accompanied with phenotypes and symptoms resembling the ones found in many mitochondrial disorders, including hearing loss, problems in vision, fatigue, muscle weakness, exercise intolerance, dementia, diabetes, cardiomyopathy and reduced fertility. Although ageing seems to be an entity itself with no other obvious "external cause" than the passage of time, a higher load of mitochondrial DNA (mtDNA) mutations have been reported in old individuals compared with young ones. Mitochondrial diseases with symptoms described earlier, are commonly associated with mtDNA deletions, depletion or point mutations, the underlying defect very often in a nuclear gene for mitochondrial protein with functions in mtDNA maintenance and replication, such as the catalytic subunit of DNA polymerase gamma (*POLG*).

POLG contains within its coding sequence a trinucleotide microsatellite repeat, which is absent in corresponding genes of *e.g*. mouse and *Drosophila*. Trinucleotide repeats are shown to have evolved in length during primate evolution. The structural properties of microsatellite repeats make their replication error-prone resulting in expansions or contractions. Mutations affecting the length of microsatellite repeats are also frequent cause of genetic disease.

All cells of higher eukaryotes except mature erythrocytes contain mitochondria, which produce the bulk of biological energy in the form of ATP in cellular respiration. Intact gene products of mtDNA are all needed for proper mitochondrial function. All cells, and especially the cells in brain, muscle, heart, liver, sensory- and reproductive organs are dependent on energy produced by mitochondria, and any defect in mitochondrial function would show up as a disease or altered phenotype. Mitochondrial theory of ageing is based on the assumption of mitochondria having the fundamental role as both producers and targets of the reactive oxygen species (ROS) which are produced as a side product of cellular respiration. Those ROS are thought to bring about more mutations in mtDNA than in nuclear DNA, not only because mtDNA resides just where the ROS is produced, but also because of the absence of protection by nucleosomes, and insufficient DNA repair inside mitochondria. The resulting synthesis of defective respiratory chain subunits would then manifest as defective respiration and impaired mitochondrial function. This would in turn lead not only to suboptimal cell function but also to more production of ROS, and thus to the *vicious cycle*. Environmental factors like radiation, pollutants or

unwanted chemicals in nutrition could act as triggers in the process, accelerating apoptosis, cell senescence and ageing.

Up to 20 % of couples are estimated to suffer from infertility and half of the cases are attributable to male defects. Male infertility can result from a multiplicity of different reasons but in many cases the aetiology remains undiscovered. Spermatozoa are heavily dependent on respiratory energy produced by mitochondria. Therefore, impaired energy metabolism, whether in mature spermatozoa or at earlier stages of male germ cell differentiation, is a long hypothesized mechanism contributing to male infertility.

This study illuminates the role of POLG and mutations in its sequence in health and disease. A major part of the experimental studies concentrated on characterizing the microsatellite region, analysing its evolution and variation in different primate species, as well as frequencies of different alleles in human populations. The possible connection between different POLG microsatellite length variants with common mitochondrial diseases characterized by mtDNA deletions, depletion or point mutations as well as with male infertility was analysed. The correlation between POLG length variation and mtDNA mutations in sperm is reported, as well as results from a preliminary screen for other *POLG* mutations found in infertile males. To study the possible phenotypic effect of a mutated POLG in an experimental animal, a transgenic mouse model was created. mtDNA point mutation load, the accumulation of mutations during lifetime, and the pattern of mutations in the mutator animals was analysed. The *POLG* genotype/mtDNA/phenotype relations are discussed.

The results of the experimental studies as well as those in the published literature are discussed in combination to see possible consensus or disagreement. This, together with some hypotheses about what might be happening could then serve as the bases for future experiments.

5. LITERATURE SURVEY

5.1 Mitochondria and mtDNA

Mitochondria are found in all higher eukaryote cells except mature red blood cells. They are thought to have originated from an endosymbiotic event in the early evolution of eukaryotic cells, since they resemble bacteria in many structural and functional ways. Mitochondria are referred to as the powerhouses of the cell because of their ability to convert the energy recovered by the oxidation of glucose to chemical energy in the form of ATP *via* the final process called oxidative phosphorylation. In electron-microscopic pictures mitochondria are round or oval-shaped structures with a visible, smooth outer membrane and an inner membrane with cristae (membrane invaginations) protruding into the matrix (Revel et al., 1963). In reality mitochondria exist as a dynamic network structure with continuous fusion and fission (Nunnari et al., 1997; Yaffe, 1999). The mitochondrial network can easily be seen in confocal microscopic pictures, as can fusion and fission in video images from living cells, where mitochondria have been visualized by expressing a mitochondrially targeted fluorescent protein (Garrido et al., 2003). The network is frequently most dense in perinuclear areas but extends also to the remote areas of cells, such as the axons of neurons (Stamer et al., 2002).

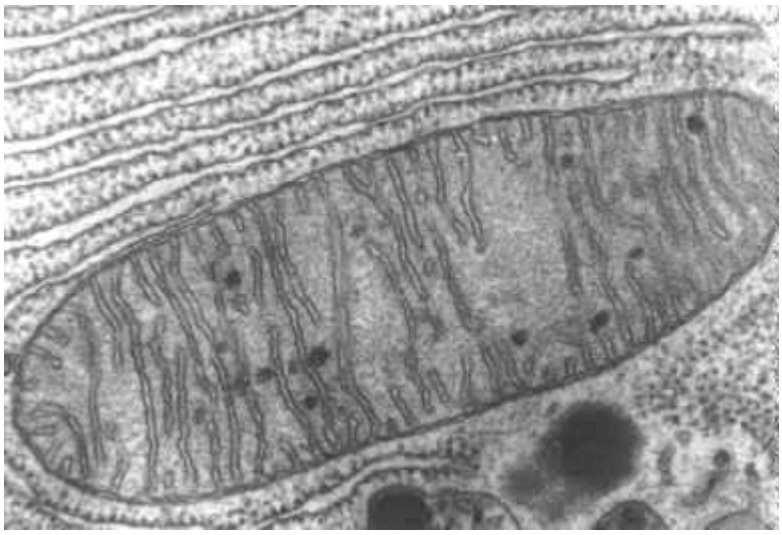


Figure 1. *This electron micrograph taken from Fawcett, A Textbook of Histology, Chapman and Hall, 12th edition, 1994, shows an oval shape mitochondrion. The organization of the two membranes is clearly visible, as are the cristae protruding into the matrix.*

The four distinct compartments of mitochondria all have their own specific functions. The outer membrane (OM) not only encloses the organelle but also harbours the main entrance gateway for protein molecules that are translocated from the cytosol, namely the Translocase of the Outer Membrane (TOM complex, Pfanner and Meijer, 1997; Kűnkele et al., 1998). The inner membrane (IM) has its own polypeptide transporters (TIM complex) and the sorting and assembly complex (SAM complex, Pfanner et al., 2004) for directing the imported proteins to the IM or into the inter-membrane space (IMS). The electron transport chain (ETC) enzyme complexes (cI-IV) and ATP synthase (cV) are embedded in the inner membrane. Between the membranes is the IMS into which the ETC pumps protons. This excess of protons maintains the membrane potential and is needed by cV to produce ATP. In the matrix are the mitochondrial ribosomes for the translation of mitochondrial mRNAs into mitochondrially-encoded polypeptides. In the matrix are located also the enzymes of the Krebs´ cycle and β-oxidation.

5.1.1 Organization of human mitochondrial DNA

Human mitochondrial DNA is a 16569 nt long double-stranded circular molecule with no introns. It encodes 13 polypeptides that all are components of the ETC plus cV, 2 ribosomal RNAs (12S and 16S) and 22 transfer RNAs (tRNAs; www.mitomap.org), all of them needed for mitochondrial protein synthesis. In addition, mtDNA has a non-coding control region, which includes the D-Loop or displacement loop (a triple-stranded region generated by the synthesis of a short piece of H-strand DNA, the 7S DNA); the major promoters for transcription of

each strand and the classical origin for replication (see text below, section 5.1.3). The D-loop sequence is highly variable between and within species. The two strands of mtDNA (the heavy and light strand) have different purine & pyrimidine contents and thus different molecular weight, and can be separated from each other by density gradient centrifugation. Ten of the mRNAs (2 of which are bi-cistronic), 14 tRNAs and both rRNAs are transcribed from the heavy strand, one mRNA and eight tRNAs from the light strand (Anderson et al., 1981).

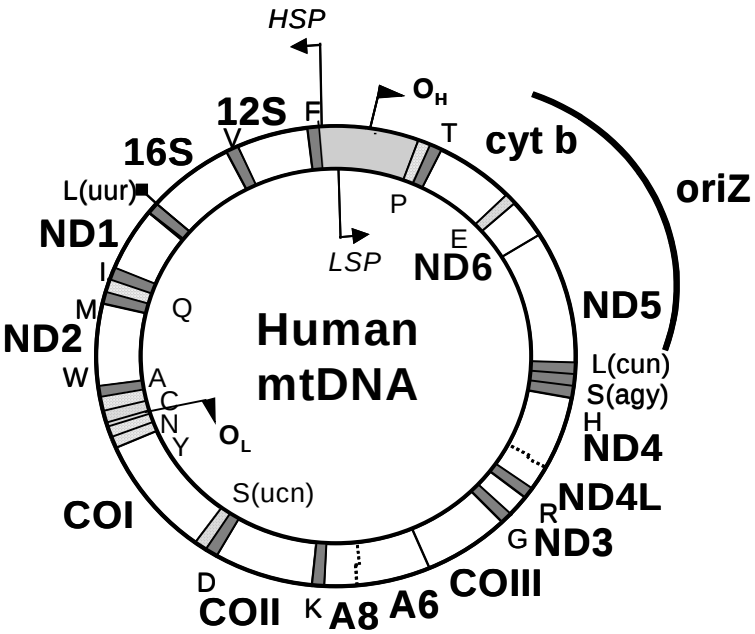


Figure 2. *Human mtDNA. Non-coding control region (CR) is between proline (P) and phenylalanine (F) tRNA genes. 12S and 16S, ribosomal RNAs; cyt b, cytochrome b; ND, NADH dehydrogenase; CO, cytochrome c oxidase; A, ATP synthase; O_H and O_L, replication initiation start sites according to the classical model; oriZ, replication initiation zone according to the latest model of Bowmaker et al., (2003). HSP; heavy strand promoter; LSP, light strand promoter. The 22 tRNAs are named according to the one letter code of the corresponding amino acid. Two different tRNAs for both leucine (L) and serine (S) are found in the human mitochondria indicated here by their codon recognition sequence.*

5.1.2 Nucleoids

Each cell contains typically thousands of mtDNA molecules organized in inner membrane-associated structures called nucleoids (Nunnari et al., 1997; Jacobs et al., 2000; Lehtinen et al., 2000), usually several mtDNA molecules (approximately 5) in each nucleoid together with proteins needed for replication and maintenance of mtDNA. The proteins found in nucleoids include

mitochondrial single stranded binding protein (mtSSB), the mitochondrial DNA helicase TWINKLE (*C10orf2*), transcription factor TFAM and both subunits of DNA polymerase γ (POLG, POLGβ). The last two are probably not structural nucleoid proteins but present only at sites of mtDNA replication or repair (Spelbrink et al., 2001; Garrido et al., 2003). Because of their capabilities for fission and to a lesser extent for fusion, and of their dynamic movements inside cell, nucleoids are suggested to be the mitochondrial units of inheritance. Segregation of these heritable units to daughter cells at cell division seems to be controlled by the nuclear genome (Lehtinen et al., 1999). In mammalian cells the discrete nucleoid structures are not seen in ρ^0 cells (cells artificially rendered devoid of mtDNA).

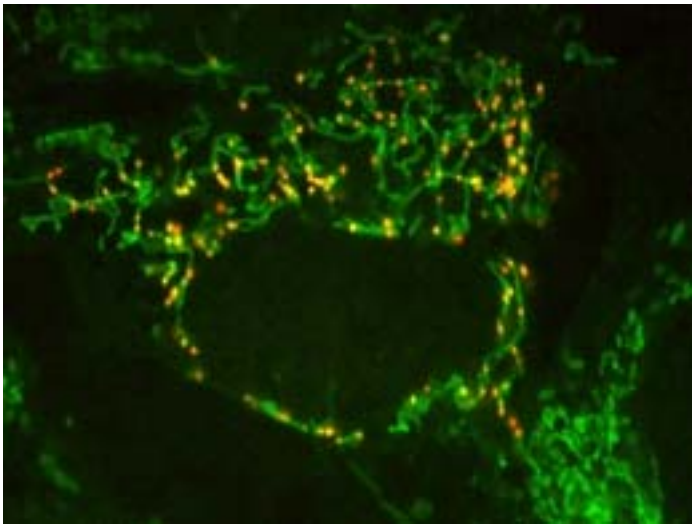


Figure 3*. Confocal microscopy picture of 143B osteosarcoma cells over-expressing fluorescent mtSSB-DsRed, a protein known to bind mtDNA. The dark area in the middle is the nucleus. The mitochondrial network is stained with Mitotracker Green. The red nucleoids co-localizing with the green mitochondria are yellow. Courtesy of Hans Spelbrink.*

5.1.3 mtDNA replication

The classical model of mammalian mtDNA replication originates from findings in electron microscopy over 30 years ago, and subsequent molecular studies (Clayton, 1982). According to the model the leading-strand replication of mammalian mtDNA maps to closely spaced, defined sites (origin of heavy strand replication O_H) located downstream from the major transcription promoter of the light-strand and proceeds unidirectionally with displacement of the parental heavy-strand until approximately two-thirds of the closed circular mtDNA has

been copied. As the replication fork passes the major origin for lagging-strand synthesis, the single-stranded form of O_L is exposed allowing it to adapt the characteristic secondary structure of this origin, thereby permitting initiation of lagging-strand synthesis. The two segregated progeny mtDNA circles are thus a duplex circle with a newly synthesized heavy strand and a gapped circle with a partially synthesized light strand. The final steps of synthesis and ligation of both new strands result in closed circular mtDNA products.

Subjecting mtDNA isolates to different nuclease treatments and 2D-gel electrophoretic analysis has recently shed new light on mtDNA replication. Analysing the migration behaviour of mtDNA restriction fragments has revealed different kinds of replication intermediates like Y-arcs, X-forms and replication bubbles. The available data indicate that mammalian mtDNA replication, at least under some circumstances, *e.g.* copy number amplification to restore mtDNA content, occur also *via* bidirectional, coupled leading- and lagging-strand synthesis. This appears to initiate from several points within a replication initiation zone (oriZ), downstream from O_H, including the genes for *cytochrome b*, *ND5* and *ND6* in the coding region in solid tissues (*Fig2*; Holt et al., 2000; Bowmaker et al., 2003), although the existence of oriZ has also been questioned using mtDNA extracted from cell lines (Fish et al., 2004). Both ribonuclease-resistant and ribonuclease-sensitive replication intermediates of mtDNA have been demonstrated, leading to the interpretation that the newly synthesized L-strand is much more ribonucleotide-rich than the H-strand, which seems to be RNase resistant. The possible role of RNA nucleotides within mtDNA is a rather controversial issue, and a proposal has been made that maybe the ribonucleotide-rich mtDNA is merely the "working copy" and the "complete" DNA genome is maintained only on low levels and serves as a master copy or "mitochondrial germ line" (Yang et al., 2002; Murakami et al., 2003).

The story of mtDNA replication and discussion concerning it is far from completed, even though it seems that the existence of the bidirectional strand-coupled replication is well documented. The two latest papers from Holt and co-workers show firstly, that in avian species mtDNA replication occurs bidirectionally throughout the genome initiating mainly from a wide initiating zone flanking the NCR (Reyes et al., 2004). In human cultured cells clear differences compared to solid tissues are seen in mtDNA replication (Yasukawa et al., 2005). Strand-coupled replication initiates bidirectionally in a narrow region of the NCR distal to O_H, compared to the broad oriZ in solid tissues. Also ribonucleotide incorporation is suppressed when mtDNA amplification is induced. Until now the majority of the results have come from one group of researchers, so a contribution to the discussion in the form of experimental results from other research groups would be appropriate.

5.1.4 mtDNA inheritance

Apart from red blood cells, where mtDNA is absent, the number of mtDNA molecules varies between 10^2-10^4 copies/cell. According to current data mtDNA is inherited purely maternally (St. John, 2000a; Marchington et al., 2002; Taylor, 2003), although there is one report (Schwartz and Vissing, 2004) describing a male myopathy patient with both maternally and paternally inherited mtDNA in his muscle tissue. The mutation most probably causing the myopathy symptoms in the patient is a 2 bp deletion in the mitochondrially encoded gene for complex I subunit *ND2*. This mutation is found only in the patients´ paternal mtDNA of haplogroup U5 but not in his maternal mtDNA pool of haplogroup H (haplogroups are discussed later in section 5.5.3). The authors hypothesize that the mutation happened spontaneously either in early embryonic development or in the father´s germ line.

During the early stages of oogenesis the mtDNA of an oocyte precursor cell goes through a so-called genetic bottleneck, such that the 1-2x10^5 mtDNA molecules of a mature ovum originate from a very small pool of precursor mtDNA molecules, perhaps as few as 200 copies (Reynier et al., 1998). In the first cell divisions of the primordial germ cells there is only partition of the existing pool of mtDNA with the cytoplasm, leading to a dramatic reduction in mtDNA copy number per cell. Later in oogenesis this pool is greatly amplified and then inherited by the offspring if the egg is fertilized and develops to a new individual.

5.2 DNA Polymerase gamma

Cellular replicative DNA polymerases fall in three different families, classified largely according to amino acid sequence alignments. The nuclear polymerases α, δ and ε all belong to the group B-family, and the bacterial replicative enzyme, DNA polymerase III holoenzyme, to the group C-family. Mitochondrial DNA polymerases, the γ polymerases, belong to the group A-family of bacterial DNA polymerases, of which the *E. coli* polI is the prototype. All γ polymerases share the common conserved exonuclease (exo I-III) and polymerase (pol A-C) motifs, but have additional γ-specific conserved sequence elements. Four of these additional elements reside between the exo and pol motifs on so called spacer region, and the remaining two are situated on both sides of pol C motif (reviewed by Kaguni, 2004).

5.2.1 POLG is a nuclear encoded mitochondrial protein

Human mtDNA polymerase is composed of two subunits, a 140 kDa catalytic subunit encoded by the *POLG* gene on chromosome 15q25, and a 55 kDa

accessory subunit (POLGβ) encoded by the *POLG2* gene on chromosome 17q23-24. The human mitochondrial DNA polymerase γ (OMIM 174763) is thought to be solely responsible for the replication, repair and recombination of the mitochondrial DNA (Ropp and Copeland, 1996; Lecrenier et al., 1997; Longley et al., 2001; Kaguni, 2004). The human POLG catalytic subunit exhibits both polymerase activity and 3′→5′exonuclease activity for proofreading, plus several RNA-associated catalytic activities, such as reverse transcriptase activity, RNA-primed DNA synthesis activity, proofreading activity with an RNA template, and ribonucleotide incorporation activity, all probably needed in the replication of mtDNA (Murakami et al., 2003). An additional catalytic activity needed in base excision repair (BER), and found intrinsic to POLG, is the 5′-deoxyribose phosphate lyase activity (Longley et al., 1998). In its amino-terminal end the POLG pre-protein has a mitochondrial targeting signal, 20 amino acids in length, which is cleaved off after translocation to the IM of the organelle (Spelbrink et al., 2000). The pre-protein is encoded by 23 exons, has 1239 amino acids and a molecular weight of 139,562 kDa.

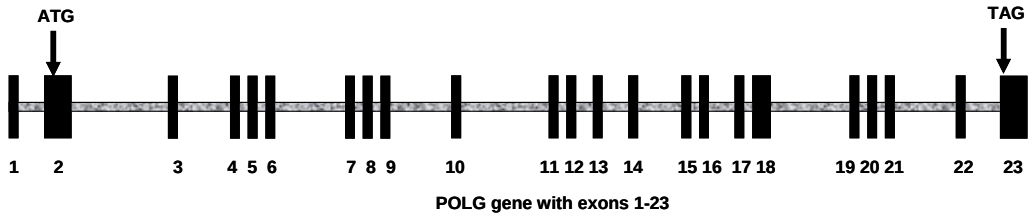


Figure 4. *POLG gene consists of 23 exons. An* ***ATG*** *in the second exon encodes the first amino acid (methionine) of the preprotein.* ***TAG*** *in exon 23 is the stop codon for translation. The last 374 nucleotides (of 500) of exon 23 are untranslated. Not to scale.*

Inside the coding region, in exon 2, POLG has a polyglutamine tract encoded by a disrupted CAG repeat $(CAG)_{10}CAA(CAG)_2$. The polyglutamine tract is not found at the corresponding position in Polg of mouse, *Drosophila* or yeast Ropp and Copeland, 1996; Lecrenier et al., 1997).

Figure 5. *Schematic presentation of the human POLG pre-protein. The mitochondrial targeting signal at the amino terminus of the preprotein is cleaved off after translocation across the mitochondrial inner membrane. The polyglutamine tract is present in the mature polypeptide. Exonuclease motifs (I, II, III) harbour the 3′→ 5′ proofreading activity, whilst the conserved polymerase motifs (A, B, C) contain the catalytic activity of the polymerase.*

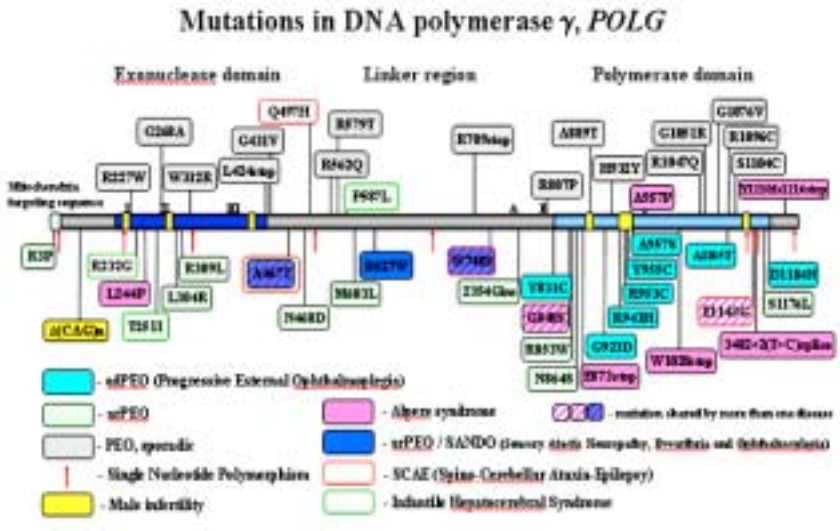
5.2.2 POLG point mutations are found in neurodegenerative disease

Point mutations in *POLG* have been connected with neurodegenerative diseases exhibiting a wide range of symptoms and severity. Progressive external ophthalmoplegia (PEO) is characterized by multiple deletions of mtDNA in post-mitotic tissues. The patients show ptosis (drooping eyelids) as the first and sometimes the only visible symptom, but also ophthalmoplegia (paralysis of the external muscles of the eye), more general skeletal muscle weakness and neurological symptoms like ataxia, neuropathy, depression and mental retardation are commonly encountered. Interestingly, in one study (Luoma et al., 2004) most women with PEO also show early menopause - before age 35 years, a symptom not typical for a mitochondrial disease. In addition, Parkinsonism and enhanced age-related accumulation of point mutations in the control region of mtDNA have been reported in patients with pathological *POLG* point mutations (Luoma et al., 2004; Wanrooij et al., 2004; Taanman and Schapira, 2005).

Both dominant and recessive inheritance of PEO as well as sporadic cases, have been reported (Van Goethem et al., 2001; Di Fonzo et al., 2003; Grazievicz et al., 2004). As illustrated in *Figure 6*, most of the dominant mutations seem to cluster in the polymerase domain, mainly in or close to the B motif. The recessive mutations are found in all parts of the coding sequence.

Another neurological, recessive, developmental disorder with mtDNA depletion and *POLG* mutation is Alper´s syndrome (Naviaux and Nguyen, 2004). The children born with this disease develop normally for the first few weeks to years. The symptoms include refractory seizures, episodic psychomotor regression, cortical blindness and liver disease, and the patients die as children or young adults. Many Alper´s patients are homozygous for the G2899T (Glu873Stop) nonsense mutation just upstream of the polymerase motif A, generating a truncated POLG protein. The polymerase activity in the patients´ cells at the late stages of the disease is still 5 %, suggesting some kind of stop-codon suppression, *i.e.* read-through of the amber codon by the ribosomes. Patients in that study were also heterozygous for the G1682A (Ala467Thr) substitution in the linker region of the protein but the possible connection to Alper´s syndrome is not known. The Ala467Thr mutation has been reported only in a heterozygous state in other conditions, and its prevalence in the Belgian population has been reported to be 0.6 % (Van Goethem et al., 2003).

Other mutations in different patients associated with MDS (mitochondrial DNA depletion syndromes) include several missense mutations in different regions of the protein, 3630C insertion creating a frame shift and subsequent stop-codon, and a 3482+2T→C splicing mutation in the 3´ exon/intron donor site of exon 21 (Ferrari et al., 2005). About 50 *POLG* disease mutations or SNPs (Single Nucleotide Polymorphism) have been reported this far (http://dir-apps.niehs.nih.gov/polg/).



http://dir-apps.niehs.nih.gov/polg/

Figure 6. *Visual overview of mutations that have been mapped to the gene for the catalytic subunit of DNA polymerase γ, POLG, and found to be associated with mitochondrial disease. This database lists known mutations in the coding region of the POLG gene with corresponding literature references. The list is maintained by the Mitochondrial Replication Group at NIEHS (National Institute of Environmental Health Sciences) and is regularly updated by Dr. William Copeland. The image presents the situation as of February 2006.* ***PEO****, Progressive External Ophtalmoplegia;* ***ar****, autosomal recessive;* ***ad****, autosomal dominant;* ***SANDO****, Sensory Ataxia, Neuropathy, Dysarthria and Ophthalmoparesis;* ***SNP****, Single Nucleotide Polymorphism. For discussion of mutations causing male infertility see results section of this thesis.*

5.2.3 POLG exonuclease deficient cell lines accumulate mtDNA point mutations

Human cell lines stably expressing POLG with an Asp198Ala (D198A) mutation in the Exo I motif (in addition to the endogenous POLG present in the cells) accumulate mitochondrial point mutations during three months´ continuous culture. In D198A cells grown for three months the mutation rate is 1/1700 bp or 5.9/10 kb (analysed in two independent cell clones), whereas cells expressing the wild type POLG or ΔQ_{10} version (CAG repeat deleted) of human POLG show no differences compared to control cells (Spelbrink et al., 2000). Exonuclease activity measured from cell extracts of D198A cells is undetectable. Mitochondrial functions in D198A mutator cell lines are not disturbed, which was suggested to be due to complementation arising from hundreds of copies of

mtDNA in each cell. We have to remember that the cells were still expressing the endogenous POLG, which might maintain enough intact mtDNA molecules in the cells. With mutations D890N or D1135A in the polymerase domain no stable cell lines could be generated, but in transient transfection experiments these mutations result in decreases in mtDNA levels.

Only human POLG is shown thus far to contain the polyglutamine repeat, so the ΔQ_{10} version of human POLG was also stably expressed in human cells to test the possible effect of its absence on protein function. Extracts from these cell lines show elevated levels of both protein and polymerase activity, compared with all other cell lines. The proofreading activity measured from cell extracts is present at control levels. Mitochondrial functions of these cells are not disturbed (measured as oxygen consumption) but the cells have a low mtDNA content compared to other cell lines in the experiment.

5.3. Trinucleotide repeats in human disease

Mutations affecting the length of microsatellite repeats are a frequent cause of a class of genetic diseases, called trinucleotide repeat expansion diseases (TREDs). Common examples of dominant or X-linked neurodegenerative disorders with coding DNA (CAG, polyglutamine) expansions are Huntington´s chorea (HD) and Kennedy´s disease, respectively, with mutations in the genes encoding huntingtin and androgen receptor, respectively. A common recessive disorder with a very large expansion of a non-coding repeat is Friedreich ataxia (GAA in intron 1). Other well-known syndromes associated with trinucleotide expansions are Fragile X syndrome (CGG, 5´ UTR), various spinocerebellar ataxias (CAG, polyglutamine), Synpolydactyly (GCG, polyalanine), and myotonic dystrophy (CTG in 3´ UTR). No expansions of GAC or GTC are found in human disease (Mitas, 1997).

In general, the longer the repeat, the more severe are the symptoms. Another feature is anticipation, the tendency to evolve ever longer repeats in each new generation. The structure of triplet repeats make them error-prone in replication, and pausing and stalling may happen, allowing the opportunity for slippage-mispairing (Michlewski and Krzyzosiak, 2004). During replication the primer and template strands transiently dissociate allowing the formation of DNA hairpin structures inside such repeats. Some hairpin structures are more stable and may escape the function of repair proteins. A DNA loop formed in a displaced neighbouring Okazaki fragment during synthesis is resistant to the 5´→ 3´ flap endonuclease. In both cases the result is a loop of extra DNA and so expansion of the repeat (Moore et al., 1999). As the repeat grows, the expansion rate increases. This can be seen in the pre-mutation alleles of HD patients´ unaffected parents (reviewed in McMurray, 1999; Gárdián and Vécsei, 2004).

The appearance of polyglutamine aggregates at the protein level is dependent not only on the length of the repeat, but the polyglutamine protein context and posttranslational modifications are also important (Holmberg et al., 2004). An expanded polyglutamine stretch can lead to conformational change and/or abnormal protein-protein interactions. In HD patients the glucose metabolism is decreased in cerebral cortex leading to the enhanced production of free radicals and oxidative damage. The expanded polyglutamine repeat of huntingtin, the protein encoded by the *HD* gene, has been shown to interact abnormally with other proteins containing short polyglutamine tracts, such as the transcriptional co-activator CREB-binding protein, CBP (Nucifora et al., 2001). Human CBP containing a stretch of 18 glutamines is not only recruited into the aggregates but also redistributed away from its normal location in the nucleus into huntingtin aggregates. The redistribution of CBP from the nucleus is dependent on the polyglutamine in CBP because CBPΔQ was not sequestered from the nucleus. A polyglutamine binding protein (PQBP1) binds preferentially to longer polyglutamines and is shown to bind mutant ataxin and huntingtin. Mutations in PQBP1 have been shown to cause X-linked mental retardation (Kalscheuer et al., 2003). Polyglutamine binding to regulatory proteins and signalling molecules leading to modified transcription may underlie much of polyglutamine-mediated pathology.

Polyglutamine aggregates are seen as inclusion bodies in both nucleus and cytoplasm of CAG-repeat disease patients´ cells. The mutant proteins are targeted into both the ubiquitin/proteasome and endosomal/lysosomal pathways for protein degradation. Polyglutamine aggregates have been shown to interfere with proteasome function by engaging the proteasome machinery for longer times than other substrates (Holmberg et al., 2004).

Not all TREDs involve polyglutamine, and the disease-causing “agent” must be the extended RNA in cases where the RNA is not translated, such as in FXTAS (Fragile X-associated tremor/ataxia syndrome) or in Friedreich ataxia. In the case of “traditional” polyglutamine disease, a *Drosophila* model however has demonstrated that the polyglutamine tract *per se* can cause visible symptoms, with both types of glutamine-encoding repeats, CAG or CAA (McLeod et al., 2005). If a stop codon is inserted 5´ of the repeat, leaving the repeat itself in the 3´ UTR, no visible symptoms appear in the fly.

5.4 Trinucleotide repeat variation and evolution

In a computational comparative analysis of 28 repeat-containing gene sequences of human and mouse, no evidence for preferential accumulation of expansion of CAG repeats in the human genome relative to that of mouse was seen (Hancock et al., 2001). There was also no evidence of any difference in the distribution of CAG repeats within coding regions between the two species, or differences in base composition between genes containing or not containing such repeats. So in

the light of this study, it seems that any evolutionary repeat expansion would be gene-specific.

The locus type (anonymous, gene-associated, disease-causing trinucleotide repeat) is the major significant factor for differences in within-population genetic variability, the disease-causing CAG repeats (in the non-disease range of repeat counts) having the highest within-population variability (Deka et al., 1999). The patterns of repeat-length variation of 29 trinucleotide-repeat loci from diverse locations of the human genome did not conform to any standard distributional form (bell shape, uniform, L shape, J shape). GC-rich gene-associated trinucleotides were found to be the least polymorphic. In general, the allele sizes are overlapping in all four populations studied (Germans, Nigerians, Chinese and New Guinea highlanders) but they show frequency differences across populations.

The first exon of the AR gene (androgen receptor) contains several trinucleotide repeats with unknown function. The glutamine-repeat beginning at codon 58 is polymorphic with certain length variants being disease-associated (see section 5.5.2). Comparison of the CAG (polyQ), and GGC (polyG) repeat lengths of AR in different primate species has been carried out in order to follow the evolution of trinucleotide repeats, and revealed the linear increase in trinucleotide repeat expansion of homologous sequences to be proportional to the time of species divergence (Choong et al., 1998). The NH_2-terminal residues 1-53 as well as residues 360-429 of the AR are completely conserved among primates studied (human, chimpanzee, baboon, macaque). The diagnostic polyQ1 (first of the three glutamine repeats in exon A of AR) sequence length is 2Q in rat, 4Q in lemur, 7-9Q in macaque, 8-10Q in baboon, 18-23Q in chimp and 22Q in human, showing a fast evolution in the repeat length after branching of old world monkeys. Serine 94 is phosphorylated in human AR and the study documented a positive correlation between the repeat length expansion and phosphorylation status of this particular serine.

Comparison of seven expanding CAG loci in four primate species (human, chimpanzee, gorilla, orang-utan) shows that in general, humans have higher mean number of repeats than other species, although this does not apply in all cases (Andrés et al., 2004). Repeat-length variance is higher in humans for the expanding loci but not for the nonexpanding loci, heterozygosity is high and very similar in humans and chimpanzees but low in gorillas and orang-utans. In corresponding loci of mouse a complete absence of the repeat or only very short tract is present. The seemingly contradictory conclusions of the two studies (Hancock et al., 2001 and Andrés et al., 2004, respectively) come more close to each other when the differences in selection of analysed gene loci and the different questions asked in the beginning of both studies are taken into account. The Hancock study focused on the question if there was any evidence of preferential accumulation or expansion of CAG repeats between human and mouse, and if the surrounding amino acid composition would be a driving force in repeat expansions. The latter Andrés study compared selected functional repeats, expanding with nonexpanding, in different primate species. Three main

conclusions from these two studies are: (i) Overrepresentation of amino acids Gln, Pro, Ser, and His in repeat-containing proteins may reflect some functional selection on these genes both in mouse and human. (ii) If the gene was prone to repeat expansion, the repeat expansion has happened during evolution. (iii) The further expansion of these repeats will continue in some disease-causing genes from one generation to another if no purifying selection was present.

5.5 Male infertility

The clinical definition of infertility is "The inability of a couple to achieve conception after one year of unprotected intercourse, or the inability to carry direct pregnancy to live birth."

Up to 20 % of couples are estimated to suffer from infertility (WHO 1987), and half of the cases are attributable to male defects. Male infertility can result from a multiple of different reasons but in many cases the aetiology behind it remains uncovered. Unlike other health problems, reproductive health problems do not show "symptoms" in young, seemingly healthy people.

It has been asserted that, “The human male is characterized by extremely poor semen quality as reflected in the number, morphology and motility of the spermatozoa and a high incidence of nuclear and mitochondrial DNA damage” (Aitken et al., 2003). This observation allows us to hypothesize that defective sperm function is a major contributor to the aetiology of human infertility. The factors contributing to poor semen quality include genetic aberrations, paternal age and environmental factors. Diminished selection for fitness may also play a role (personal hypothesis).

5.5.1 Chromosomal abnormalities in male infertility

The 22 pairs of autosomes and two sex chromosomes, XX in females and XY in males, are the 46 chromosomes found in normal human cells. In infertile males chromosomal abnormalities are found in 5.8 % of cases and of these, sex chromosome abnormalities account for 4.2 % and autosomal abnormalities for the remainder. For comparison, the incidence of chromosomal abnormalities in newborn male infants is 0.38 % and the corresponding numbers for sex chromosome and autosomal abnormalities 0.13 % and 0.25 %, respectively (reviewed by Hargreave, 2000).

Disorders of the chromosomes can be numerical or structural. Numerical polyploidy means that the whole set of chromosomes is multiplied (triploid = 69 chromosomes, tetraploid = 92 chromosomes), and is essentially incompatible with life. Aneuploidy means a loss or gain of one or more chromosomes, the most common in the context of male infertility being 47XXY or Klinefelter syndrome. Structural disorders of chromosomes may be deletions or duplications

of material, or rearrangements of material from one chromosome to another. Chromosomal aberrations are more common in males with azoospermia (no spermatozoa in semen fluid) or with low sperm counts (< $2x10^7$ /ml), than in males with normal sperm counts (Vogt, 2004; Clementini et al., 2005).

Klinefelter syndrome is the most frequent sex chromosome abnormality (0.07 % of male births). It is found in different variants, (47XXY; 48XXXY; 49XXXXY) with increasing number of X chromosomes shifting the phenotype in the female direction. Mosaic karyotypes 45X; 46XY/47XXY; 47XYY; 45X/46XY also lead to reduced fertility through inhibited or faulty meiotic pairing in germ cell development.

Chromosomal rearrangements affecting the Yq11 region of the Y chromosome lead to infertility, manifesting as azoospermia or oligozoospermia because of disruption of the AZF (Azoospermia Factor) locus. AZF is subdivided into three spermatogenesis loci, AZFa, AZFb, AZFc, and the deletions of each of these interrupt spermatogenesis in different phases, respectively, resulting in complete absence of germ cells, meiotic arrest at pachytene or mixed atrophy in testicular tubules.

5.5.2 Other genetic anomalies in male infertility

The CF-locus in 7q31.2 (OMIM 602421) encodes the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene. The basic function of the protein is a low-voltage, cyclic-AMP-regulated trans-membrane channel for chloride ions. *CFTR* gene defects cause Cystic Fibrosis (CF), which is mainly characterized by chronic and severe infection in the airways. The majority of adult males with CF (99 %) have congenital bilateral absence of *vas deferens* (CBAVD, Jarzabek et al., 2004) and because of that the transport of mature spermatozoa through the testicular and epididymal structures is blocked. The increasing number of *CFTR* mutations defined and tested within CBAVD men brings the number of mutation carriers close to 100 %.

The androgen receptor (AR) gene located at Xq11-12 (OMIM 313700) encodes a transcription factor, which is a member of the steroid receptor family (Lubahn et al., 1988). Androgen binding in the cell cytosol activates the receptor. After translocation to the nucleus the AR binds to androgen response elements (ARE) in the promoter regions of androgen-responsive genes, causing specific gene transcription. In the trans-activation domain in exon 1 the AR protein has a polyglutamine tract encoded by $(CAG)_n$, as discussed earlier. The normal length variants are 22+/-3 glutamines (Tut et al., 1997). In the case of shorter polyglutamine tracts the risk of prostate cancer increases but the risk of infertility decreases, probably because of greater “androgenicity” that boosts germ cell proliferation and over-stimulates the growth of prostatic tissue. On the other hand, individuals with repeat lengths more than 24 often present with oligozoospermia, and lengths over 26 can manifest as azoospermia (Mifsud et al., 2001). With a CAG repeat-length of 40-66 the patients start to show

symptoms of Kennedy´s disease (X-linked SBMA, Spinal Bulbar Muscular Atrophy), a neurological disorder. The symptoms include progressive weakness of muscles of the face, neck and shoulders, low virilization, reduced sperm production, testicular atrophy and infertility (La Spada et al., 1991). Point mutations affecting the linker regions located between the structural helices of the AR ligand-binding domain (LBD) can result in male infertility in minimal AIS (androgen insensitivity syndrome, Yong et al., 1998).

Myotonic muscle dystrophy (DM) is a dominant inherited disorder with myotonia, a condition in which the muscles contract but have decreasing ability to relax. The patients can also present with motor and sensory neuropathy. The major gene involved is DM-1 or DMPK (Dystrophia Myotonica Protein Kinase, OMIM 605377) on chromosome 19q13.2-3. DM is caused by an expansion of the CTG repeat in the 3´ UTR of the RNA (Brook et al., 1992). The mutated RNA accumulates in the cell nucleus causing a series of downstream consequences, including mental deficiency, hair loss, cataracts, hypogonadism and infertility. Unaffected individuals have 5-35 copies of the CAG-triplet. Minimally affected DM patients have at least 50 repeats, while severely affected patients can have an expansion of thousands of repeats. Infertile DM patients have testicular atrophy, loss of libido and of potency, and a complete loss of capacitation and acrosome reaction of sperms.

The list of other genetic disorders resulting in male infertility is extensive. In many of them the underlining gene defect is unknown, but there are also numerous infertility syndromes with polygenic inheritance patterns. The cellular phenotypes are highly variable, including for example, immotile sperm because of complete loss of dynein arms or round-headed sperm with malformed acrosome (reviews by Vogt, 2004 and Hargreave, 2000).

5.5.3 Mitochondrial haplogroups and infertility

The uniparental inheritance and relatively high mutation rate compared to the nuclear DNA has generated heritable variants of human mtDNA, spread throughout the human population. Specific maternal lineages or haplogroups can be identified by sets of shared mtDNA sequence polymorphisms. The major haplogroups are defined by ancient polymorphisms and characterized by considerable variance. Haplogroups L0, L1, L2, L3 and L4 are the most ancient and common amongst the African populations, whereas haplogoups A, B, C, and D predominate amongst Native Americans. The European population is mainly distributed among haplogroups H, I, J, K, T, U, V, W and X, whereas the Asian populations carry mainly haplogroups A, B, C, D, F, G and certain sub-clusters of haplogroups M and N (Macaulay & Richards, 1999; 2000; 2002).

Several studies of haplogroup frequencies in different populations, as well as possible distribution differences correlating with certain diseases or longevity have been published (Schurr and Wallace, 2002; Jacobs et al., 2005; Niemi et al., 2005; see also *Table 1*)). In an analysis of sperm motility and haplogroup

correlation in the Spanish population (Ruiz-Pesini et al., 2000a) a higher proportion (7.4 %) of haplogroup T was found amongst asthenozoospermic (AP) subjects compared to the non-asthenozoospermic (non-AP) males (2.2 %). Similarly a higher proportion on haplogroup H (59.4 %) was present in non-AP compared to the AP subjects (44.9 %). Both complex I and complex IV showed high relative enzymatic activity in samples carrying the H mtDNA haplogroup and the lowest relative activity in samples harbouring the T mtDNA, although the 23 % and 29 % reductions, respectively, were not statistically significant by the ANOVA test. From the increased frequency of haplogroup T in the moderate AP population, and the reduced cI and cIV activities in T samples, the authors inferred that haplogroup T represents a weak genetic background that can predispose to asthenozoospermia. The accumulation of the H mtDNA haplogroup in the non-AP population, on the other hand, could be taken as an indication of a robust genetic background preventing against some OXPHOS weakness in spermatozoa.

5.5.4 Sperm mtDNA content and infertility

In mammalian sperm the number of mtDNA molecules is greatly reduced during spermatogenesis (Rantanen et al., 2001). The copy number of mtDNA in mature sperm varies from as low as none or a few molecules/cell to 200-1200 molecules/cell depending on the state of maturity and mobility of the sperm, as well as from the method used in the studies (Diez-Sánchez et al., 2003a; May-Panloup et al., 2003). All mitochondria (and so all mtDNA molecules) of spermatozoa are located in the mid piece and enter the egg after sperm-egg fusion. They are, however, destroyed during the first cell divisions following fertilization, probably by the ubiquitin-proteasome system, and paternal mtDNA is no longer detectable in the preimplantation embryo (Sutovsky et al., 1996; 2000). The mitochondrial ribonucleic acids (mtRNAs) in sperm are not translated any more in ejaculated human sperm. This means that the OXPHOS system components are all synthesized and assembled during sperm maturation and the ETC activities fixed long before they are measurable (Diez-Sánchez et al., 2003b).

The most motile sperm seem to have the least mtDNA (May-Panloup et al., 2003.) After fractionation of the sperm by Puresperm density gradient centrifugation, the mtDNA/β-globin gene ratio in the “100 % layer” containing the most motile sperm was 1.4 for normal sperm, 6.1 for sperm presenting at least one abnormal criterion (from sperm count, motility and morphology) and 9.1 for sperm samples with two or more of these abnormal criteria. In the 40 % density layer containing the most abnornal sperm from a given sperm sample, the numbers were much higher, and the mean ratio for mtDNA/β-globin gene was 17.1. The low mtDNA copy number in mature sperm is probably one means to prevent paternal inheritance, and thus contributes to the purely maternal

inheritance of mtDNA. Most probably the one sperm that fertilizes the egg will be from the pool of the most motile.

5.5.5 Sperm mtDNA mutations and fertility problems

Both oocytes and sperm carry mtDNA deletions at a low level (Reynier et al., 1998). A Southern blot from different single-cell oocyte PCR amplification from the same donor showed a distinctive pattern of zero to several mtDNA deletions. In the case of sperm samples the tendency seems to be for more deletions in the less motile, less fertile sperm fractions (Kao et al., 1998), with an absence of correlation between the age of the patient and the occurrence of deletions, such as is commonly seen in other tissues. Reduction of sperm mtDNA content (depletion) has also been reported in men with asthenozoospermia (Kao et al., 2004). Biochemical studies have shown a correlation between the mitochondrial functionality and seminal quality, especially sperm motility (Ruiz-Pesini et al., 1998; 2000b).

The data above, together with numerous other studies, describe more defects in mtDNA quality and quantity, and subsequently in mitochondrial functions, in sperm cells of infertile/subfertile individuals than fertile males. The flawless mitochondrial function thus seems to be a prerequisite for optimum fertility. The available data is anyhow mainly descriptive, and the molecular mechanisms behind *e.g.* the increased amounts of mtDNA mutations in sperm cells are not clear.

5.6 Tumour protein p53 or TP53

Wild type *p53* is a recessive tumour suppressor gene and a transcription factor involved in several and diverse cellular mechanisms, while mutant forms can act as dominant oncogenes. p53 protein amount is usually low and regulated by MDM-2 protein, which ubiquitinates p53 and thus targets it to proteasomal degradation. p53 is generally considered as “guardian of the genome” because of its role in sensing DNA damage and responding accordingly by initiating events that lead, depending on the level of the damage, either to cell cycle arrest and DNA repair, permanent cell senescence or apoptosis. This pleiotropy is probably due to several different signal transduction pathways acting in different cellular contexts and developmental stages. Over 35 000 articles, including many comprehensive reviews, are immediately listed by a search for p53 in PubMed. I am now going to briefly review some of them (Harris and Hollstein, 1993; Liu and Kulesz-Martin, 2001; Campisi, 2001; 2005) to summarize the main functions and signalling responses of p53, with the later discussion about checkpoints in mind (see sections 5.7.6 and 5.8).

The protein consists of 393 amino acids and contains domains for trans-activation, DNA-binding and tetramerization, a basic domain, and sites of binding for cellular (MDM-2) and oncoviral proteins (SV40 T-antigen and human papillomavirus E6 and adenomavirus E1b proteins). The activated p53 has a binding activity for DNA, both to specific and to non-specific sequences (el-Deiry et al., 1992). The specific DNA sequence recognized and bound by the protein, termed the p53 responsive element (RE), consists of two copies of the 10 bp motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by a spacer of 0-13 nucleotides. In addition, p53 is capable of binding DNA non-specifically at sites such as DNA duplex with free ends, nucleotide insertion-deletion lesion mismatches, Holliday junctions, triple-stranded DNA, double-stranded DNA with a gap, nicked DNA, or DNA damaged by γ-radiation; all having ssDNA adjacent to dsDNA, and all representing intermediates of DNA damage and DNA repair. This non-specific DNA binding is mapped to the C-terminal end of the protein, as is the ssDNA-binding activity of p53.

Stress signals are transmitted to p53 by post-translational modifications brought about by mediator molecules, including phosphorylation by protein kinases, acetylation by co-activators with acetyltransferase activity, deacetylation by corepressors with deacetylase activity, ubiquitination by ubiquitin ligases MDM-2, Cop-1 or Pirh-1 that direct p53 to degradation by the proteasome, or sumoylation by SUMO-1. These modifications contribute to p53 regulation by affecting its DNA binding, degradation, localization and/or oligomerization. Association with different cellular factors, and a combinatorial set of modifications mentioned above may well be specific to cell type and to the type of stress in the cell. The stress signals include DNA damage, telomere shortening, hypoxia, ribosome biogenesis problems, rNTP depletion, spindle damage, heat or cold shock, nitric oxide (NO) produced by tissue inflammation, and oncogene activation. Posttranslational modifications lead to activation of p53 as a transcription factor that initiates cellular programs leading to cell cycle arrest (G1 or G2), senescence or apoptosis. P53 protein mediates the apoptosis checkpoint in response to DNA damage, and the end result might be dependant on the threshold of the ratio of [sdsDNA (partially single-, partially double-stranded DNA)] + [p53] / [DNA repair proteins]. If there is an excess of functional p53, apoptosis will happen, but if p53 amount or activity are not elevated the cell will survive and continue replication after DNA repair. In addition to protein modifications, other factors influencing cell fate (cell cycle arrest, senescence or apoptosis) include the nature of the stress signal and pattern of gene expression occurring in the specific tissue undergoing the p53 response.

There are several positive and negative autoregulatory feedback loops in the p53 response (deduced from results obtained using cultured cancer cells), several of which act through the MDM-2 protein. Each of these loops creates a network composed of proteins whose activities or rates of synthesis are influenced by the activation of p53, and this in turn results in the alteration of p53 activity in a cell. The positive feedback loops include PTEN-AKT, $p14/19^{ARF}$ and RB. The negative feedback loops modulating p53 activity down may function through

MDM-2, Cop-1, Pirh-2, p73 delta N, cyclin G, Wip-1 or Siah-1 proteins (for details, see Harris and Levine, 2005).

p53 is the most commonly mutated gene in human cancers, with the frequency exceeding 50 % (Harris and Hollstein, 1993). Most mutations that inactivate p53 in cancer cells are missense mutations within the evolutionary conserved DNA-binding domain of the p53 protein, producing a protein that fails to activate transcription of p53-responsive genes. A mutation in p53 can lead not only to a loss of function, but also to a gain of oncogenic functions, or adoption of a dominant-negative conformation able to inactivate the protein product of the normal allele by heterotetramerization.

A fraction of p53 localizes into mitochondria upon cisplatin-induced DNA damage, which has been implicated in bringing about cell death by apoptosis (Yoshida et al., 2003). A putative p53-binding sequence has been identified in mtDNA (Heyne et al., 2004), starting at position 1553, by co-expression experiments and *in vitro* binding assay, but no p53-mtDNA complexes have been detected thus far in ´chromatin` immunoprecipitation assays. However, p53 has been shown by coimmunoprecipitation experiments to interact physically *in vivo* with mitochondrial transcription factor A (mtTFA) and to regulate its binding to DNA (Yoshida et al., 2003). Furthermore, p53 seems to enhance mtTFA binding to cisplatin-modified but not oxidised mtDNA, probably reflecting the different affinity of mtTFA to mtDNA with different conformations. p53 -deficient mouse embryos show a secondary reduction in mitochondrial 16S RNA transcript amounts during the embryonal switch from anaerobic to aerobic metabolism (Ibrahim et al., 1998).

POLG has been suggested to play a role in mtBER (base excision repair) *in vitro,* based on the identification and analysis of its 5´-deoxyribose phosphate lyase activity (Longley et al., 1998). Basal concentrations of p53 may function in the nucleotide incorporation/termini-processing step in mtBER. POLG nucleotide incorporation activity is lower in mitochondrial extracts from p53 deficient mice. Recombinant p53 has also been shown to enhance POLG activity in a dose-dependent manner (de Souza-Pinto et al., 2004). In another paper (Stuart et al., 2004) the same group claims that an unknown activity, factor or interaction is important in BER and is deficient in ρ^0 mitochondria from 143B cells. These results were obtained by using mitochondrial lysates and oligonucleotide substrates containing uracil residues or 8-oxodG. No direct interaction between p53 and POLG has yet been demonstrated, but POLG can be stimulated by PCNA, which interacts directly with p53 (Taguchi et al., 1995).

5.7. Cellular senescence and ageing

Senescence and ageing

Senescence is the eventual and inevitable result of cell differentiation. Hayflick suggested already forty years ago (Hayflick, 1961; 1965) that there is a certain replicative lifespan for cells, and proposed that this ageing process occurs intra-cellularly. His work showed for normal human fibroblasts in culture a replicative capacity of 60-80 population doublings and he postulated that only cancer cells can have the property of immortality. Cellular senescence and development of cancer are always discussed together and described as the two extreme ends of ageing. On the other hand, ageing and tumours are considered to be as connected as a pot with its lid.

Fibroblast cultures derived from aged individuals have an earlier onset of cell-culture senescence and a shorter *in vitro* lifespan compared to parallel cultures of young donors (Schneider and Mitsui, 1976). The differences observed in cell cultures from old and young donors are qualitatively distinct from differences observed in early and late passages of WI-38 cells (modelling *in vitro* ageing).

Telomeres are repetitive DNA sequences (TTAGGG) that with specialized proteins cap the ends of linear chromosomes, and are essential for chromosomal integrity. During each cell division the telomere length is reduced by 50-200 bp due to the biochemistry of DNA replication, and telomere length is proposed to predict the replicative capacity of human fibroblasts. A recent study (Valdes et al., 2005), investigating white blood cells (WBC) of over 1100 Caucasian women aged 18-76 years found that, the telomere length decreases steadily with age at a mean rate of 27 bp per year. Cigarette smoking and obesity increase the rate of telomere loss significantly. The difference in telomere length between being lean and being obese corresponds to 8.8 years of ageing; smoking corresponds on average to 4.6 years of ageing; and smoking one pack of cigarettes per day for 40 years corresponds to 7.4 years of ageing.

A reverse transcriptase, telomerase, that is responsible for *de novo* telomere synthesis, has been shown to be able to restore telomere length. The enzyme is not present in most normal human cells, but is shown to be highly active in cancer cells and in immortal cells. Interestingly, telomerase is also active, although at a much lower level, in human fetal tissue, normal bone marrow stem cells, testes, peripheral blood lymphocytes, skin epidermis and intestinal crypt cells, which all are in a continuously replicating pool of differentiating cells (Ulaner and Giudise, 1997). Telomere shortening may thus be the molecular determinant of longevity determination and not ageing.

It has been suggested that ageing is a stochastic process that occurs after reproductive maturity in animals that reach a fixed size in adulthood, leading to

molecular disorders that represent the genesis of age-associated changes. As repair mechanisms gradually become overwhelmed, the escalating molecular fidelity ultimately exceeds the repair capacity and results in increasing vulnerability to age-associated pathology (for debate and arguments, see Hayflick, 2003; 2004; Holliday, 2004). Longevity, on the other hand, is proposed to be genetically driven, species specific, and linked to the time needed for reproductive maturation. The two terms are considered as different entities even though in common language their usage is not accurate. The stochastically driven ageing process, including ROS-induced damage to chromosomes and mtDNA, acts to perturb the fidelity of the molecules present at reproductive maturity. In young animals repair processes are proposed to be capable of managing these losses in molecular fidelity to ensure reproductive success.

On the cellular level the term senescence (meaning terminal cell cycle arrest) is used when talking about ageing, and two different mechanisms with descriptive names are proposed (Mathon and Lloyd, 2001). Replicative senescence is induced as a result of the countdown of an intrinsic mitotic counter – the only known counter mechanism being telomere shortening. However, it is probably only a contributory factor: lower organisms, consisting mostly of post-mitotic cells, still age, as do the post-mitotic tissues of higher organisms. Premature senescence is induced by extrinsic factors that can act at any point of a cell´s replicative history. These factors include oncogenic Ras protein, DNA damage, oxidative stress, or cumulative trauma of *in vitro* cell culture.

5.7.1 The mitochondrial theory of ageing

Free radical theory of ageing

The "free radical theory of ageing" was introduced already 50 years ago (Harman, 1956) based on findings that endogenous oxygen radicals were generated in cells and that those reactive species would result in a cumulative damage to cells over years. Harman´s theory was later supported by the documented lifespan-prolonging effects of antioxidant enzymes, like superoxide dismutase (SOD, Sohal et al., 1996). Since mitochondria are the site of cellular respiration and main producer of the reactive oxygen species (ROS) and free radicals, the theory has evolved into the "mitochondrial theory of ageing". According to the theory, cell senescence and ageing occurs because of mutations, inactivation or loss of mitochondrial DNA either leading to and/or resulting from ROS production. Oxygen radicals are produced as by-products of respiration during the transfer of electrons from NADH or $FADH_2$ to molecular oxygen through the quinone cycle (Finkel and Holbrook, 2000). Mitochondria consume ~90 % of delivered oxygen and estimations of ROS production vary between 0.1-2 % of consumed oxygen to be converted to free oxygen radicals (Lopez et

al., 2000; Wright et al., 2004). The possible lack of fully operational DNA repair systems in mitochondria, or non-programmed but unavoidable oxygen radical damage to the membranes and genome of mitochondria in terminally differentiated cells is proposed to result in cellular senescence. Cell differentiation triggers, and metabolic rate modulates the rate of senescence.

Often used synonymously is "the rate-of–living hypothesis" (ROL), describing the following cascade: the higher the metabolic rate, the greater the ROS production and the shorter the lifespan (Finkel and Holbrook, 2000). The theory, itself originating from Aristotle´s text, has been under discussion from the beginning of the 20th century and was recently reviewed extensively by Speakman (2005). The theory is based on findings that, across species, one gram of tissue on average expends about the same amount of energy before it dies (measured as resting metabolic rate, RMR, lifetime expenditure of energy per gram of tissue) regardless of species. One problem in applying the theory is that animals and humans expend enormous amounts of energy on things other than RMR. Other problems arise *e.g.* on definitions and data recording of life spans (wild animals *vs.* captives, average *vs.* maximum life span), and finally, birds and bats, as well as primates, deviate from other species because they tend to produce fewer ROS at a given metabolic rate. Rates of mitochondrial superoxide and H_2O_2 formation are shown to correlate negatively with maximum lifespan whereas basic metabolic rate (BMR) is poorly correlated with lifespan (Wright et al., 2004).

mtDNA mutations in ageing (see also Table 1.)

Mitochondrial DNA deletions and point mutations accumulate during ageing in several different species ranging from worms and flies to rodents (respectively, Melov et al., 1995; Yui et al., 2003; Tanhauser and Laipis, 1995) and humans (Liu et al., 1998; Michikawa et al., 1999; Wang et al., 2001). One of the early reports in humans was the report of accumulation of the common 5kb deletion (Kearns-Sayre syndrome or KSS deletion or mtDNA^{4977}) in normal heart and brain of adult individuals but not in corresponding fetal tissues (Cortopassi and Arnheim, 1990). There seems to be tissue-specific differences in accumulation of the common deletion, the skeletal muscle having the most and myocardium and kidney much less of the mtDNA^{4977} (Liu et al., 1998). Skeletal muscle of rhesus monkeys (*Macaca mulatta*) has been shown to accumulate deleted mtDNA molecules and develop electron transport system (ETS) abnormal fibre segments that are COX^{-}/SDH^{++} in an age-related manner (Lopez et al., 2000). The length of the region exhibiting ETS abnormalities within a fibres increase with age and results in fibre atrophy. The KSS deletion is also shown to be present in mtDNA of both human oocytes and preimplantation embryos, but the percentage of the deletion was significantly reduced after fertilization (Brenner et al., 1998).

Single-cell oocytes from a given individual are shown to carry mtDNA with several different deleted molecules and sperm samples of different males to carry

distinct patterns of deletions (Reynier et al., 1998). No correlation between the mtDNA deletions in human gametes and patient age is reported although an association between mtDNA deletions and sperm motility and fertility has been suggested (Kao et al., 1998). Clonally expanded, deleted mtDNA molecules found in aged human tissues and lacking the origin of light strand replication (O_L) are partially duplicated molecules, similar as found in certain mtDNA disorders (Bodyak et al., 2001). Even though the age-associated accumulation (starting around 40 years of age) and pattern of the deleted mtDNA molecules seems to be tissue specific, with skeletal muscle having the most before heart and kidney, not all tissues in the same individual, or not all individuals seem to have these deletions (Liu et al., 1998). Other studies have shown a low frequency existence of sublimons (re-arranged mtDNA molecules with deletions and partial duplications) with variable sizes in several human tissues (Kajander et al., 2000; 2002). Sublimons are tissue-specific and accumulate with age, but at such a low rate that they would probably never become a significant contributor to age-related pathogeneses.

Similar findings of differences in accumulation of deleted mtDNA molecules (dmtDNA) between individual animals or tissues in one animal are reported in rodents (Tanhauser and Laipis, 1995), although in mouse, liver seems to accumulate more deletions than other tissues such as brain, muscle or heart. Small amounts of diverse mtDNA deletions can be detected in DNA extracted from the thorax (containing lots of muscle tissue) but not from whole-body extracts of ageing fruit fly *D. melanogaster* (Yui et al., 2003) as well as in a single worm PCR/Southern blot of lysed, ageing nematode *C. elegans*. The frequency of deleted mtDNA (ΔmtDNA) in a longer-living *age-1* mutant nematode was much less than in controls of similar chronological age, demonstrating that the accumulation of ΔmtDNA can be modulated by genetic background, at least in this experimental model.

Specific point mutations accumulate in the control region of mtDNA isolated from muscle tissue, as well as fibroblast cultures derived from aged people (Michikawa et al., 1999; Wang et al., 2001) but not in ageing mice (Song et al., 2005). The specific point mutations in the region containing the two evolutionary conserved sequence blocks (CSB2 and CSB3) of the non-coding region were found to accumulate in humans aged 60 years or older, whereas small amounts of these specific mutations, random substitutions, deletions and (CA)n length variations could be detected at any age. One must note however, that not all (even old) individuals carry point mutations in their mtDNA, and that the mutation frequency varies between individuals. An increase in mtDNA copy number and/or mitochondrial volume is seen in many cases of pathogenic mtDNA mutations, suggesting that cellular response mechanism compensates such defect in energy production by increasing the number of functional units. However, Miller et al., (2003) found no significant change in mtDNA copy number with age in humans.

The overall conclusion from these and many other papers is that the theory of oxidative stress in ageing, however attractive in principle, is still supported by

very little hard evidence in spite of a large group of supporters and a growing list of publications around the subject.

Table 1. *A sample of published data on different mtDNA variants associated with ageing or infertility in humans and some experimental models.*

MtDNA variant	Comment	reference
haplogroup J	northern Italian centenarians	De Benedictis et al., 1999
150T, 150T/489C/10398G	aged Finnish and Japanese	Niemi et al., 2005
subhaplogroups J2, D5, M7b		
T285C, A368G, 383i, T414G	fibroblast cultures from old humans	Michikawa et al., 1999
A189G, T408A, T414G	muscle tissue from aged humans	Wang et al., 2001
specific point mutations on CR	NOT found in ageing mouse	Song et al., 2005
multiple deletions	increase w/age in wt *C. elegans* but not in age-1 mutant	Melov et al., 1995
common deletion A3243G	ageing human muscle, heart, kidney, tissue specifically	Liu et al., 1998
multiple deletions	ageing *D. melanogaster* thorax	Yui et al., 2003
multiple deletions	ageing mouse liver, brain, heart	Tanhauser and Laipis, 1995
copy number	NO change in human skeletal muscle with age	Miller et al., 2003
re-arrangements deletions, depletion	ageing human cardiomyocytes, diaphragm, skeletal muscle	Bodyak et al., 2000
multiple deletions	human sperm w/ primary infertility, oligo/asthenozoospermia (OA) NO correlation w/age	Kao et al., 1998
common deletion	low levels in human adults	Cortopassi and Arnheim, 1990
deletions	mosaic pattern in ageing monkey muscle	Lopez et al., 2000
deletions	individual-specific human control and OA sperm, oocyte specific, NO age correlation	Reynier et al., 1998
deletions	human oocytes- NO correlation w/age, human embryos-levels lower than in oocytes	Brenner et al., 1998 Perez et al., 2000
sublimons (re-arrangements)	several human tissues, accumulate w/age in heart	Kajander et al., 2000; 2002
depletion	human sperm in asthenospermia	Kao et al., 2004
increased content	with increasing sperm abnormalities in humans	May-Panloup et al., 2003
copy number	increased in non-progressive human sperm	Diez-Sánchez et al., 2002
point mutations	accumulation w/time in POLG mutator cell line	Spelbrink et al., 2000
point mutations	accumulation w/age in PEO patients	Wanrooij et al., 2004

5.7.2 Mouse as an experimental model of ageing

Obvious advantages in using mice as experimental animals in ageing studies are firstly, that they are mammals with close evolutionary relationship to humans and secondly, that they have a life span of 2-3 years, short enough to perform ageing studies within a reasonable length of time. The various inbred lines are thoroughly characterized, the mouse genome is available (http://www.ncbi.nlm.nih.gov/genome/guide/mouse/) and mouse genetics is in a very advanced state. The ability to manipulate the mouse genome through transgenic and knockout methods is unsurpassed in mammalian systems. In addition to all this, adult mice have mitotic compartments of self-renewing stem cells, whereas somatic cells in adult worms and flies are virtually all post-mitotic.

5.7.3 Delayed ageing

Mouse models with delayed ageing (reviewed by Donehower, 2002) include the *Snell dwarf* mouse with a spontaneous mutation in *Pit-1* (pituitary-specific transcription factor 1) gene, the *Ames dwarf* mouse with an IR-induced mutation in Prophet of Pit-1 (*Prop-1*) gene, *Little* mouse with a spontaneous mutation in growth hormone releasing hormone receptor (*GHRH*), and growth hormone receptor knockout ($GHR^{-/-}$) mouse. All of these affect growth hormone production and the increase in longevity probably involves the growth hormone/IGF-1 axis, the cellular mechanisms for delayed ageing being similar or related to calorie restriction. $P66^{Shc-/-}$ knockout mice show extended lifespan and impaired p53 apoptotic response. $P66^{Shc}$ is an adaptor protein important in the signalling response to reactive oxygen species, and cells lacking this protein show an increased resistance against apoptosis induced by UV light or hydrogen peroxide treatment.

5.7.4 Calorie restriction

Calorie restriction or other dietary manipulation as a means to prolong lifespan has been demonstrated in several organisms, including mice, rats, the roundworm *Caenorhabditis elegans*, fruit fly *Drosophila melanogaster* and non-human primates (Fernandes et al., 1976; Lemon et al., 2005; McShane and Wise, 1996; Lakowski and Hekimi, 1998; Houthoofd et al., 2002; Lopez et al., 2000; Zainal et al., 2000; Wanagat et al., 1999; Cirelli et al., 2005; Mair et al., 2005; Bauer et al., 2004). Even interviews with human centenarians give an impression (with no scientific value as such) that the secret behind their long life is partly due to moderate nutrition.

A short-lived, autoimmunity –susceptible (NZB x NZF)F_1 (B/W) mouse was used to investigate the influence of different diets on longevity. These mice

develop an autoimmune renal disease early in life and die between 8 and 15 months of age. Their lifespan was systematically prolonged by calorie-restriction, even though the effect was most prominent with no interventions on protein or fat content. In another short-lived strain, DBA/2, the calorie restriction *per se* had no effect, but life expectancy was prolonged by a diet restricted with respect to protein (Fernandes et al., 1976). The authors claim that their results confirm previously reported profound effects of dietary manipulations on immunity functions, shown here by the longer survival of the immune-compromised mice.

Work by Lemon and co-authors (2005 and references therein) describes the positive, longevity-extending effect of a complex dietary supplement on transgenic growth hormone mice (TGM), as well as a similar but milder effect on normal mice. TGM have a 50 % reduced lifespan with early appearance of several age-associated symptoms like decline in learning and memory, reduced replicative potential of cells in *vitro*, early-onset arthritis, elevated ROS production that increases with age, elevated inflammatory processes and abnormal mitochondria in ageing heart. All of these characteristic age-related pathologies were ameliorated in the dietary supplemented groups and this was hypothesized to be the main contributing factor for the extended lifespan.

Gene silencing, a process where entire regions of chromosomes are inactivated, has also been suggested to regulate ageing. This silencing in yeast is carried out by a complex of silent information regulator (Sir) proteins acting to deacetylate histones associated with selected gene segments, like telomeres and mating-type genes. Histone-deacetylation has been shown to expand life in experimental models like yeast and nematode. Sir2 functions at the "crossroads" of ageing and metabolism, and its involvement in human ageing has also been suggested. Sir2 directly binds and deacetylates human and mouse p53 in response to DNA damage and oxidative stress. This may influence longevity in mammals in part through p53-dependent effects (Donehover, 2002; Guarente and Kenyon, 2000).

5.7.5 Premature ageing

A mutant mouse strain created by random transgene insertion (Kuro-o et al., 1997) exhibits shortened longevity and premature ageing-related phenotypes, such as short lifespan, arteriosclerosis, osteopenia, ectopic calcification of various tissues, pulmonary emphysema, impaired maturation of sexual organs, senile atrophy of the skin and hearing impairment. The mouse, as well as the affected gene, is called *Klotho* (OMIM 604824), the name coming from one of the three fates in Greek mythology (spinner of the thread of a life). The *Klotho* mice die prematurely around 8-15 weeks of age with no obvious single cause of death. KLOTHO is a β-glucosidase found in two alternative splice variants, a 1012 amino acid membrane form and a 549 amino acid secreted form. The protein is expressed in muscle, pancreas, testes, urinary bladder, ovary, placenta,

and faintly in the brain, and it is proposed to be a member of the negative regulatory circuit of Vitamin D metabolism (Nabeshima, 2002). The human functional variant of *KLOTHO* (KL-VS or V-allele) with two amino acid substitutions, F352V and C370S, has been proposed to associate with reduced longevity when homozygous, and to be an independent risk factor for early onset coronary artery disease, both in the heterozygous and homozygous state (Arking et al., 2003). Serum levels of KLOTHO decline with age in humans and cognitive defects have been reported in both KL^{-} mice and in humans with *KLOTHO* V/V genotype (Nagai et al., 2002; Deary et al., 2005).

One can find several reports in literature of experiments using telomere shortening as a trigger for ageing in mouse (reviewed by Mathon and Lloyd, 2001). The telomerase deficient $mTR^{-/-}$ mouse is phenotypically normal for several generations, arguing against a direct role for telomere shortening and replicative senescence in the process of ageing. Only after four or more generations of breeding the $mTR^{-/-}$ mice, telomere shortening with various defects in mouse phenotypes can be observed. The late-generation animals develop both male and female infertility and no offspring are produced in G^6 intercrosses. The male mice have defective spermatogenesis with increased apoptosis and decreased proliferation in the testis. The females exhibit a decrease in the number oocytes upon ovulation and poor early developmental progression of G^7 wild-type embryos. These results combined to a compromised long-term renewal of haematopoietic stem cells of bone marrow and spleen (Lee et al., 1998) indicate that long telomere maintenance is essential in maintaining diverse cell types with high proliferation rate. The late generation telomerase-deficient mice also show shortened lifespan, reduced capacity to respond to stresses, an increased incidence of spontaneous malignancies and higher amounts of activated p53, which can promote apoptosis or cell senescence depending on the cell type (Rudolph et al., 1999). The question still remains how relevant these studies are, given that rodent cells, differently from most human cells, do express telomerase and maintain long telomeres and yet senesce in culture (Prowse and Greider, 1995) indicating an additional, telomere-independent mechanism to limit the proliferative lifespan of certain cell types.

p53 tumour suppressor activation has been linked to cellular senescence. Increased phosphorylation-status independent p53 activation, affecting both DNA binding and transcriptional activity (but not in the amount of p53 protein or mRNA), is reported when human diploid fibroblast senesce (Atadja et al., 1995). A transgenic mouse model ($p53^{+/m}$ mouse), expressing a truncated C-terminal fragment of p53 that augments wild-type p53 activity, shows enhanced tumour resistance, but reduced longevity and early ageing, in contrast to a $p53^{-/-}$ mouse which shows a very high incidence of tumours and reduced lifespan (Tyner et al., 2002). The tumour incidence in $p53^{+/+}$, $p53^{+/-}$ and $p53^{+/m}$ mouse is >45 %, >80 % and 6 %, respectively, and none of the 35 $p53^{+/m}$ mice develop overt, life-threatening tumours. Both the median and maximum lifespan of the $p53^{+/m}$ mouse was reduced by 20 % despite of the lower incidence of tumours. In addition, they showed early ageing phenotypes with weight loss, lordokyphosis

and an absence of vigour. Spleen, kidney and testes, but not brain, were reduced in mass, which was due to overall decrease in cellular mass. The mice also showed reduction in mean dermal thickness, hair sparseness and reduced wound-healing, probably all as a result of a reduction in proliferating stem cells. The early ageing and tumour resistance in $p53^{+/m}$ mice is consistent with the idea that senescence is a mechanism of tumour suppression (Campisi, 2001; Donehower, 2002). A role for p53 in regulating ageing and longevity in mouse is proposed, and ageing-related reduction in stem cell proliferation may have a more important role in longevity than previously recognized. Over-expression of p53 in mice results in embryonic lethality, as does over-expression of Mdm2, the protein that directs p53 to degradation, suggesting that cancer resistance, ageing phenotypes and evolutionary fitness may all be dependant on p53 dosage.

The progeroid Werner syndrome in humans can be caused by several different mutations in *RECQL2* (*WRN*) gene, which all either create a stop codon or cause frame-shifts that lead to premature terminations in the synthesis of the Werner helicase. *Wrn* deficient mice are phenotypically normal, but late-generation mice null with respect to both *Wrn* and *Terc* (encoding the telomerase RNA component) develop typical Werner syndrome phenotypes, and Wrn/p53 bideficient mice have accelerated tumour incidence and reduced lifespan compared to parental p53 deficient mice (Lombard et al., 2000; Chang et al., 2004).

5.7.6 Checkpoint genes and ageing

In case the genome of an organism becomes damaged, checkpoint mechanisms ensure that the damage is detected and removed (Lengauer et al., 1998). In the cell cycle the mitotic spindle checkpoint genes *MAD2*, *BUB1* and their numerous functional homologs ensure proper sister chromatid separation (Cahill et al., 1999) and mutations in spindle checkpoint have been suggested to be the genetic basis in cancers with aneuploidy (an aberrant number of chromosomes, Lengauer et al., 1998; Lengauer and Wang, 2004). Another checkpoint protein, tumour suppressor p53, senses multiple types of DNA damage and brings about cellular responses (see above) to prevent propagation of DNA defective cells (Liu and Kulesz-Martin, 2001). This can happen through cell cycle arrest at G1 and DNA repair, permanent cell senescence or apoptosis. Mutations in both alleles of p53 are needed for this protective function to fail and result in development of cancer. The DNA damage checkpoint machinery contains numerous proteins, and co-operate with the DNA repair machinery to suppress genomic instability and hence cancer. In case of failure of this process the result is senescence or apoptosis (Motoyama and Naka, 2004), as a default mechanism to prevent cancer.

A mathematical model by Wodarz (2004) explains the relation between checkpoint genes, ageing and the development of cancer. The model developed

takes into account the dynamics of both healthy tissue and tumour, and the interaction between these two cell types. The model finds two types of behaviour. (i) The higher the checkpoint competence, the lower the rate of cancer incidence and progression, but the earlier onset of ageing. A reduction in checkpoint competence prevents early onset of age-related symptoms, but results in faster development and progression of tumours. (ii) Reduced checkpoint competence can result both in a reduced ageing of tissue and in a reduced incidence of tumours. The established tumours would however develop with an accelerated rate. This will apply only if the cost for increased cell death in the absence of repair is higher than the benefit from avoiding cell cycle arrest upon damage.

Mutations in DNA-damage checkpoint genes, including *ataxia telangiectasia mutated* (*ATM*, ataxia telangiectasia being a premature ageing syndrome), the ATM-related gene (*ATR*), *BRCA1*, *BRCA2* and *p53* have been implicated in tumorigenesis (Motoyama and Naka, 2004). Tumour development (as a result of genetic instability), apoptosis and senescence are often discussed in relation to each other. A take-home message from current data is that maintenance of genomic integrity and tumour suppression need communication between DNA repair and checkpoint molecules.

5.8 The "Mitocheckpoint"

Mitochondria have a central role in multiple cellular functions including energy production, diverse reactions of metabolism, maintaining intracellular homeostasis of inorganic ions and apoptosis, and they also are the main source of ROS and oxidative stress. Recently Singh (2005) suggested the idea of a mitochondrial damage checkpoint or mitocheckpoint, which would coordinate and maintain the proper balance between apoptotic and anti-apoptotic signals. According to this hypothesis the mitocheckpoint monitors the functional state of mitochondria and responds accordingly when mitochondria are damaged or become dysfunctional, and is thus comparable to the cell cycle checkpoints. The hypothetical mitocheckpoint can adjust the cell cycle response and gene expression to help repair damaged mitochondria to restore normal mitochondrial function. In the case of severe, persistent damage to mitochondria, such as extensive mtDNA mutation, the checkpoint could fail and the result would be a nuclear mutator phenotype, seen as increased cell survival in yeast and cancer in humans. This theory is based on several findings, which I now summarize.

Cells lacking mtDNA (ρ^0 –cells) and yeast cells with deleted mtDNA molecules (ρ^- -cells) are widely used in mitochondrial research, and have shown evidence for cross talk and functional inter-dependence between mitochondrial and nuclear genomes. Mitochondrial impairment affects nuclear genome stability resulting in a nuclear mutator phenotype by both ROS-dependent and ROS-independent pathways, including a mitochondrially mediated error-prone DNA

repair pathway (Rasmussen et al., 2003). If oxidative phosphorylation is blocked in yeast cells by antimycin A (cIII inhibitor) the result is an increase in intracellular ROS, whereas ρ^- and ρ^0 -cells with inactive ETC have decreased intracellular ROS levels. In both cases the cells have a nuclear mutator phenotype with ~2-3 –fold higher mutation frequencies in the nuclear genome compared to untreated and wild type control cells, respectively.

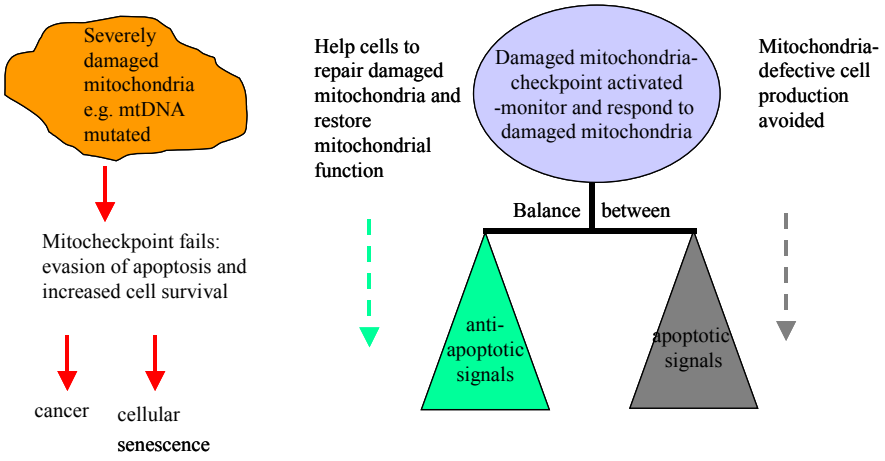


Figure 7. *The proposed "mitocheckpoint" (right diagram) coordinates and maintains the proper balance between apoptotic and anti-apoptotic signals so that mitochondria-defective cells whose function cannot be repaired are directed to apoptosis to be destroyed and so the integrity of cell population is retained. In case of a severe damage to mitochondria, like extensive mtDNA mutation (left diagram), the "mitocheckpoint" may fail and result in evasion of apoptosis and a nuclear mutator phenotype because of reduced ATP-dependant DNA transcription-, replication- and repair activities. The surviving cells in a tissue would undergo cellular senescence or could lead to cancer.*

A reduction in intracellular ATP concentration affects ATP-dependent pathways in DNA replication, repair and recombination. Reduction in ATP can also lead to O_2 deprivation or uncoupling, and result in ATP hydrolysis. Mitochondria are also involved in nucleotide biosynthesis, and imbalance in the nucleotide pool can contribute to nuclear mutagenesis.

The idea of a mitochondrial checkpoint directing cell fate has also been discussed elsewhere. Caspases need ATP, and reduction in the amount of ATP switches cell fate from apoptosis to necrosis in human lymphocytes, suggesting that mitochondrial membrane potential is a crucial checkpoint of T-cell fate decisions (Perl et al., 2004). A short pulse of high dose staurosporin, a tyrosine kinase inhibitor and widely used apoptosis inducer, causes pre-apoptotic chromatin condensation (PACC) with associated double-strand breaks and a general yet partial decrease in transcription, which occurs before mitochondrial membrane permeabilization (MMP, Audreau et al., 2004). MMP inhibitors Bcl-2 and vMIA not only inhibit apoptosis but also stimulate the repair of DNA lesions, meaning reversal of PACC. The rate of neurodegeneration correlates with maximum lifespan potential and ROS-formation, and the intrinsic

mitochondrial cell-death pathway directly or indirectly regulates cell death in these disorders (Wright et al., 2004).

5.9 In conclusion

Mitochondria and mtDNA are essential for a multitude of cellular functions, especially respiration and ATP production, and therefore are also the main source of reactive oxygen species (ROS). There is only one polymerase, POLG, currently known and proposed to be responsible for the replication and maintenance of mtDNA. Human *POLG* carries in its sequence a microsatellite repeat which is absent in mouse and fruit fly. Microsatellite expansions in other genes are a frequent cause of human neurodegenerative disease. Mutations in *POLG* result in deletions, depletion or point mutations in mtDNA, which are further presented as diverse, more or less severe phenotypes in humans, ranging from infertility and hearing loss to severe, even life-threatening neurodegenerative diseases and suggested age-related phenotypes. There is cross talk between the nuclear and mitochondrial genomes, and mitochondrial dysfunction is suggested to lead to a nuclear mutator phenotype. Mitochondria are proposed to possess a similar kind of checkpoint role as the well-known cell-cycle checkpoints. Depending on the severity of mitochondrial dysfunction, the end result can be apoptosis, cellular senescence or cancer.

Based on the data available in the beginning of this study, and the data accumulating during it, this study has focused on gathering more information about the role of *POLG* and its sequence variants in health and in particular in male infertility and ageing. The co-evolution of the CAG repeat with primate species evolution was also followed in order to look for evidence that it was under selection for specific length variants. Finally, the work has led to an important advance in understanding the role of mitochondrial DNA mutations in ageing.

6. AIMS OF THE STUDY

The aim of this study was to analyse the role of mitochondrial DNA polymerase gamma and its mutations in healthy and diseased individuals, and the effect of one specific *POLG* mutation on mitochondrial DNA and organismal phenotype in an experimental animal model.

More specifically the aims can be listed as follows:

1. To analyze the length and variation of the POLG polyglutamine repeat in different control populations and in individuals with common mitochondrial diseases.
2. To analyze the possible role of the CAG repeat length in complex, heterogeneous disorders with suggested mitochondrial involvement, specifically in type 2 diabetes and male infertility.
3. To trace back the evolution and sequence variance of the *POLG* CAG repeat in primates based on its existence in humans and absence in mouse and *Drosophila*, for clues of the selection forces acting upon it.
4. To analyze mtDNA point mutation load and accumulation in a knock-in mouse model with a mutation in the *POLG* exonuclease domain. This experiment was addressed to enlighten the role of mtDNA mutations seen in aged individuals. The mice showed a premature ageing phenotype and were, because of the introduced transgene, theoretically prone to mtDNA mutations.

During the research, an association of *POLG* genotype with a certain type of male infertility was found which led to further analysis of mtDNA samples, as well as a search for other possible mutations in *POLG* of infertile males. The accumulation of point mutations on different regions of mouse mtDNA was analysed in more detail. Finally, I set out to test whether the loss of mtDNA integrity in the mouse leads to nuclear mutator phenotype. These latter experiments are discussed briefly as unpublished results.

7. MATERIALS AND METHODS

7.1 Human infertile patients and controls

All samples were taken with informed consent following the ethical guidelines of the medical centre in question. Infertility for the purposes of this study is defined as lack of conception after at least 12 months of unprotected intercourse. Volunteers for the study, as authorized by the local ethical committee, were recruited from amongst infertile men attending a clinic in Oxford, and semen samples obtained by masturbation were stored frozen in liquid nitrogen for further study (kindly supplied by Dr. J. Poulton, University of Oxford). Azoospermic individuals (approx. 20 % of the total) were excluded, as were any for whom a clear, established aetiology (hormonal, developmental, karyotypic or traumatic) could be found to account for their infertility. Semen was also collected from sperm donors, for use as fertile controls in the study. Seminal plasma was collected from similarly defined infertile males in Tampere, (Dr. L. Wichmann, Medical School, University of Tampere) with ethical approval No. R00104/2000.

Following ethical committee approval, 494 consecutive male patients attending the assisted conception (HARI) unit at the Rotunda Hospital, Dublin, for treatment with ICSI between July 1996 and June 1999 were studied (kindly provided by Dr. D. Barton, University College, Dublin, Ireland). In all cases cytogenetic screening was carried out and 13 patients with an abnormal karyotype were excluded from the present study. The men ranged in age from 21 to 55 years (mean 35.3 years). All but 39 men provided at least one semen specimen, each after three days´ abstinence from sexual intercourse. Blood samples were provided by otherwise healthy volunteers amongst laboratory personnel and associates, who were not screened for fertility. Controls also included 270 unselected males from a previously studied birth cohort from England (Poulton et al. 1998).

Somewhat different criteria were applied in the collection of patients in the various centres participating in the study. Blood DNA samples of the Scottish patients (provided by Dr. D. Elliot, Edinburgh, Scotland) included a high proportion of azoospermic individuals and those with severe oligozoospermia (sperm concentration $< 5 \times 10^6$ /ml). Blood DNA that was provided by the German (Dr. H-C Schuppe, University of Duesseldorf, Germany) and Irish (Dr.

R. Harrison, Rotunda Hospital, Dublin, Ireland) groups, included many with only a mild abnormality of sperm concentration, motility and/or morphology. The Taiwanese samples (provided by Y-H Wei, National Yang-Ming University, Taipei, Taiwan) consisted of cases of 'pure asthenozoospermia', with sperm concentrations well into the normal range (generally >20 x 10^6 /ml), and the DNA was isolated from sperm. Samples from Australia (provided by Dr. J. Cummins, Murdoch University, Western Australia) included sperm DNA of patients with various sperm abnormalities, plus 17 from whom testicular biopsies were taken for analysis but about whom no other clinical information was available.

DNA from Chinese patients with acute amino glycoside ototoxicity was kindly donated by Dr W-Q Qiu (Tiedao Medical College, Shanghai, China), from UK myotonic dystrophy patients by Dr Helen Harley (University of Wales, Cardiff), from Spanish subjects with the nt 1555 mtDNA mutation by Dr Ignacio del Castillo (Hospital Ramon y Cajal, Madrid, Spain), and from members of Finnish pedigrees carrying the nt 3243 mtDNA mutation by Dr Kari Majamaa (University of Oulu, Finland).

Human ρ^0 A549.B2 cells, human male lung carcinoma cells artificially devoid of mtDNA (later B2ρ^0, Bodnar et al., 1993), were grown in a humidified incubator having +37 °C and 8 % CO_2 in DMEM medium containing 4.5 % glucose and 1 mM Na-Pyruvate, supplemented with 10 % FCS, 2 mM Glutamine, 100 units/ml of Penicillin and 100 µg/ml of Streptomycin (all from Cambrex, East Rutherford, NJ) and 50 µg/ml of Uridine (Sigma-Aldrich, St Louis, MO).

The DNA samples from two African Pygmies, 5 Mbutis from North-Eastern Zaire and 5 Biakas from Central-African Republic were a kind gift from Dr. John Armour, University of Nottingham, UK.

7.2 Primate samples

Primate genomic DNAs were obtained from collaborating laboratories or extracted by standard methods from blood, tissue samples or commercial cell-lines. All work complied with the requirements of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, see http://www.cites.org). Hairs from individual specimens were processed as for human hair (see section 7.4). Stock DNA concentrations varied from 50– 400 ng/µl.

7.3 Mouse DNA samples

A knock-in mouse expressing a proof-reading deficient version of PolgA (mtDNA mutator mice) was created by collaborators at Karolinska Institutet in Stockholm. The mutation was a D257A substitution changing a conserved amino acid in the second exonuclease domain. Total DNA was extracted with standard methods from the brains of 4 and 8 week old wild-type, heterozygous and mtDNA-mutator mice, as well as head parts of E13.5 embryos and heart tissue of 40 week old mice, both wild type and mutator. Enriched mtDNA was extracted from the isolated mitochondria (prepared by differential centrifugation) of brain, heart and liver tissue of 25 weeks old mice and hearts of 40 weeks old mice. Mouse ρ^0 cell DNA was a kind gift from Prof. Jose A. Enriquez, University of Zaragoza, Spain.

7.4 DNA isolation

From samples collected in Tampere the blood DNA was isolated from 50 µl capillary blood, which was suspended in 500 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA) and micro-centrifuged at 13,000 g_{max} for 5 minutes. After removal of the supernatant mononuclear cells were washed twice more with TE. The cell pellet was re-suspended in 100 µl of K buffer (50 mM KCl, 10 mM Tris/HCl pH 8.0, 2.5 mM $MgCl_2$, 0.5 % Tween 20) containing 100 µg/ml of fresh Proteinase K (Finnzymes, Espoo, Finland). The samples were incubated at 56 °C for 45 min, followed by 10 min at 95 °C to kill the enzyme. DNA was prepared from frozen sperm or seminal plasma similarly, but the cells were collected from an aliquot of 50 µl and 400 µl, respectively, of each sample by micro-centrifugation at 13,000 g_{max} for 5 min, after which the pellets were washed twice with PBS. Prior to PCR, the DNA extracts were micro-centrifuged for 15 s and 1 µl of clear lysate used as template. B2 ρ^0 DNA was isolated similarly from $5x10^5$ cultured cells.

Other sperm and blood DNAs, prepared by similar or standard methods, were obtained from clinical sources in the various centres (listed in section 6.1), along with clinical information and sperm analysis data, supplied anonymously.

For extraction of hair root DNA four hair roots were collected in a clean test tube and DNA was extracted as follows: 100 µl of BuccalAmp™ QuickExtract™ DNA Extraction Solution 1.0 (Epicentre, Madison, Wisconsin) was added. The incubation protocol was: 10 s vortex, 30 min 65 °C, 15 s vortex, 8 min 98 °C, 15 s vortex, 8 min 98 °C, 15 s vortex followed by a short spin to collect the solution on the bottom of the tube. DNA was stored at -20 °C. 1 µl of DNA solution was used as template in a 25 µl PCR reaction.

Some human control samples were also obtained as bloodstains and a published protocol (Walsh et. al., 1991) with small modifications was used to

isolate the DNA. The spot was cut out of card, added to an eppendorf tube containing 1ml of sterile distilled water, vortexed well and incubated at ambient temperature for 15-30 min with shaking every 5 minutes. Samples were centrifuged at 13,000 g_{max} for 3 min. The supernatant was removed leaving the fibre residue in the tube and the pellet was re-suspended in 1 ml of sterile distilled water. Centrifugation was repeated and the supernatant removed. The pellet with the fibre residue was resuspended in 170 µl of 5 % Chelex 100 (BioRad) and Proteinase K (FinnZymes, Espoo, Finland) added to final concentration of 100 µg/ml. Tubes were incubated at 56 °C for 10-15 min followed by boiling the samples for 8 min. Samples were centrifuged at 13,000 g_{max} for 3 min and the supernatant transferred to a clean tube. Five µl was used as template in PCR.

7.5 Fluorescent PCR and fragment analysis

Fluorescent PCR (F-PCR) of the *POLG* CAG microsatellite region (see *Figures 8 & 9*) was performed in a 25 µl reaction using primers mip31 or mip32 with a 5´-ROX-labelled mip51 (Genset, Paris, France), giving a fluorescent PCR product of 126 bp and 165 bp, respectively, based on the reference sequence. AmpliTaq Gold® DNA Polymerase, 1 U/reaction, was used together with Gold Buffer containing 25 mM $MgCl_2$ (both from AppliedBiosystems, Foster City, CA). The enzyme was provided in an inactive state requiring a 5-10 min pre-PCR heating step to allow an efficient hot-start, which increases the specificity of the reaction. All four deoxynucleotides were used at 200 µM final concentration (AmershamPharmacia Biotech, Upsala, Sweden) and pyrogen-free MilliQ-H_2O was filtered and autoclaved in our own laboratory. The reaction mixtures were subjected to PCR cycling consisting of an initial 10 min denaturation/enzyme activation step, 30-35 cycles of denaturation for 1 min at 95 °C, annealing for 45 s at 62 °C, and extension for 2 min at 72 °C , with 5 min extension in the final cycle.

Figure 8. *The polyglutamine region of human POLG, with the polypeptide sequence denoted in the one-letter amino acid code under the first DNA nucleotide of each corresponding codon. Flanking primer sites mip31, mip32 and mip51 used in amplification of the region are underlined. The CAG microsatellite is marked with a horizontal line over the nucleotide sequence. The mitochondrial targeting sequence is in light grey 5´ of the mature polypeptide sequence. GenBank accession No: NM_002693. The presented sequence starts from the first amino acid of the mitochondrial targeting sequence, which corresponds to nucleotide 283 in the cDNA according to Lecrenier et al., 1997.*

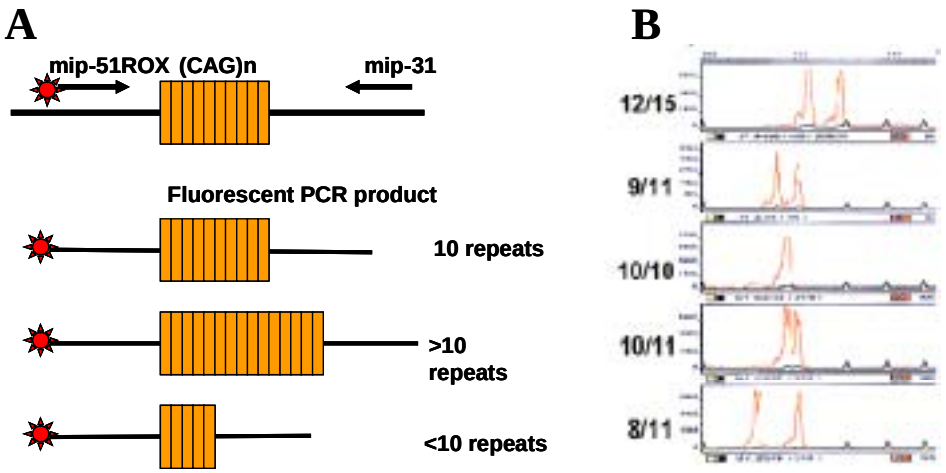


Figure 9. *Principle of fluorescent PCR. (**A**) The length of the PCR product depends on the number of repeats in the template sequence. (**B**) ROX-labelled 5´primer enables the detection of the length of the PCR product by ABI310. The numbers on left side of the electropherograms represent the number of consecutive CAGs in alleles of that given genomic DNA.*

For fragment analysis, 1 µl of F-PCR product and 0.5 µl of 350-TAMRA molecular weight markers (AppliedBiosystems, Foster City, CA), were added

into 12 µl of high purity Formamide. The mixture was denatured for 2 min at 92 °C, cooled on ice and loaded onto the ABI310 Genetic Analyzer. Separation of the products was performed under denaturing conditions following the manufacturers´ instructions using the data collection software of the instrument. Electrophoresis conditions were: 36 cm capillary, POP4 Performance Optimized Polymer as separation medium and Running Buffer w/EDTA (all from AppliedBiosystems), oven temperature 60 °C, injection 15 kV during 5 s and filter set A for detection of ROX as red and TAMRA as yellow. Separation time was 24 min and the results were auto-analyzed with AppliedBiosystems GeneScan analysis software.

7.6 PCR, cloning and sequencing

7.6.1 PCR

PCR for POLG sequencing was performed as F-PCR but with non-labelled mip51 primer or other exon-specific primers. PCR for mouse, as well as human mtDNA sequencing was performed under standard conditions using 2.5 units of Pfu DNA polymerase (Promega, Southampton, UK) per reaction and 18-20 cycles for amplification of mouse DNA samples and 25-35 cycles for human samples. For the mouse p53 nuclear gene PCR primers mp53-51 and mp53-31 amplified a 237 bp long fragment. 2.5 U of Pwo SuperYeld DNA Polymerase with supplied buffer (Roche Applied Science, Mannheim, Germany) was used in a 50 µl reaction for 31 cycles was used under otherwise similar conditions as for other experiments.

Table 2. *Primers used in F-PCR, PCR and sequencing. For details see text. Sequence co-ordinates are numbered according to the following references: POLG, Transcript ENST00000268124 Ensembl; mouse mtDNA, NC_005089 (Bayona-Bafaluy et al., 2003); human mtDNA, AB055387 (revised Cambridge reference sequence, Andrews et al., 1999).*

name	Sequence 5´to 3´	location	notes
M13F	GTAAAACGACGGCCAG	plasmid	
M13R	CAGGAAACAGCTATGAC	plasmid	
Mip51	CCAGCTCCGTCCCCGCGTCCGACC	362-385	Human POLG
Mip51-ROX	ROX- CCAGCTCCGTCCCCGCGTCCGACC	362-385	5´ ROX labelled Human POLG
Mip31	GCTGCCCGCCCTCCTAGGATAGCAC	463-487	Human POLG
Mip32	CTCTCGAGAGCATCTGGATGTCCAATG	500-526	Human POLG
mmtDNA 14078L	CACAGCATTCAACTGCGACC	14078-14098	Mouse cyt b F
mmtDNA 14912H	GTAGTTGTCTGGGTCTCCTAG	14881-14912	Mouse cyt b R

mmtDNA 15363L	GAAGGAGCTACTCCCCACCACC	15363-15384	Mouse CR F
mmtDNA 138H	CACCGGTCTATGGAGGTTTGC	117-138	Mouse CR R
mmtDNA 16192H	GAACTACTAGAATTGATCAGG	16177-16197	Mouse CR R
mmt12S-1F	CTTAAGACACCTTGCCTAGC	199-218	Mouse 12S F
mmt12S-2R	GGTGTGTGCGTACTTCATTG	888-907	Mouse 12S R
mp53-51	TCTTTGTCCCATCCACAGCCATCACCTC	Exon4	Tre53e4F mouse p53
mp53-31	GGGGCCCACTCACCGTGCACATAACAG	Exon4	Tre53e4R mouse p53
hmtDNA 14682L	CACGGACTACAACCACGACC	14682-14699	Human cyt b F
hmtDNA 15516H	GTATAATTGTCTGGGTCGCCTAGG	15493-15516	Human cyt b R
hmtDNA 35L	GGAGCTCTCCATGCATTTGG	35-54	Human CR F
hmtDNA 611H	CAGTGTATTGCTTTGAGGAGG	591-611	Human CR R
2aF 2aR 2bF 2bR 222F	CTCCACGTCTTCCAGCCAGTAAAA TTCTGCAGGTGCTCGACGCT GGGCTGCACGAGCAAATCTT AGCCCGTAACAGGACCTCAGAAAA GCCAAGCTGGAGCCCAAAG	838> <1418 1335> <1845 1031>	Human POLG exon 2
3F 3R	TAGGTGTGCAGTGGTTGTTG AACCACTGAGATTAGGGCTC	4494> <4833	Human POLG exon 3
4F 4R	TCCACACCACCAAGCAGTGGT AGAGGGGGTCCCAAGCACTAT	5654> <5953	Human POLG exon 4
5F	CCGGTGGGCAGGAGCATAGTG	5918>	Human POLG exon 5
6R	GGGCCCGGGTACCAGGAACACA	<6449	Human POLG exon 6
7F 7R	GTGGCTGGGTTGAGGCTTGCTAAA CAGTCCCCTGCCTAGATCCTG	7403> <7720	Human POLG exon 7
8F 8R	GCCTTAGCCCTGCCCTAGTG ATCTGCTCCCACCCGCTCATCT	7749> <8055	Human POLG exon 8
9F 9R	AGATGAGCGGGTGGGAGCAGAT GAATGGCAGCAGGTCAAT	8034> <8361	Human POLG exon 9
10F 10R	GGGACATTGTGAGAGAGAGA ACTCTTTCCACTAGCCTGAG	9079> <9481	Human POLG exon 10
11F	GAGTGGGCATCTGGTAATCA	10568>	Human POLG exon 11
12R	AAGAACTAGGTGGGCAAGAGGAAG	<11107	Human POLG exon 12
13F 13R	ATGGCCCTTGCTGAATGCAG TGGGCCTTGAGCAGAATGAG	11274> <11498	Human POLG exon 13
14F 14R	GCAGGTACTCACGTTGGTTC CTGTGGGAATCCAGGGTTAG	11869> <12188	Human POLG exon 14
15F	ATGGTGGGATGTGGGATAGATT	12763>	Human POLG exon 15
16R	GAGGAAGAGCAGGGGCCAGAGGTA	<13148	Human POLG exon 16
17F	AAAGGCTCTAGCTCTTTCTCAG	13501>	Human POLG

17R	CAGAACTGTCAATATGCTGAGG	<13776	exon 17
18F 18R	CCTCAGCATATTGACAGTTCTG GTAATGGGCAGGAGATAGAACA	13755> <14176	Human POLG exon 18
19F 19R	AGCCGTTTCTTCCTCTGA GACCAGAAACAGAGGGCAGAC	15344> <15740	Human POLG exon 19
20F 20R	CAG AAGAGCTTGGATGCTTTG ACTCTGGCCCTACCTACAAACATT	15669> <16013	Human POLG exon 20
21F 21R	GCTTCTACCCTGGAGTTAATTG CAAGGAACGCTCACCCAAAG	16029> <16374	Human POLG exon 21
22F 22R	GTCATTGCTCCAGGAGTGAT CTGTTCTCCAAGACCCACTT	17237> <17543	Human POLG exon 22
23F 23R	GCTCCTTTGCTCACTTCTGA CTACTGAAAAATGGCTGGCC	17944> <18220	Human POLG exon 23

POLG, DNA Polymerase Gamma, mmtDNA, mouse mitochondrial DNA; CR, control region; cyt b, cytochrome b; 12S, 12S ribosomal RNA; R, reverse; F, forward; L, light strand; H, heavy strand; Trp53e4, mouse p53 gene exon 4.

7.6.2 Gel electrophoresis

Before cloning or sequencing, the PCR products together with GeneRuler™ 100bp DNA Ladder (Fermentas, Vilnius, Lithuania) were separated on 22 cm agarose gels (1, 1.5 or 3 % depending on fragment sizes, Pronadisa, Madrid, Spain) containing 0.5 µg/ml of ethidium bromide (Sigma-Aldrich, St.Louis, MO) using TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA) run at 3-5 V/cm and visualized and documented under UV-light by video print.

7.6.3 Cloning and sequencing

For sequencing analysis the region of interest was amplified with PCR and subjected to either direct sequencing, or cloning and sequencing of the purified plasmid DNA. PCR products were cloned using TOPO TA Cloning® Kit or Zero Blunt® TOPO® PCR Cloning Kit according to the manufacturer's provided manual (Invitrogen, Paisley, UK). 2 µl of PCR product was mixed with 1 µl of TOPO TA -vector, 1 µl of manufacturer's salt solution and 2 µl of H_2O and incubated 15 min at ambient temperature. 2 µl of the cloning mixture was transformed into 50 µl of competent TOP10 cells (Invitrogen), incubated on ice for 30 min, followed by a heat-shock at 42 °C for 45 s. 250 µl of SOC medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to cells and the tubes were placed at 37 °C for 45 min to recover. One hundred µl of transformed bacteria were plated on LB plates (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 1.5 % agar), containing 100 µg/ml of ampicillin (Sigma-Aldrich, St.Louis, MO) or 25 µg/ml of Zeocin™ (Invitrogen) as a selective antibiotic and 40 µg/ml of X-gal (Fermentas, Vilnius, Lithuania) as a substrate for β-galactosidase to distinguish the positive clones from those with empty vector. Individual white or light blue colonies were picked and grown in 2 ml of LB_{amp} (as plates but with no agar) or

TB_{Zeo} (1,2 % tryptone, 2.4 % yeast extract, 0.4 % glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) at 37 °C on an orbital shaker over night.

Plasmid DNA was purified manually using the Wizard® Plus SV Miniprep DNA Purification System (Promega, Madison, WI) or using NucleoSpin®Robot-96 Automated Plasmid DNA Purification Kit (Machery-Nagel, Düren, Germany) on a Tecan Genesis RPS100 pipetting robot. The principle in both methods is the same: 1.5 ml of bacterial suspension is harvested by centrifugation, re-suspended, lysed and neutralized. The cleared lysate obtained either by centrifugation or filtration is transferred and bound to a kit-provided spin-column. After two washes with the kit wash solution, supplemented to contain 70 % ethanol, the DNA is eluted in 100 µl of ddH_2O.

In the case of direct sequencing the PCR products were purified either manually using the PCR Purification Kit (Qiagen, Hilden, Germany) or with the GFX 96 PCR purification Kit (AmershamBiosciences, Upsala, Sweden) using a Tecan Genesis RPS100 pipetting robot. Briefly, the PCR product was bound to kit columns, washed with the kit washing buffer containing 70 % ethanol, and the DNA eluted in 50 µl of ddH_2O.

The ABI PRISM® BigDye™ Terminator Ready Reaction Kit (AppliedBiosystems, Foster City, CA) utilizing fluorescent-labelled terminator ddNTPs and PCR-cycling was used for sequencing reactions. The sequencing primers (Genset, Paris, France) were M13F or M13R in the case of plasmid DNA and one of the PCR primers in the case of direct PCR sequencing. Briefly, 2-4 µl of purified DNA, 1 µl (1.6 pmol) of primer, 4 µl of Template Ready Reaction Mix and 1-3 µl of ddH_2O were combined and subjected to 25 cycles of 96 °C for 30 seconds, 50 °C for 15 s, 60 °C for 4 minutes. Amplified products were ethanol-precipitated to remove unused labelled terminators.

For separation and analysis on the ABI310 or ABI3100 Genetic Analyzer the purified products were re-suspended in 12 µl of Template Suppression Reagent or HiDi Formamide (AppliedBiosystems), denatured for 2 min at 92 °C and cooled on ice. The products were separated under denaturing conditions following the manufacturers´ instructions and using ABI310 Data collection software and auto-analyzed with ABI Sequencing Analysis software.

7.7 SNP analysis

The frequency of an unpublished single nucleotide polymorphism on POLG exon 8 in infertile and control samples was analyzed by using the ABI PRISM® SNaPshot™ Multiplex Kit according to the kit manual. Briefly, an exon 8-containing fragment was amplified from genomic DNA by using primers 8F and 8R. PCR fragments were purified as described using the GFX purification kit (AmershamPharmacia) and Tecan Genesis RPS100 pipetting robot. For primer extension reactions 1 µl of SNP mix, 4 µl of sequencing dilution buffer (AppliedBiosystems), 3 µl of purified PCR product and 2 µl (0.2 µM final) of

G517Vfor primer (CAGCAAGTTGCCCATCGAGG) were combined and subjected to 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 30 s. To digest the unincorporated labelled dideoxynucleotides, the extension products were incubated at 37 °C for 1 hour with 1 unit of Calf Intestinal Phosphatase (FinnZymes, Espoo, Finland) followed by 15 min at 75 °C to inactivate the enzyme. 1 µl of each purified product was combined with 0.5 µl of GeneScan 120 LIZ size standard and 10 µl of Hi-Di Formamide (both from AppliedBiosystems), denatured at 95 °C for 5 min and placed on ice. Fragments were separated on an ABI3100 Genetic Analyzer with 22 cm capillary array, POP4 Polymer, and Data Collection program and analysed with GeneScan program according to the instrument manuals. The final analysis of electropherograms (the allele calls) was performed manually.

7.8 Statistical analysis

Chi-squared testing was applied to determine whether the frequencies of different genotypes in infertile, control and fertile groups were significantly different. Post-hoc analysis was based on standardized residuals. Sequence comparisons were done by using SeqMan™II sequence analysis software (DNASTAR Inc). Other statistical tests were used when appropriate as described in results and discussion sections.

8. RESULTS

8.1 *POLG* microsatellite region in different human samples (I, II, III, unpublished results)

Fluorescent PCR combined with hot-start polymerase was used to genotype the *POLG* CAG region in infertile and control samples, and appeared to be a rapid, reliable and sensitive method for fragment length analysis. Before starting the actual allele call, the values were standardized using test samples by comparing the GeneScan results to the sequencing results, which also confirmed that the amplified product was in fact the *POLG* CAG region. The final allele calls were done manually from the printed electropherograms. The 126 bp fragment was auto-analyzed by the instrument as 124 bp, the difference coming probably from both the sequence content and the different retention characteristics of the two fluorescent dyes used (ROX in the PCR products and TAMRA in the molecular weight markers).

Seminal plasma samples from Finland were originally stored for biochemical analyses and were thus almost devoid of sperm. Nevertheless it was possible to isolate DNA from these samples and amplify it by PCR. DNA from blood samples was successfully amplified, as was that from sperm and hair. Initial experiments were carried out by amplifying the region with non-fluorescent primers and separating the products on 3 % agarose gels, as used later for cloning. To reveal possible differences in nucleotide sequence, the PCR products were sequenced after fragment analysis. Homozygous samples were sequenced with gene-specific primers whereas heterozygous samples were cloned, and a minimum of ten clones were sequenced to get at least three successful sequences of each allele. In human samples heterozygosity was shown in every case except five to be due to different number of CAGs in the main repeat tract.

8.1.1 High frequency of the common POLG allele in diverse populations (I, II, III, unpublished data)

Fragment analysis and sequencing of DNA samples from different populations revealed a high frequency of the common ten-repeat allele in different populations. In a total sample of 1210 *POLG* alleles (605 unrelated individuals)

of unselected human control samples from several European countries, from Australia and Taiwan, the majority was homozygous for the common allele. 19.2 % were heterozygous having one common 10-repeat allele and a second allele with a repeat length of 5-14, the next most common variant alleles having 11 and 9 CAGs, respectively. Only 1.2 % of control individuals lacked the common allele (later called homozygous mutants), having instead two identical or nonidentical variant alleles. Homozygosity for the common allele was 79.7 % in this group of people, mainly of Caucasian origin. Our small sample of Chinese individuals, 21 controls and 56 asthenozoospermic males, showed similar or even higher numbers for the common allele and the controls were thus included in this control group.

DNA isolated from ten African pygmies, five Mbutis from North-Eastern Zaire and five Biakas from Central African Republic, was analyzed to ascertain if the high frequency of the common allele was present in deep human branches. Both groups showed a high frequency of heterozygous individuals with similar allele lengths to Caucasians. The most common alleles had 10 and 11 CAGs. The genotypes by GeneScan were one of each, 11/11, 11/13, 10/10, 10/11 and 10/13 for Mbutis and 4 cases of 10/11 and one 11/11 for Biakas.

Table 3. *Genotypes and allele frequencies of POLG in a mixed group of human control samples. Pygmies are presented in a separate column with both tribes combined because of the small number of samples. Percentages are given to a maximum of 2 d.p.; R denotes arginine; X, any length variant other than ten CAGs.*

Allele/ genotype	number of analyzed alleles (% of all)	Alleles in Pygmies (tribes combined)
3R(CAG)7	2 (0.17)	-
3R(CAG)8	5 (0.41)	-
3R(CAG)9	22 (1.8)	-
3R(CAG)10	1045 (86.4)	7 (35)
3R(CAG)11	99 (8.2)	10 (50)
3R(CAG)12	28 (2.3)	-
3R(CAG)13	4 (0.33)	2 (10)
3R(CAG)14	1 (0.08)	-
4R(CAG)9	4 (0.33)	1 (5)
Alleles total	1210	20
10/10	79.7 %	-
10/X (het)	19.2 %	60 %
X/X	1 %	30 %
Heteroz. with 4R variant	0.66 %	10 %

Sequence analysis verified that in virtually all cases the differences in the allele length in all sample groups were due to length variation of the CAG repeat. One new allele was found, where the first CAG was changed to CGG and thus had four arginines instead of three, and nine CAG glutamines instead of ten, and was named as POLG-4R (EMBL Accession AY377897). This allele was found in five cases out of all the analyzed individuals, always in combination with another allele having three arginines. The allele combinations with the new allele were $4R(CAG)_9/3R(CAG)_{10}$ (in one Mbuti and in one control sample each, from Scotland and England), $4R(CAG)_9/3R(CAG)_{11}$ and $4R(CAG)_9/3R(CAG)_8$ (both in German controls). This means that all five individuals were heterozygous for the variant sequence, two of them being heterozygous also for length and three homozygous for length.

8.1.2 Lack of the common POLG allele associates with male infertility (II)

Fluorescent PCR with custom primers, and sequencing to confirm the results if needed, was used to genotype the infertile and control individuals for *POLG* CAG repeat length. Using sperm DNA from individuals in whom azoospermia and severe oligozoospermia were excluded, 9 out of 99 infertile males (9 %) from Finland (6/59) or England (3/40) were found to be homozygous for the absence of the common $(CAG)_{10}$ allele. In contrast, the common allele was present in all 98 fertile males studied as well as in all but 6 of 522 healthy controls whose blood DNA was analyzed at the same time (*Tables 3 and 4, Fig 10*).

The frequency of the common allele ranged from 70.6 % in Australian testicular biopsies to 99.1 % in Chinese asthenozoospermic patients. The frequency of the common allele amongst British, Finnish and German combined controls was 87.1 % and in infertiles from the same backgrounds 82.2 %. Based on standard Hardy–Weinberg prediction (based on random mating), the X/X genotype (absence of the common allele, whether or not this reflected homozygosity for a particular non-ten allele) should be found in approximately 1.7 % of individuals. In my study it was found at a frequency slightly below expectation (1.2 %) in the general population, although this deviation is not statistically significant. In contrast, my finding that the X/X genotype occurs in 9 % of infertile males but 0 of fertile males is highly significant based on two different statistical tests (see section 8.1.3 below). I also found a higher frequency of heterozygosity in infertile men (35 %) than in fertile men (18 %) or in the general population (24 %): some infertile men may be compound heterozygotes, with a second mutation elsewhere in the gene.

Table 4. *POLG genotype of infertile men and control groups.* [a]*Excluding a low proportion (approx. 15 %) of infertile men from whom semen DNA failed to produce PCR products despite several repetitions of the analysis.* [b]*I here define the 'wild-type' allele as the one carrying 10 CAG repeats. Heterozygotes carry one copy of the wild-type and one other allele. Non-ten individuals carry copies of either two different alleles (x/y) or a single (x/x) allele with a different number of repeats. Percentages are quoted to the nearest whole number.* [c]*Combined data for 59 Finnish (6/59 non-ten) and 40 English (3/40 non-ten) infertile patients, compared with 65 Finnish and 33 English fertile males, P < 0.0001 (see statistical analysis).* [d]*Finns plus ethnically mixed population, both genders.* [e]*Males born in Hertfordshire 1920-1930, as studied previously (Poulton et al. 1998).* [f]*Note that the 522 controls included in the population surveys were not scored for fertility, and are therefore likely to include some infertile males.* [g]*Hardy-Weinberg predictions in %, to 1 d.p., based on allele frequencies in the combined control groups (no selection based on fertility) of 0.87 for the wild-type allele and 0.13 for all mutant alleles combined. Observed genotypes in the control groups were close to these predictions.*

Phenotype	Total number[a]	Homozygous wild-type[b]	Heterozygous[b]	Non-ten individuals[b]
Infertile males[c]	99	55 (56 %)	35 (35 %)	9 (9 %)
Fertile males[c]	98	80 (82 %)	18 (18 %)	0
Controls (mixed)[d]	134	105 (78 %)	28 (21 %)	1 (1 %)
Controls (English, male)[e]	270	196 (73 %)	70 (26 %)	4 (1 %)
Controls (German, male)	118	90 (76 %)	27 (23 %)	1 (1 %)
All controls combined[f]	522	391 (74.9 %)	125 (23.9 %)	6 (1.2 %)
Frequency expectation[g]		75.7 %	22.6 %	1.7 %

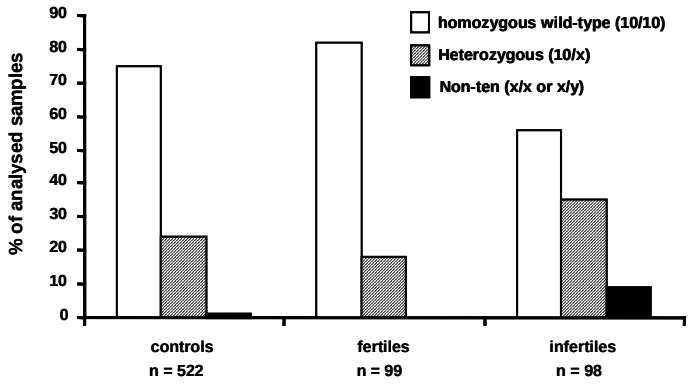


Figure 10. *POLG genotype frequencies in infertile and fertile males, plus control individuals. The lack of the common allele was in 9 % of infertiles compared to 1.2 % of unselected controls and 0 amongst fertile males. * indicates the observed statistical significance when comparing infertiles to either unselected controls or known fathers.*

Many different repeat-length alleles were found, either singly or in combination, in individuals lacking the common allele, with a frequency distribution otherwise similar to that in the general population (*Fig 11*). The results indicated that it is the absence of the common allele, rather than the presence of any particular

alternate allele, that is associated with infertility. Allele lengths of 5-15 CAGs were found, the second most common being one repeat unit longer than the common allele. No very long, expanded alleles were found.

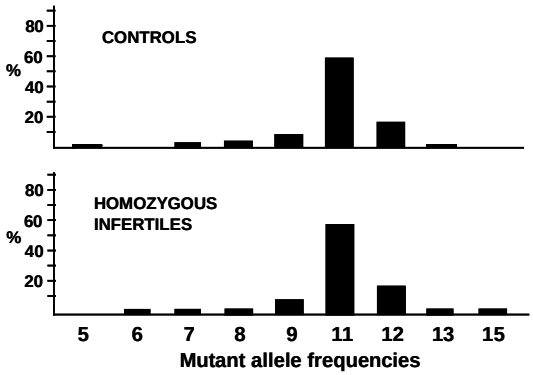


Figure 11. *Frequencies of different mutant alleles (other than CAG_{10}). Top panel; unselected controls (125 heterozygotes plus 6 homozygotes, 137 alleles together), lower panel; infertile men lacking the common allele (66 alleles, all clinical sources combined).*

8.1.3 Statistical analysis confirms the plotted data for the significance of the lack of the common allele (II)

Two statistical tests were applied (Poisson analysis and chi-squared test). Using standard Poisson statistics (assuming a mean population frequency of 1.7 % to the non-ten genotype), the probability of finding 9 or more such individuals out of a sample of 99 is 6×10^{-5}. When fitting the model of homogeneous distributions using the chi-square statistic to the cross-tabulated data presented in *Fig 10*, the result was highly significant ($P < 0.0001$), *i.e.*, the distributions were not homogeneous. The *post-hoc* analysis, (carried out by professor Pekka Laippala) based on the standardized residuals, which have a standardized normal distribution, showed that the reason for significance was the infertile group, where the homozygous wild-type class was significantly under-represented ($z = -2.0$) and the heterozygous and 'homozygous mutant' classes significantly over-represented ($z = 2.1$ and $z = 4.8$, respectively), when compared to the control and the fertile groups. Between the last two groups there was no significant difference.

8.1.4 Non-ten genotype does not correlate with severe sperm phenotype (II)

Sperm cells are highly dependent on energy produced by the mitochondria in their maturation and function. To establish the type of infertility associated with *POLG,* I studied blood DNA collected from three groups of infertile men in Scotland, Ireland and Germany, as well as sperm DNA collected from men in Taiwan and Australia for whom extensive phenotypic data, including clinical profiles and sperm analysis, were available. The varying criteria applied in the selection of those samples are explained in *Table 5*. Homozygosity for the lack of the common allele was not associated with severe phenotypes like azoospermia or severe oligozoospermia based on three different populations studied (Scottish, Irish and German). The overall frequency of mutant genotypes in this group (3/318 or 0.9 %) was similar to that in the general population.

To study the possible correlation of *POLG* mutant genotype and isolated asthenozoospermia, a sample of 56 blood DNAs from such Chinese patients was analyzed. I found no cases of the lack of the common allele and only one heterozygous individual ($CAG_{9/10}$) was present. All the other patients were homozygous for the common allele. Data on Scottish and German patients (blood DNA) were combined because similar information was available for the two groups. Patients in this combined group with a known external cause for infertility were excluded from the analysis. Two additional non-ten individuals were detected in the combined group, but were not included in the data shown: one Scottish individual for whom sperm data were unavailable, plus one German who also had Klinefelter syndrome. Data on the Irish patients (blood DNA) are presented separately, since information concerning other probable causes of infertility (affecting approximately 40 % of the individuals studied) was not available on a case-by-case basis. As a result, the proportion of *POLG* variants detected in this group was about half of that seen in other groups of infertile men with comparable sperm counts.

Table 5. *POLG genotype in different categories of infertile males. [a]Individuals lacking the common, ten-repeat allele. [b]Sperm DNA, [c]Blood DNA, data on Scottish and German patients are combined, but data on Irish patients are presented separately because information on other causes of infertility was not available on a case-by-case basis. The proportion of POLG variants detected in this group is thus about half that seen in other, comparable groups. [d]Includes 62 men with azoospermia plus 73 with severe oligozoospermia (<5×10^6 sperm/ml). [e]Sperm DNA, excludes one additional on-ten individual with a sperm count in the low normal range.*

Population	Phenotype	Total	Non-ten individuals[a]	%
Finnish& English[b]	Excluding azoospermia and severe oligozoospermia	99	9	9.1
Scottish &	Azoospermia or severe	135	0	0

German[c]	oligozoospermia (<5x10^6 sperm/ml)			
Scottish & German[c]	Moderate oligozoospermia (>5x5x10^6 sperm/ml) with or without other defects	113	8	7.1
Chinese[b]	Asthenozoospermia	56	0	0
Australian[e]	Combined oligo- and asthenozoospermia	13	1	7.7
Australian	Unspecified (testicular biopsies)	17	2	11.8
Irish[c]	<5x10^6 sperm/ml	183	3	1.6
Irish[c]	>5x5x10^6 sperm/ml or no data	287	10	3.5

8.2 Mitochondrial DNA sequencing of males lacking the common *POLG* allele (unpublished results)

To reveal if a homozygous *POLG* CAG repeat variation could function as an mtDNA mutator, segments of the cytochrome b gene (cytb) and the non-coding CR of sperm or blood DNA of infertiles and control individuals was amplified, cloned and sequenced. Several samples gave clones with the same sequence of an apparent nuclear pseudogene for mitochondrial cytb, showing 99 % identity with the *Homo sapiens* chromosome 5 genomic contig NT_023148.12|Hs5_23304. The same sequence has also been amplified from other human samples in our laboratory, and appears to be present in both test samples and controls. In case of 8 samples out of 36 analysed, the CR sequencing resulted in a mixed pattern of clones, each of which had several nucleotide changes per clone compared to the reference sequence but no similar sequences were found when these clones were blasted against the human genome. If all the base substitutions in all clones of those samples were counted, the samples had as much as 53 mutations per 10 kb. Most (75-90 %) of the nucleotide changes in these clones were at sites reported to be polymorphic or at sites also found by others to be mutated in ageing human tissues, but mutations on other sites were also present. Because of the extraordinary pattern of these mutations and therefore a need for further and extensive analysis, these clones were left out of the mutation rate calculations in this instance.

As a whole, when leaving off clones suscepted to be pseudogene-derived, as well as the highly mutated CR clones discussed above, the cytb region contained ~0.5 mut/10 kb in each analysed group (*Fig 12*). The control region seemed to have more mutations than cytb in all analysed sperm sample DNAs (1.08-2.74 mut/10 kb) but not in blood DNA from azoospermic males with *POLG* CAG X/X genotype. A large variation between different individuals was detected in each group and no statistics was applied.

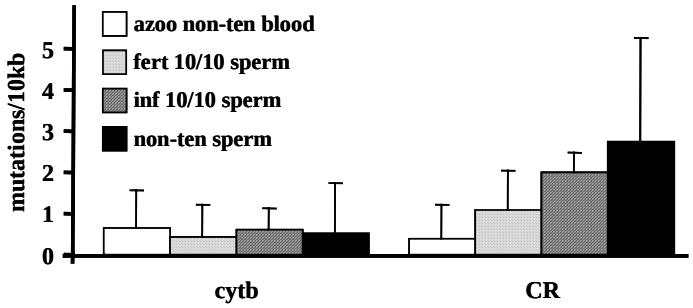


Figure 12. *mtDNA mutation accumulation on cytb and control regions analysed from blood DNA of azoospermic individuals with POLG CAG X/X genotype (5 cases) plus fertile 10/10 sperm (13), infertile 10/10 sperm (3) and infertile X/X sperm (12) DNA. Error bar presents standard deviation and shows the large variation inside each experimental group.*

Table 6. *A typical example of mutation pattern in a homozygous variant (CAG 11/11) male sperm mtDNA. Left column; clone number, first row; nucleotide change, rows 2-4; mutation described in connection with. All so called wild type clones of this person were 73G, 152C, 195C, 263G on these polymorphic sites. P, polymorphic site; age, described in aged tissue; ca, carcinoma; ov, ovary; or, oral; lu, lung.*

	G67T	**73G**	G92A	A123T	**152C**	A189G	**195C**	T199C	G203A	T204C	A214T	T250C	C253T	G255A	**263G**	A300G	i314-21	C338T	C404A	T408A	C418T	iT455	d456	d513-4	A519G	d540	iCn567
	PEO	PEO				PEO	MNGIE				PEO		PEO	MNGIE						PEO						PEO	PEO
					ov ca		lu ca	ov ca		or ca														ov ca			
		P/age	P/age		P/age	P/age	P/age	P	P	P	P	P			P		P	P		P/age			P	P			P
F9		G			T	x	T	x	x	x		x			G							x		x			x
H19		G			T	x	T	x	x	x		x			G								x	x			x
G39		G			T	x	T	x	x	x		x			G								x	x			x
A7		G			T		T	x	x	x		x			G							x					x
H29		G			T	x	T	x	x	x		x			G												
D29		G			T		T	x	x	x					G												
E59		A			T	x	T	x				x			G	x			x				x	x			x
A10		A			T		T				x				G			x					x	x	x		
F10		A			T		T						x		G			x					x	x			
C11		A			T		T				x				G			x					x	x			
G11		A			T		T				x				G			x						x			
F69		A			C		T	x	x	x					G												
C19		A			T		T								G			x									
D10		A			C		T								G												
D8		A			T		C				x				G			x		x			x	x			
C10		A		x	C		C								G						x						
G10		A			C		C								G						x		x				
C9		A			C		C								G		x										
F11		A			C		C							x	G												
B12	x	A			C		C								G												
F133		A			C		C								A												
H233		A	x		C		C								G												
F433		A			C		C								G									x			
F633		A			C		C			x					G												
G19		A			C		C								G											x	
C59		A			C		C			x					G												

8.3 Point mutations in *POLG* of heterozygote infertile males (unpublished results)

A screen for other possible mutations in *POLG* gene of infertile males heterozygous for the CAG repeat was initiated by PCR amplification and sequencing of *POLG* exons with flanking exon-intron junctions. 75 of such patients from Germany, 9 from Britain and 46 from Finland were subjected to

the analysis. Although not all exons from all patients have yet produced a good PCR product and/or readable sequence (about 15 % of all exons), several mutations or polymorphisms, both already described and novel, were detected.

A novel mutation G7849T resulting in an amino acid change Glutamine 517 to Valine (G517V) was found in heterozygosity in three German infertiles who were all 10/11 heterozygotes for the CAG repeat. After this finding, all German infertile (309) and control (115) samples were screened for the mutation with SNP analysis and using the new, specific G517Vfor primer. The mutation was found to be present similarly in the heterozygous state also in three control samples, all of which (as the infertiles) were 10/11 for the CAG. The same amino acid change was also present in two infertiles from Britain, similarly 10/11 for the CAG repeat, and in 7/44 Finnish infertiles with CAG genotypes 10/11 (2), 10/9 (4) and 10/8 (1) but was absent in 44 Finnish control fathers and in 46 British controls, all of which were also screened by using the same SNP analysis protocol.

Two German ($CAG_{10/8}$ and $CAG_{10/11}$) and one British ($CAG_{10/8}$) infertiles were found to be heterozygous for the base change G4664C resulting in the amino acid change Gly268Ala. This mutation has previously been described in a PEO patient as a compound heterozygote with the Ala467Thr mutation (Del Bo et al., 2003; Di Fonzo et al., 2003). One German infertile ($CAG_{10/11}$) was found to be heterozygous for the Tyr831Cys (G15790A) mutation in exon 16. This mutation has been described previously as autosomal dominant in connection with PEO and Parkinsonism (Mancuso et al., 2004). Seven German infertiles were found to be heterozygous for the Gly1236His (G18033T) mutation which has previously been described as a polymorphism present at frequency of 4 % (Gene SNPs). It is also found in PEO and in ataxia-myopathy *in cis* with Arg627Gln and *in trans* with Ala467Thr (Di Fonzo et al., 2003 Luoma et al., 2005). A silent G15790A mutation in Threonine 1066 was detected in two German individuals.

A single G nucleotide insertion at 18098 in the 3´ UTR, and thus probably harmless, was found in all but one of the British samples. Several mutations affecting introns 4, 8, 9, 11, 17, 18, 19, 21 and 22 were seen. The most common of them was the AGGT insertion after nucleotide 13708 in intron 17, which was present in over half of the analysed samples, either as homozygote or heterozygote. None of the intron mutations was situated very close to the splice junction. The nucleotide numbering is based on ENST00000268124 Ensembl, where the start point of exon 1 (87678908) was in our analysis given nt number 1.

8.4 *POLG* CAG repeat polymorphism analysis of patients with a mitochondrial disease (I) and those with non-insulin-dependent diabetes

To find out if polymorphism or expansions of the *POLG* CAG repeat could be related to common mitochondrial diseases, several groups of patients were studied, including 51 unrelated myotonic dystrophy (DM) patients from the UK and a group of 12 unrelated Chinese patients with acute aminoglycocide ototoxicity (AAO), one of which had the nt 1555 mtDNA mutation. A group of 46 patients with a variety of mtDNA deletions, depletion or different mtDNA point mutations leading to disorders like PEO, MELAS, MERFF, NARP, myopathy and deafness, were also screened for *POLG* CAG repeat length. No expanded alleles were found, and the frequency of the common allele amongst all patients combined was not significantly different from the group of combined controls. In all 109 individuals studied the next commonest allele was of 11 repeats (7 %), with other, rarer alleles between 5 and 13 repeats comprising the remainder.

Previously, an association with a common mitochondrial DNA T16189C variant with insulin resistance in adult life was reported (Poulton et al., 1998). I now wanted to reveal if any *POLG* CAG repeat variation different from control samples was present in those samples. There was no difference in *POLG* CAG length variation, either when comparing the diabetics with non-diabetics or individuals having the T16189C variant with those not having it (data not shown).

8.5 Same *POLG* genotype in different tissues (unpublished result)

A mutation disturbing the development or function of a certain tissue, like testis in case of infertility, could also arise somatically and be found only in this specific organ or cell type. Blood, sperm and hair root DNA were used as template in the analysis of *POLG* CAG repeat length. Similar frequencies of different alleles were present in both blood and sperm samples. Also the same genotype was present in both blood and hair root DNA in a given control individual. No longer or shorter alleles were found in any tissue, and the proportion of heterozygotes and mutant homozygotes varied rather according to the phenotype of the patient, not by the source tissue of DNA. One has to remember here that from the azoospermic males, only blood but not sperm DNA was available.

8.6 *POLG* CAG repeat in primates (III)

The primers designed for human *POLG* amplified also all the primate samples, but no PCR product with human primers was obtained from feline, bovine or pig DNA (unpublished results, not shown).

8.6.1 POLG polyglutamine repeat evolution

The POLG polyglutamine repeat is absent from the corresponding gene in yeast (*MIP1*), *Drosophila* and mouse Polg. To see at which point of mammalian evolution the tract appeared, DNA from 42 gorillas (*Gorilla gorilla*), 16 unrelated common chimpanzees (*Pan troglodytes*), 14 unrelated bonobos (*Pan paniscus*), a total of 7 orang-utans from Sumatran and Borneon subspecies (*Pongo pygmaeus pygmaeus and P.p. abelii,* respectively), 6 species of Old World Monkeys (OWM, Rhesus macaque = *Macaca mulatta;* Crab-eating macaque = *Macaca fasicularis;* Bear macaque = *Macaca arctoides;* Baboon = *Papio hamadryas;* Vervet monkey = *Cercopithecus aethiops;* Greater spot-nosed monkey = *Cercopithecus nictitans*) and 2 species of New World Monkeys (NWM, Common Marmoset = *Callithrix jacchus;* Cotton-top tamarin = *Saguinus oedipus*) could be amplified with human primers, genotyped with GeneScan and further sequenced, either directly from PCR product or through a cloning step.

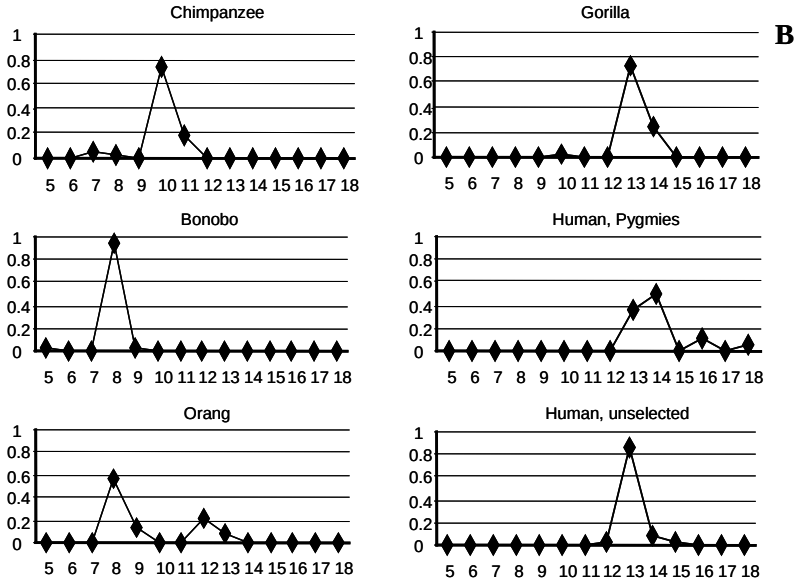
The length and nucleotide sequence of the microsatellite of *POLG* showed rapid evolution from a short glutamine-proline-lysine rich region in OWM and NWM to the clean tract of 13 glutamines in humans. The flanking sequences (16 amino acids upstream and 12 amino acids downstream of the repeat) showed high conservation with only minor changes between species (*Fig 13A)*. In orang-utans, human glutamine 38 (numbering according to ENSP00000268124) is altered to glutamate and lysine 62 is a proline, as in all monkeys analysed. In most ape alleles the stretch of three arginines is present, although a change of glutamine 43 to a fourth arginine is seen in all gorilla alleles, in all orang-utan alleles and in one human and one chimpanzee allele variant.

8.6.2 POLG alleles in primate species

All primate species had several different alleles, with variation in both nucleotide sequence and/or repeat length. Considering only the overall *POLG* microsatellite region length (amino acids 39-60 in human common variant), each great ape species has its own common length variant: in humans and gorillas of 22, in chimpanzees of 19 and in bonobos of 17 amino acids. The next common length variant in each case was one amino acid longer, and these two comprised over 90 % of all alleles seen in each particular species (*Fig 13B).*

Species	allele	Amino acid sequence
Human	10	SSSVPASD PSDG QRRR------QQQQQQQQQQQQQQPQQPQ VLSSEGGQLRHN
	5	SSSVPASD PSDG QRRR----------QQQQQQQQQPQQPQ VL
	15	SSSVPASD PSDG QRRR-QQQQQQQQQQQQQQQQQQQPQQPQ VL
	$4R(CAG)_9$	SSSVPASD PSDG QRRRR------QQQQQQQQQQQQQQPQQPQ VL
Gorilla	A	PSDG QRRRR------QQQQQQQQQPQQPQQPQ VL
	B	PSDG QRRRR-----QQQQQQQQQQPQQPQQPQ VL
	C	PSDG QRRRR------QQQQQQQQQQP---QQPQ VL
P.p.abelii	P	PSDE QRRRR-------QQQQQQQQQQQQQPQQPQ VP
P.p.abelii	Q	PSDE QRRRR----------QQQQQQQQQPQQPQ VP
P.p.abelii	R	PSDE QRRRR------QQQQQQQQQQQQQPQQPQ VP
P.p.pygmaeus	S	PSDE QRRRR----------QQQQQQQQQQQPQ VP
P.p.pygmaeus	T	PSDE QRRR----------QQQQQQQQQQQPQ VP
Chimpanzee	U	PSDG QRRRR--------QQQQQQQPQQPQQPQ VLSSEGGQPRHN
	V	PSDG QRRR----------QQQQQQQQPQQPQ VLSSEGGQLRHN
	W	PSDG QRRR---------QQQQQQQQQQQPQQPQ VLSSEGGQLRHN
	X	PSDG QRRR---------QQQQQQQPQQPQQPQ VLSSEGGQLRHN
	Y	PSDG QRRR---------QQQQQQQPQQPQQPQ VLSSEGGQPRHN
	Z	PSDG QRRR----------QQQQQQQQPQQPQ VLSSEGGQLRHN
Bonobo	B1	PSDG QRRR----------QQQQQQQQPQQPQ VLSSEGGQLRHN
	B2	PSDG QRRR----------QQQQQQQQQPQQPQ VLSSEGGQLRHN
	B3	PSDG QRRR----------QQQQQQQQPQQPQ VLSSEGGQLRHN
	B4	PSDG QRRR-------------QQQQQPQQPQ VLSSEGGQLRHN
OWM	1	PSDG RRR---------------QQQQLQQPQ VP
OWM	2	PSDG RRRR--------------QQQQLQQPQ VP
NWM		PSDG RR----------------QQLQQQPPQ VP
MOUSE	ASSVLDPV	PSDG RP----------------------PSQ MPSSENGQLRLN
RAT	SSSVLDPV	PSDG QP----------------------QSQ MPSSENGQLRLN

A



B

Figure 13. (A) *Several different POLG alleles were found in different primate species. Four human alleles are shown as reference, as well as mouse and rat sequences to compare. Differences from reference sequence are underlined and CAA-glutamines are presented in bold. Other alignments are possible.* ***(B)*** *High frequency of a species specific allele length for POLG microsatellite region in each ape species, chimpanzees, gorillas, bonobos and orang-utans, as well as in the two groups of unselected humans; mainly Caucasians and African Pygmies, respectively. X-axes; number of codons, human common allele* $(CAG)_{10}CAA(CAG)_2$ *as a reference giving number 13 to show the length of the polyglutamine stretch . Y-axes; proportion of all analysed alleles.*

In gorillas 3 different alleles were found, allele A being the most common. The common chimpanzee had a total of 6 different sequence variants with frequencies of 15-35 % each, but only four different length variants, one being predominant in this collection of samples. In bonobos four different alleles were found, the most common allele B1 with a frequency of 91 %. There are two different subspecies of orang-utans, Sumatran and Borneon, and I was not precisely informed if the samples belonged to either or both of them. I found five different alleles in these samples and, subtracting from pieces of information and *POLG* sequences, concluded tentatively that I had samples from both subspecies. From seven individuals six were heterozygous and no allele could be assigned as more common than any other. Sequencing of six different species of OWM resulted in two different alleles otherwise similar but with either three or four arginines. The two different species of NWM were both homozygous for the same allele. A full list of alleles with amino acid sequences is presented in *Fig 13A.*

8.7 Mutator mouse (IV, V, unbublished results)

A knock-in mouse carrying the D257A amino acid change in the second exonuclease domain of Polg was created by our collaborators to test the mitochondrial theory of ageing and the causative effect of the predicted accumulation of mtDNA point mutations on animal phenotype. Homozygous mutant mice (mut/mut) were produced by mating two heterozygous (het, +/mut) animals, and their homozygous wild type (wt, +/+) littermates were used as controls.

8.7.1 mtDNA point mutations accumulate linearly in mutator mouse

Total DNA was extracted from brain tissue of mutator and control mice of 4, 8, 25 and 40 weeks of age, as well as from 13.5 days old embryo (E13.5) head parts. From 25 weeks old animals liver and heart tissue were also analysed in addition to brain. mtDNA point mutations were analysed by PCR amplification, cloning and sequencing of both non-coding control region (CR) and the gene encoding the cytochrome b (cytb) subunit of cIII. From 25-week old animals a fragment of the gene encoding the ND1 subunit of cI and a long segment covering the gene for 12S ribosomal RNA, the CR and cytb gene was analysed. All primers used are listed in *Table 2*. Typically readable sequence was obtained from 45-48 (of 48) clones/animal/gene fragment. Plasmid inserts were sequenced starting from both reverse and forward M13 priming sites in the plasmid, respectively, to give sequence of the full insert with considerable overlap in the middle. The very same mutation could appear in one or several clones in one

tissue of a given animal, but each mutation was counted only once in each such sample.

The mutator mice accumulated mtDNA mutations already as embryos and further during their whole life (*Fig 14*). At embryonic day E13.5 the head part contained 7.98 mut/10 kb of analysed sequence in the *cytb* region, compared to the 0.91 in their wt littermates. The corresponding numbers in brain tissue at 40 weeks of age were 15.73 for the mutator and 5.36 for the wt. The mutation frequency in the non-coding control region (CR) was considerably lower being 2.71 at E13.5 and 9.28 at 40 weeks of age. Three different tissues, brain, heart and liver, of 25 weeks old animals had 10.14, 13.59 and 14.03 mut/10 kb on cytb and 4.07, 6.99 and 5.75 mut/10 kb in the CR, respectively (*Fig 15*). The mutation frequency of the heterozygous animals did not differ markedly from that of their corresponding wt littermates.

The amount of background (mutations created by the polymerase during the experiment) was analysed by subjecting one individual, already analysed at both cytb and CR, to a similar PCR, cloning and sequencing protocol as the experimental samples, and comparing the obtained sequences to the sequence of the original clone. The mean value, 0.88 mutations/10 kb was subtracted from the final results.

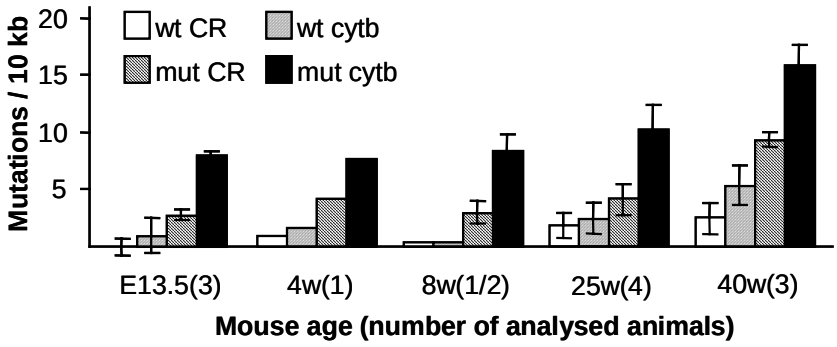


Figure 14. *Mutation accumulation in mutator animals and their control wild type littermates. Total DNA from mouse embryo E13.5 head parts and brain tissue from mice of 4, 8, 25 and 40 weeks of age was analysed for the mitochondrial DNA mutation load. Values are expressed as mutations/10 kb. The number of analysed animals is in brackets and the error bars (sd.) are plotted where more than one animal was analysed*

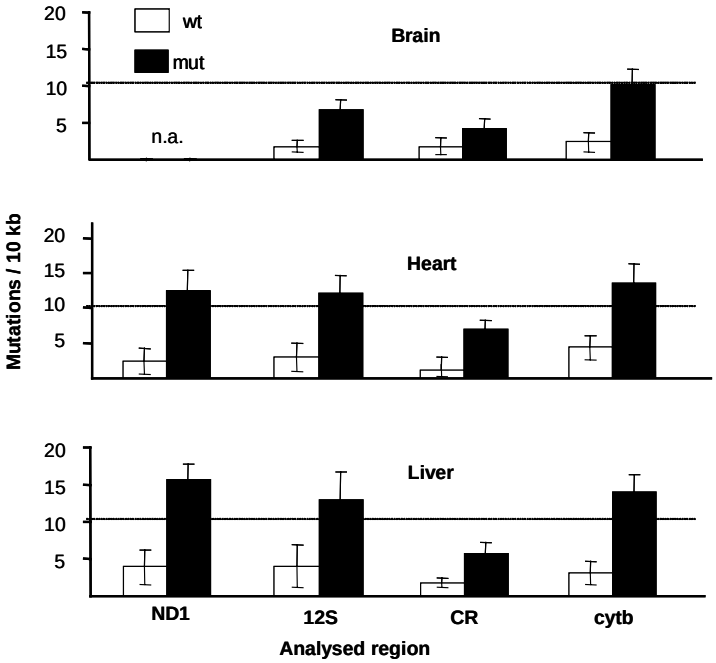


Figure 15. *Amounts of mutations in different mitochondrial sequence segments in three different tissues. Mutation levels of gene fragments coding for respiratory chain subunits ND1 and cytb, the ribosomal 12S RNA gene and the non-coding control region of brain, heart and liver of 25 weeks old mutator and wt animals were analysed and expressed as mutations / 10 kb of analysed sequence. Y-axis has the same range in each graph. The dotted line at 10 mutations / kb was drawn to emphasize the differences between tissues. n.a., not analyzed.*

8.7.2 Mutation type and distribution along mtDNA

The nature and distribution of the mutations were analysed from the 25-week old mutator animals. Combining all mutations found (964 in total) in all three tissues (brain, heart, liver) and two regions (cytb and CR) the most common mutations were transitions A/G (0.20), C/T (0.23), T/C (0.19), G/A (0.09) making 71 % of all the mutations (*Table 7*). The most common transversion A/T was present in 9.6 % of cases. Deletions and insertions accounted 3.3 % of all mutations. There was no significant difference (t-test, data not shown) in the mutation type between different tissues or between the different sequence segments, the cytb gene and the non-coding control region. Also, the region 15449-16048, with low mutation rate compared to other regions, showed a similar pattern of base changes (data not shown).

Table7. *Mutation bias in 25-week old mutator mice. All 964 mutations found in brain, heart and liver tissue and both cytb and CR segments of mutator mouse were analysed for the frequency of each type of mutation. The table shows the base changes as % of all. Transitions are underlined. The most common transversions (A/T and T/A) are in italic.*

	to	A	C	G	T	del	ins
from	A	0.0	2.6	20.2	*10.0*	1.0	0.2
	C	2.3	0.0	1.3	22.5	0.1	0.1
	G	9.6	0.4	0.0	0.8	0.1	0.2
	T	*6.8*	18.5	1.6	0.0	1.3	0.2

To ascertain if the mutations were evenly scattered along the analysed sequence, the liver DNA of four mutator animals was sequenced along the whole region covering nucleotides from 14099 through 16299/0 to 887 (on average 46 clones / animal / segment) and analysed in successive 300 nt blocks with 150 nt overlap. Sequence segment 15449-16048 of the CR containing extended termination associated sequences (ETAS1 and 2) had only 15-40 % as many mutations as the adjacent regions. The rest of the CR (16048-37) containing conserved sequence blocks CSB1-3 accumulated fewer mutations than the cytb or 12S ribosomal RNA genes (*Fig 16A*). A further analysis of narrower windows of sequence revealed that the region covering ETAS1-2 (15450-15560) had only 2.10 mut/10 kb, whereas the region of CSB1-3 (16035-16131) had 12.78 mut/10 kb. The sequence of tRNA Thr (15299-15355), which already in initial analysis by eye looked like a hot-spot, contained 50.84 mut/10 kb (*Fig 16B*).

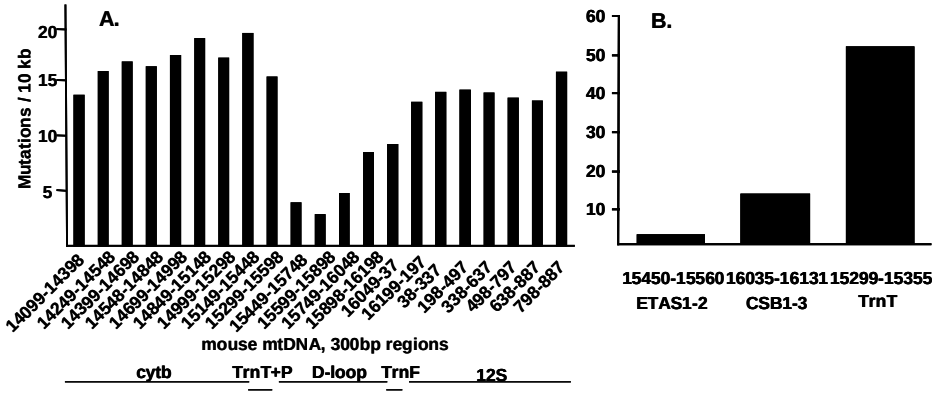


Figure 16. *Mutation rates expressed as mutations/10 kb in mouse mtDNA regions covering nucleotides 14099 through zero up to 887 in liver tissue of four 25-week old mutator animals. (**A**) Each bar represents 300 nt region with an overlap of 150 nt with its neighbours. The solid lines represent a gene on the region: cytb, cytochrome b; 12S, 12S ribosomal RNA; TrnT, P and F, transfer RNAs threonine, proline and phenylalanine, respectively. D-loop area contains ETAS1-2 and CSB1-3 sequences. (**B**) A closer look of more defined areas of ETAS1-2, CSB1-3 and TrnT. Note the different scale of the Y-axes of (**A**) and (**B**).*

The distribution of the mutations between the three different codon positions in cytb gene was analysed (*Fig 17*). Mutation load at heart codon positions one and three differed slightly from other data points but using chi-squared calculations in combination with a z-test to compare the detected values to the expected values, the differences were not statistically significant.

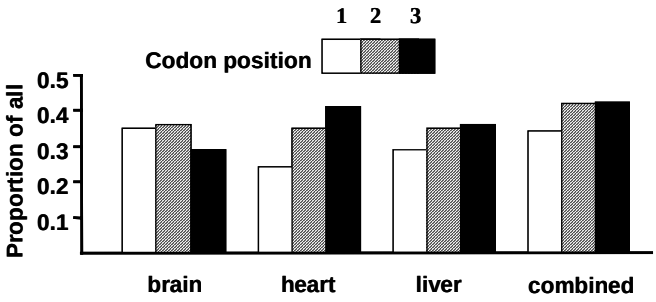


Figure 17. *Relative proportions of mutations hitting each codon position in cytb gene. 583 mutations in total were analysed separately in each of the three tissues, brain (161), heart (201) and liver (221), of four 25 weeks old mutator animals.*

8.7.3 Mutations in nuclear Trp53

To determine whether the nuclear genome was also affected secondarily *e.g.* through ROS production by defective mitochondria, a 237 bp long segment of exon four of the mouse Trp53 (tumour suppressor p53) was amplified from brain DNA of four 25 weeks old animals with the highly accurate Pwo polymerase, cloned and sequenced. In total ~240 000 nucleotides were sequenced from both wt and mutator animals giving an average of 0.99 mut/10 kb for mutator animals and 1.19 mut/10 kb for their wt littermates. The difference between the groups is not significant (t-test). The method-created background for Pwo polymerase was not analysed, so the results are calculated as actual values with no background deduction, although, according to the available information from company catalogues and other sources, the error rate for Pwo polymerase is comparable or slightly higher than that of the Pfu polymerase used in mitochondrial DNA sequencing. These values for Trp are therefore hardly above background.

8.8. Pseudogene involvement exclusion

The possible interference of nuclear mitochondrial pseudogenes (NUMTs) in mtDNA sequencing of both human and mouse samples was ruled out by a similar PCR – cloning – sequencing experiment as used with the actual samples. This was done even though the mitochondrial primers were designed so that they

should not amplify anything from the nuclear genome. The template was human B2 ρ^0 -cell line DNA or mouse ρ^0 -cell DNA, respectively. All mitochondrial primer pairs in each case were tested using nuclear primers as a positive control. In both cases after 30 cycles of PCR the nuclear target gene plus one band with mitochondrial cytb primers was amplified, but no other bands were visible (*Fig 18*). The nuclear mip -primers to amplify the polyQ region of human POLG exon two did not give any band with the Pfu polymerase, which was an expected result. This microsatellite region of *POLG* typically needed 30-35 cycles, and was always best amplified with the AmpliTaq Gold® DNA Polymerase, which allows a hot start for PCR. Amplification with primers for *POLG* exon three resulted in a clear band, thus serving as a positive control for PCR.

The human cytb pseudogene band had a 99 % identity with the *Homo sapiens* chromosome 5 genomic contig NT_023148.12|Hs5_23304. As described earlier, in some individuals this sequence was present in 2-4 % of the analysed mtDNA clones, but was clearly distinguishable from mitochondrial cytb by contig alignment. The mouse NUMT fragment amplified by cytb primers showed 100 % identity to a sequence segment on mouse chromosome 4, database location AL773519.5 62072 to 62902 (+), genomic location: 4 79065147 to 79065977 (+), but only 93 % identity to mouse mitochondrial cytb. These pseudogene segments were clearly identifiable and did not contribute to any of the mtDNA sequence variation reported in this thesis.

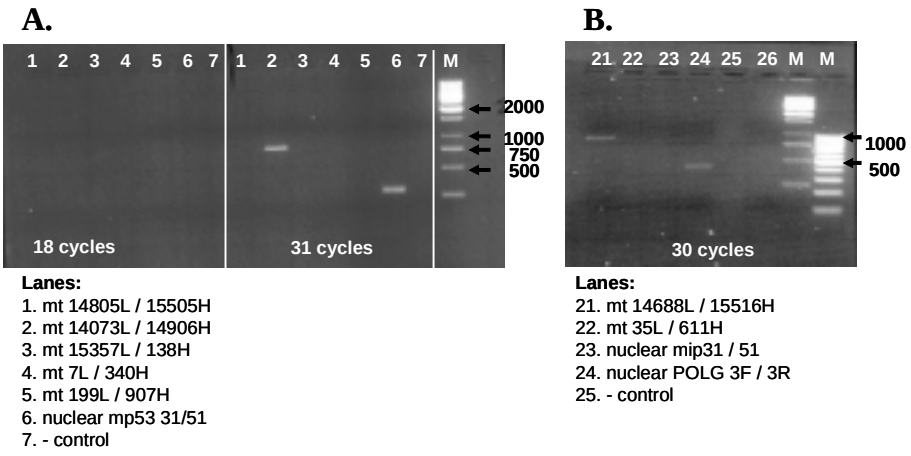


Figure 18. *PCR amplification of ρ^0 DNA with mitochondrial and nuclear primers as described in the figure. M, molecular size marker. (**A**). Mouse ρ^0 cell DNA. (**B**). Human B2 ρ^0-cell line DNA.*

9. DISCUSSION

9.1 The main experimental findings

The main findings of the experimental work in this study were:

- The common allele of human *POLG* with ten CAGs is maintained at high frequency in all populations studied.
- The microsatellite region of POLG has evolved during primate evolution from a short arginine-glutamine-lysine-proline-rich region in monkeys to the stretch of thirteen glutamines in the human common allele.
- Each higher primate species has its own common length variant of POLG maintained by high frequency.
- Length variation of the *POLG* CAG microsatellite is not associated with common mitochondrial disease.
- Lack of the common allele but not appearance of any specific variant allele is associated with male infertility characterized by oligozoospermia and other sperm defects, but not with severe conditions, such as azoospermia or isolated asthenozoospermia.
- Infertile males carry, in addition to the CAG length variant, additional point mutations in their *POLG* gene.
- *POLG* mutant males accumulate point mutation in their sperm mtDNA control region.
- Knock-in mice carrying a homozygous mutation in the exonuclease domain of *Polg* accumulate high loads of mtDNA point mutations in mtDNA and exhibit a premature ageing phenotype and reduced lifespan.
- The amount of mtDNA point mutations in Polg mutator mice is high already in embryonic state and accumulates linearly during lifetime along the whole mitochondrial genome.
- Accumulation of the mtDNA mutations, however, is not even. Specific segments of the non-coding control region seem to be selectively protected “cold-spots”, and region encoding TrnT is a prominent “hot-spot”. As a whole, coding regions accumulate more mutations than the non-coding CR.

9.2 *POLG* polyglutamine allele lengths

9.2.1 Species-specific allele lengths are maintained at high frequency

In the beginning of this study the polyglutamine tract of the second exon of POLG was known to be present in the human protein but not in other organisms like mouse or *Drosophila* (Ropp and Copeland, 1996; Kaguni, 2004). With the knowledge from the literature about the involvement of trinucleotide repeat expansions in human disease, and their usefulness as genetic markers, the first interest was to look at the allele lengths and frequencies of *POLG* CAG repeat in human populations. This was done by developing a fluorescent PCR/fragment analysis method on ABI310 Genetic Analyzer for high-throughput screening. The method was proven to be reliable by analysing the first batches of samples also on conventional, 3 % agarose gels and finally by DNA sequencing.

The polyglutamine tract, as well as the nucleotide sequence coding for it, appeared to be very stable and probably has relatively little value as a genetic marker. The common $(CAG)_{10}$ variant was present at a frequency of 0.89 in the genetically isolated and bottlenecked Finish population and 0.88 in an unselected control group of mainly Caucasian non-Finns, with homozygosity values of 79 % and 78 %, respectively; close to equilibrium predictions. Although several variant alleles were present both in controls and in infertile males, none of them exceeded a frequency of 0.08, and no large expanded alleles were found, even though the experimental setting was shown to be able easily to detect expanded CAG repeat alleles of the *DMPK* gene of over 500 repeat units. The data indicated that the predominant POLG allele length is either inherently stable or maintained by some sort of selection. Just recently a similar suggestion of a purifying selection acting against deleterious POLG allele lengths was suggested, based on results of the analysis of a total of 1330 individuals from 12 different ethnic groups from northern Eurasia, where a similar, high frequency of the common $(CAG)_{10}$ allele was shown to be present (Malyarchuk et al., 2005).

The result obtained from sequencing of the polyglutamine region in a small sample of African Pygmies, 5 Mbutis from North-Eastern Zaire and five Biakas from Central African Republic, showed more heterozygosity than was present in the large group of mainly Caucasian people, and had 11 CAGs as the most common variant. Other studies have shown that these two populations differ genetically (based on mtDNA, Y-chromosome and ABI Linkage Mapping Sets) not only from other populations but also from each other (Knight et al., 2003; http://info.med.yale.edu/genetics/kkidd/tree.html). The Mbutis and Biakas show also high within population microsatellite variation (Zhivotovsky et al., 2000). From such a small sample as I had in my hands, it is not possible to make any definite conclusions, if in case of POLG polyglutamine repeat the Pygmies are

significantly different from other people. The same applies the Chinese, amongst whom the common variant was present in over 90 % of individuals I studied.

Deka and co-workers (1999) analysed 29 different trinucleotide loci and found out that, compared to other types of loci, small GC-rich gene-associated tri-nucleotides show less within-population variance of allele sizes, less polymorphism with less heterozygosity, a smaller number of alleles and one predominant allele per locus in each analysed population. The allele frequency distribution in these loci does not confirm to any standard distributional form (bell shape, uniform, L shape or J shape). The disease causing trinucleotide loci on the other hand, were shown to have the highest level of within-population variation in terms of all measures listed above. This all fits with my analysis concerning the role of *POLG* CAG repeat in common mitochondrial diseases, although not in case of male infertility as discussed later.

These data prompted me to look at the same gene region in our closest relatives, the primates. In the androgen receptor gene both the CAG-I repeat and the glycine repeat are shown to have expanded during divergence of higher primates (Choong et al., 1998). The expansion of the *POLG* CAG repeat from an arginine-glutamine-proline rich region of monkeys to the present polyglutamine repeat in humans was not a surprise. The more interesting finding was that, as humans, each primate species had its own common allele length which was present at high frequency (*Fig 13B*), based on the results from the reasonably large sample of each higher primate species in my analysis. It seems also that the evolution of the repeat, similarly as in case of the CAG repeat in AR, has been fast and species-specific (*Fig 19*). This might reflect that the interacting proteins in the mtDNA replication complex have also species-specific properties. Results from cell culture experiments indicate that the human POLG catalytic subunit needs the human accessory POLGβ subunit for its function, at least when over-expressed in mouse cells, but is functional alone when expressed in human cells where the endogenous human POLGβ is present (Sanna Horttanainen and Johannes N. Spelbrink, unpublished results). The mouse Polgβ protein is only about 73 % identical to the human counterpart, which difference might be enough to confer the species-specificity.

In the AR the increased CAG length correlates with its function and alters the extent of serine 94 phosphorylation (Choong et al., 1998). Nothing is known about post-translational modifications of POLG, but there are several serines on both sides of the repeat, which in NetPhos 2.0 Server phosphorylation site prediction give values from 0.809 to 0.999, and are thus good candidates for phosphorylation sites. POLG is reported to have several both RNA and DNA dependent enzymatic activities (Kaguni, 2004). How these activities are regulated in response to different cellular needs is not known. A common strategy for such a regulation is, amongst others, protein phosphorylation.

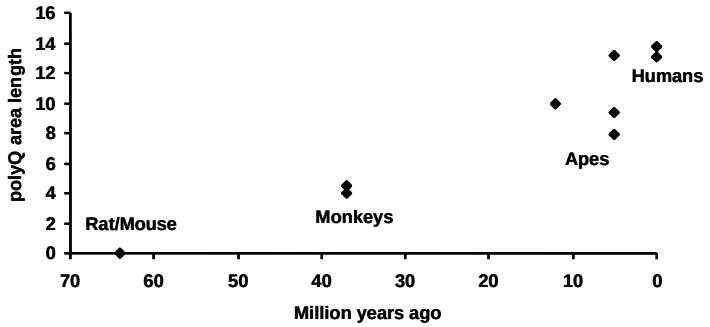


Figure 19. *Evolution of the polymerase gamma polyQ length. X-axis, divergence times in millions of years; Y-axis, number of glutamines. Apes contain Bonobo, Chimpanzee, Orang-utan and Gorilla. The two data points of humans are; lower: mixed population of mainly Caucasian, higher: African Pygmies represented by Mbutis and Biakas.*

9.2.2 POLG polyglutamine repeat expansions are not a common cause of mitochondrial disease

Polyglutamine repeats are a common cause of neurological diseases as discussed earlier in this thesis. Combining the existing data that POLG with the polyglutamine repeat in its coding region is thus far the only known enzyme to replicate mtDNA in human cells, and that many neurological diseases are associated with mtDNA deletions, depletion or point mutations, prompted me to look for the possible role of *POLG* CAG repeat expansions in mitochondrial disease. One explanation for the neurological symptoms in mitochondrial diseases could have been, by analogy with other polyglutamine expansion diseases, a polyQ aggregation interfering directly with cell functions. Alternatively the repeat expansion could act by affecting POLG activity, or sequestering the enzyme away from its physiological location and function. It turned out that the CAG repeat plays no role as a common cause of mitochondrial disease but based on the work of others (http://dir-apps.niehs.nih.gov/polg/ and references therein) rather, the main contributors to certain types of mitochondrial disease which include different forms of PEO, Alpers´ syndrome and SANDO, are the various point mutations detected in recent years all over the *POLG* coding sequence. It seems that extreme length variants of the polyglutamine repeat of POLG are either not formed at all or are not compatible with life. Alternatively neither I nor anybody else have yet looked at the right group of patients.

9.2.3 Lack of the common POLG allele associates with male infertility

Some polyglutamine expansions, like AR–linked Kennedy´s disease, are associated, in addition to neurodegenerative disease, also with infertility (Mifsud

et al., 2001). POLG is the only known DNA polymerase inside mitochondria. Mutations in *POLG* have been reported in neurodegenerative diseases characterized by multiple mtDNA deletions (Van Goethem et al., 2001). Multiple mtDNA deletions have also been reported in low quality sperm (Kao et al., 1998). The high frequency (0.88) of the common $(CAG)_{10}$-allele of POLG suggested that the common allele may be maintained by selection and, as already discussed, expansion of the repeat was ruled out as a common cause of mitochondrial disease. I now wished to ascertain if there was any evidence for involvement of POLG repeat expansion or even just repeat length variation in a complex, heterogeneous disorder, such as diabetes or male infertility. Diabetics gave similar allele lengths and frequencies as the control population but potentially, an effect on fertility of non-canonical variants would be a good selection method for maintaining any preferred gene variant.

My study, revealing an association between the lack of the common $(CAG)_{10}$-allele and male infertility did not follow the common trend of other polyglutamine diseases or other types of trinucleotide repeat expansion diseases. It rather implies that there is a common, favourable allele of the repeat, which is characteristic for *POLG*, and which may indeed be maintained by selection. This association of infertility with the lack of the common allele rather than the appearance of any specific variant allele resembles a loss of function mutation, even though the possible function of the repeat area is currently not known. The affected males do not seem to have any other symptoms than overall bad quality semen: oligozoospermia combined with defects in motility and morphology. Everything else, except this highly tissue-specific cell proliferation/maturation, was fine. As shown by other studies (Jensen et al., 2004; Aknin-Seifer et al., 2005), infertile couples having this *POLG* male factor can be helped with artificial reproduction techniques suggesting that, in case no other reason for couple infertility can be found, the male *POLG* genotype should be analysed. If the male is found to be homozygous mutant, ICSI should be offered as the first and efficient therapy to avoid any expensive and ineffective trials.

9.2.4 Optimal length of the protein may maintain optimal function

In genes causing polyglutamine disorders with severe phenotypes a “favourable” allele length(s) is also present in healthy individuals, although the variance of the normal range is larger, and the disease-causing alleles are typically very long compared to controls.

In the case of AR, the polyglutamine length limits corresponding to the different phenotypic outcomes are quite narrow, and the symptoms depend on the direction of evolution of the length of the repeat (Tut et al., 1997). This again shows that there is a certain allele length that maintains not only optimal protein function but also the person’s healthy status, and the disease-causing variants can be either shorter or longer than the average normal range.

The CAG repeat of the common allele of *POLG* is only half of the size of the predominant AR allele. The other *POLG* CAG repeat lengths that were found in both controls and infertile subjects were between five and fifteen triplets, the second most common being eleven CAGs. In the graph of all *POLG* alleles, the frequency distribution does not confirm to any standard distributional form, but leaving the common $(CAG)_{10}$ allele out, the distributions of the variant alleles of both controls and infertiles make similar bell shape graphs. This, together with the statistical analysis, points to the importance of the correct length allele.

Neither we, nor anybody else, as far as we know, have seen very long or very short (<5) CAG repeat alleles in human *POLG*. There are at least three possible reasons for that: (i) They simply are never formed, (ii) they are not compatible with life, or (iii) nobody has just looked amongst the right sample of individuals. The lack of the common allele was found to be associated with a certain kind of infertility, characterized by moderate oligozoospermia and defects in sperm morphology and/or motility, *i.e.* low quality sperm. The lack of the common allele was not associated with drastic conditions like azoospermia or isolated asthenozoospermia, and no correlation with any specific repeat-length was found in any type of infertility in this study.

9.2.5 Possible function(s) of the POLG polyglutamine tract

Any mutation affecting male reproductive success should be diluted out from a population. Such mutations would anyhow be transmitted from females to the next generation if they cause no defect in females. Such variants could also be preserved at a low frequency, if the mutation was deleterious or harmful only in the homozygous state. A third possible reason for finding a mutation in case of repeated sequences is that new variants are constantly formed by errors in DNA synthesis. The polyglutamine tract of human *POLG* is encoded by the sequence $(CAG)_{10}CAA(CAG)_2$, making it a good template for CAG expansion, even though the CAA codon in the stretch might have a stabilizing effect by disturbing the hairpin formation of the repeat, which has been suggested as one mechanism by which such repeats are expanded (Moore et al., 1999).

This study shows that lack of the common POLG length variant is associated with male infertility characterized with oligozoospermia and other sperm defects. It also describes an accumulation of point mutations in sperm mtDNA CR of those infertile male. To present any symptoms in patients, the load of mitochondrial mutations has to be very high or the individual has to encounter some sort of stress. Most mitochondrial diseases are late-onset type and it may well be that POLG infertile males were fertile in their early adulthood but during a period of years their fertility has declined. Often the symptoms of a mitochondrial disease reflect problems in very specialized organs like auditory hair cells or neurons, or high energy-demanding tissues like heart or skeletal muscle. Sperm is a very specialized cell type produced in a very specialized organ, the testis, and both would be good candidates for susceptibility to any

problems in mitochondrial function and energy production. In line with its lack of association with severe diseases, the lack of the common allele is not associated with dramatic semen phenotypes like azoospermia or isolated asthenozoospermia, suggesting a different aetiology behind these outcomes.

Polyglutamine tracts are commonly regarded as interfaces of protein-protein interactions. The crystal structure for human POLG protein is not yet available, and as mentioned before, the possible functions and/or interaction of the repeat, either at the RNA or protein level, are not known. Many possibilities remain open to account for an association of POLG polyQ length variants with infertility. These include impaired protein or signalling molecule interaction, malfunctioning of POLG itself, or involvement of another polyQ protein sequestering POLG. One good candidate is the already earlier mentioned interaction between the catalytic and accessory subunits of POLG.

CREB–mediated gene transcription promotes cell survival, and CBP, a co-activator of CREB-mediated transcription, has been shown to be sequestered from the nucleus to polyglutamine aggregates (Nucifora et al., 2001) in TREDs such as HD. Polyglutamine aggregates on the other hand have been shown to be inefficiently degraded and interfere with proteasome function (Holmberg et al., 2004). Trinucleotide repeats are capable of forming secondary loop-structures (Mitas, 1997; Sinden, 1999; McMurray, 1999) which may not only be sites of expansion mutations but may also interfere with transcription if not resolved by nucleases. Any of the above-mentioned mechanisms, if applying to POLG, could conceivably compromise the function of such an active and specialized tissue like the testis.

Sperm maturation is highly dependent on the function of the surrounding tissue, and therefore Sertoli cells have a key role in spermatogenesis. The developing spermatozoa stay interconnected and surrounded by the nurturing Sertoli cells until released from the seminiferous epithelium. A junctional complex between adjacent Sertoli cells separates the spermatogonia and young spermatocytes into the basal compartment, whereas the early and late spermatids are found in the ad-luminal part of the seminiferous epithelium. The young spermatogonia must pass the junctional complexes to enter the ad-luminal part of the seminiferous epithelium. The Sertoli cells secrete fluids and restrict movements of molecules establishing microenvironments essential for the development of spermatozoa. Sertoli cells also phagocytose degenerated spermatogenic cells and the residual bodies (Larsen, 1993; Print and Loveland, 2000). If Sertoli cell functions were compromised by a defective POLG, one could expect it to be seen as defects in sperm maturation.

The CAG repeat is absent from mouse and Drosophila *Polg*. Extracts from cell lines expressing the ΔQ_{10}-myc variant of human POLG (CAG repeat deleted human POLG with C-terminal myc-tag to enable detection of the expressed protein with antibody, Spelbrink et al., 2000) consistently show elevated levels of both protein and polymerase activity, although the cells grow well with no difference from cells transfected with the vector alone. The elevated DNA polymerase activity of the ΔQ_{10}-myc variant, together with the finding that sperm

with higher mtDNA content present at least one abnormal criterion (out of sperm count, motility and morphology, May-Panloup et al., 2003) suggests other possibilities. Maybe POLG with a variant length allele has elevated polymerase activity resulting in a higher content of (perhaps mutated) mtDNA, finally resulting in low quality spermatocytes. Alternatively, the ratio of polymerase to exonuclease activities might be crucial in regard to the accuracy of POLG. The variant enzyme may be only a weak mutator (as indicated by the analysis of sperm mtDNA from POLG mutant infertiles), but this weak mutator may nevertheless result in loss of mtDNA integrity below a critical threshold in a tissue which undergoes many rounds of cell division.

9.3 Other *POLG* mutations in infertile males (unpublished results)

As discussed in the literature review, tens of *POLG* point mutations have already been discovered associated with a variety of diseases and with considerable cross-overlaps between mutations and phenotypes. In this study the higher proportion of POLG CAG repeat length heterozygotes amongst the infertiles compared to either fertile males or unselected controls suggests that at least part of those individuals could carry another mutation somewhere else in the *POLG* gene. If this hypothetical other mutation was *in cis* to the CAG variant allele, it would probably be a dominant gain-of-function mutation to show the phenotype. If the other mutation was *in trans* to the CAG variant allele, this other mutation could also be a loss-of-function mutation which would make those individuals compound double mutants and comparable to the homozygous polyglutamine mutants. Finding other such mutations in heterozygotes would raise the number of *POLG* mutants amongst infertiles perhaps as high as 45 % of all cases (excluding azoospermic and severe oligozoospermic). Already the ~10 % of cases that are homozygous polyQ mutants represent a significant fraction of male infertiles with unknown aetiology, excluding cases of azoospermia or severe oligozoospermia.

The screening of *POLG* CAG heterozygous infertile individuals has already shown that indeed, a considerable fraction of CAG heterozygote infertile males carry, in addition to the variant polyQ length, another base substitution in the *POLG* gene. This screening and more detailed analysis is still in process, but the fact that some of the mutations found have previously been described as disease-associated, strengthens the suggestion that defective POLG is a rather common contributing factor to male infertility/subfertility. Additional supporting evidence for this theory comes from other data, discussed later.

9.4 Congruence with other studies on *POLG* CAG repeat and infertility

Similar studies on *POLG* CAG repeat association with infertility have been reported subsequently, with variable results. In one quite well conducted study (Jensen et al., 2004) an association with the lack of the common allele and unexplained male sub-fertility with normal spermiograms was reported. With seven of nine affected couples, treatment with ICSI was successful and resulted in a healthy baby. In case of one couple a subsequent ICSI resulted in another child. In case of three males homozygous for the lack of the common allele treated with IVF, the spermatozoa retained the ability to penetrate the egg but the fertilization rate was low. According to this result ICSI (but not IVF) seems to be a successful therapy for infertility associated with a male POLG defect.

In a multi-centre French study (Aknin-Seifer et al., 2005) the authors came to conclusion that "there is no relationship between the polymorphic CAG repeat in the *POLG* gene and male infertility". Their way of grouping the patients was, however, different from my analysis. Regrouping and analysis of the data shows that only 26 moderate oligozoospermic patients were included in their study, from which they found 2 (7,7 %) to be homozygous mutants for the *POLG* CAG repeat. That number amongst French / Spanish infertiles comes very close to the 9 % found in my own study for infertiles in Finland and UK, even though the group of patients in the French study was small. Aknin-Seifer and co-workers did not find any homozygous mutants in the group of isolated asthenoteratozoospermic patients, which is in accordance with my results. Furthermore, all of their homozygous mutant cases with no female factor involved could be helped by ART to produce a healthy baby.

Another study made in northern Italy (Krausz et al., 2004) also "failed to confirm any influence of the CAG repeat length of the *POLG* gene on the principal sperm parameters" and that "the analysis of the CAG repeat tract from *POLG* gene does not appear to have any clinical diagnostic value". In that study only 33 of the 195 infertile patients fell in the category of moderate oligozoospermia, and of those 2 (6 %) were homozygous mutants. 147 of the 195 infertile patients fell in the categories of azoospermia or severe oligozoospermia, in which groups I did not find any association with the lack of the common *POLG* allele. Thus their sample group and the experimental setting were different from ours. Of their control group of 190 normospermic males they found 6 (3.2 %) to be homozygous for the lack of the common allele, but only 90 of those 190 were known fathers, making any comparison again difficult. If the remaining 100 were infertile with unknown aetiology, and all 6 homozygous mutants fell in that group (which we do not know), it would make 6 %. The authors anyhow reported a reduction in sperm concentration in the normospermic control group from $80+/-49x10^6$ /ml in 10/10 to $62+/-19x10^6$ /ml in homozygous mutant individuals, although both numbers are well inside the normal range.

9.5 Defective *POLG* results in mutations in mtDNA

9.5.1 Lesson from the mutator mouse

Cultured human cells expressing a POLG protein with crippled exonuclease activity accumulate mtDNA point mutations, reaching a maximum level after three months of culture, after which time-point the mutation amounts start to go down (Spelbrink et al., 2000) as a result of negative selection against the mutator. Cells grow normally at low mtDNA mutator load without any visible problems and the simplest interpretation is that at a certain threshold the most severely affected cells die and are diluted out from the population.

To see the effects of such a *POLG* mutation in a whole organism, a knock-in mouse carrying a D257A mutation in the second exonuclease domain of POLG was created. An accumulation of point mutations in the homozygous animal tissues was documented in this study. Accumulation of mtDNA point mutations in elderly human tissue has been reported previously, so the premature ageing phenotype of the mice was rather expected, although a phenotype resembling more closely a conventional mitochondrial disease would not have been a surprise either.

Similarly to many mitochondrial diseases of late-onset type, the mutator mice were born normal and developed normally for the first weeks of their lives suggesting that any phenotype appearing would need time to develop. Also similarly to human patients with heteroplasmy, the mice showed that tissues can tolerate a certain amount of mtDNA point mutations before starting to express any phenotype. It became evident that there is a clear connection between the accumulation of mtDNA point mutations and ageing related phenotypes. Furthermore, the mutator mice not only had all the symptoms familiar to ageing humans, like weight loss, reduced subcutaneous fat, osteoporosis, kyphosis, alopecia, anaemia, heart enlargement and severe reduction of fertility, but they also had a reduced lifespan. Another mouse model with a complete PolgA deficiency caused an early developmental arrest between E7.5-8.5 associated with severe mtDNA depletion, showing that polymerase gamma function is essential for mammalian embryogenesis (Hance et al., 2005).

Another Polg exonuclease-deficient mouse was reported 14 months after ours (Kujoth et al., 2005). These mice resemble ours in regard to the late phenotype and mtDNA point mutation accumulation. The ageing phenotype in these mice is evident slightly later and they live longer, which may be because of different mouse strains used in the experiments.

De Magalhães and co-workers (2005) have analysed data statistically from published experimental ageing research to find out which of the genes that are reported to influence ageing, either by delaying or promoting it, have a true effect on ageing events *per se*. They pointed out that many disease-causing gene

defects can affect lifespan without having any direct influence on ageing process. Their analysis used Gompertz equation to model the ageing process, and they found statistical evidence that PolgA does influence ageing in mice and thus is a good model for ageing research. In the analysis concerning 20 mouse models stated either to accelerate or delay ageing, only seven appeared in reality to influence ageing directly, the rest affected lifespan mainly *via* age-independent mortality.

9.5.2 Mutations do not accumulate evenly along mtDNA

In the Polg mutator mouse, mutations did not accumulate evenly across the analysed mtDNA regions. Instead, there was a prominent cold spot at the so-called extended termination-associated sequences ETAS1-2, short conserved segments of the non-coding control region (NCR). ETAS1 is suggested to contain a recognition signal for the termination of the nascent DNA or RNA chain, and ETAS2 to contain binding sites for terminators (Sbisà et al., 1997). The conserved sequence blocks 1-3 (CSB1-3) had slightly less mutations than genes for 12S RNA or cytb, but were not significantly spared. In ageing humans the mtTFA binding sites, which are flanking but distinct from the CSBs, have been shown to accumulate point mutations (Michikawa et al., 1999). CSBs1-3 is proposed to contain regulatory signals for the processing of the RNA primers for H-strand replication. The threonine tRNA is a clear hotspot in the mutator mouse, containing 5 times as many mutations as other coding sequences. To account for this phenomenon I do not have any explanation or example from literature, but in theory every fourth nucleotide of tRNA threonine in the ageing Polg mutator mouse is predicted to carry a mutation, which will most probably affect translation seriously. Other work in our lab has shown that both synthesis, extent, aminoacylation and processing of $tRNASer^{(UCN)}$ are impaired by the structurally "mild" 7472insC mutation in mtDNA (Toompuu et al., 2002; 2004), and many other reports in the literature document the deleterious effects of pathological mutations of mitochondrial tRNAs. Six tRNA threonine mutations in mtDNA are reported to be associated with human diseases including mitochondrial myopathy, lethal infertile mitochondrial myopathy, encephalomyopathy and Multiple Sclerosis (www.mitomap.org).

The reported point mutations in ageing humans are mostly described to be found on the CR, just opposite to the mouse model. Similar accumulation of point mutations on CR only was in this study seen in POLG infertile males, as discussed later. These mutations are very often on sites that are reported as polymorphisms and/or seen in patients with POLG/PEO. Finding of these ageing related mutations on polymorphic sites raises a question: How to define which base substitution in a given individual should be considered as a normal polymorphism and which as a mutation?

One explanation for differential accumulation of the mutations on different mtDNA regions in the mutator mouse *versus* ageing humans could be two

different polymerases acting on different regions of mtDNA or in different physiological conditions. No other DNA polymerase has been found active in mitochondria this far, and PolgA knock-out mice die as early embryos devoid of mtDNA, arguing against the existence of any other replicative mtDNA polymerase, at least in early embryonal development (Hance et al., 2005). On the other hand, a residual polymerase activity has been found to be present in cell extracts of patients with a homozygous G2899T nonsense POLG mutation creating a stop-codon just upstream of the polymerase motif (Naviaux and Nguyen, 2004).

9.5.3 Ageing is caused by energy deficit with no ROS involvement

The free radical theory of ageing presented by Harman already 50 years ago proposes that the main reason for ageing is reactive oxygen species harming DNA and proteins during lifetime. The idea of the so-called *vicious cycle* has become almost an article of faith, albeit with virtually no convincing supportive evidence. Data from the phenotypic and biochemical analyses of the mutator mice performed in Stockholm, combined with my mutation analysis do not support this hypothesis, but rather indicate a direct link between the accumulation of mtDNA mutations, ageing-related phenotypes and reduced life span. The mutation load is already quite high in mid gestation, and increases linearly up to 40 weeks of age, at which point the mice start to die. Superoxide and hydrogen peroxide levels in MEFs isolated from E13.5 embryos were not elevated and there was no increase in hydrogen peroxide induced cell death (Trifunovic et al., 2005). The oxidative damage to proteins as well as antioxidant defence enzyme expression was not different from wt animals. Similarly Kujoth et al., (2005) did not report any signs of oxidative stress in their mice, supporting the direct link between mtDNA mutation loads and ageing phenotype without involvement of accelerated ROS production or any *vicious cycle*.

Elevated ROS production is suggested to introduce mutations also in the nuclear genome, but the mutation load in the p53 gene of the mutator mouse was not different from wt, further supporting the argument against extensive involvement of oxidative stress in the premature ageing caused by the Polg mutator. Just when the manuscript of this thesis was close to submission, a paper was published showing that insertion of a human disease-causing linker region mutation in the corresponding place in the yeast *S. cerevisiae mip1* gene results in elevated DNA damage both in mitochondria and the nucleus of that particular yeast strain (Stuart et al., 2006). The similar nuclear mutator effect was not present in yeast strains expressing mip1 protein carrying analogously a polymerase domain mutation or an exonuclease domain mutation.

The age-associated increase of mtDNA deletions, and subsequent appearance of COX^{-}/SDH^{++} segments is associated with fibre atrophy in rhesus monkey muscles and would suggest a continuous nuclear response to electron transport

system (ETS) abnormalities as a secondary effect to a deficit in cellular energy (Lopez et al., 2000). The similar fibre atrophy in ETS abnormal regions could explain partly the lean mass loss in mutator mice, especially when we know that the respiration of Polg mutator mice derived MEFs is severely affected.

Age-related reduction in stem-cell proliferation leads to accelerated loss of cellular mass in many organs, especially those with high turn-over, and earlier loss of maintaining homeostasis or normal cell numbers (Donehower, 2002). The cellular loss can be a result of (i) loss of stem cell number due to increased apoptosis or cell cycle arrest, (ii) reduction of self-renewal capacity or (iii) cells that can maintain self-renewal capacity but suffer a reduction in their ability to differentiate. Any of these mechanisms can be active in bringing about the phenotypes like loss of sperm or anaemia in mutator mice. In their recent review Ho and co-authors (2005) remind us, that a living organism is as old as its stem cells and that age represents the main variable and worst prognosis factor for clinical outcome in transplantation.

Defective DNA repair mechanisms are suggested to contribute to senescence and ageing, and we have now shown in an animal model that proofreading deficient Polg results not only in a premature ageing phenotype but also in a severely reduced life span in mouse. Symptoms such as loss of body mass, anaemia, lack of sperm production, osteoporosis and hair loss signal loss of cellular capacity to maintain these processes of normal life through reduced stem cell proliferation. Heart enlargement, with very large, malformed mitochondria can be interpreted as a physiological response mechanism aimed at compensating energy deficiency. In humans, mutated *POLG* results in multiple mtDNA deletions, depletion and point mutations accompanied with symptoms, which resemble age related diseases (especially premature menopause in women and Parkinsonism, Luoma et al., 2004). The mutator mice accumulate not only mtDNA point mutations but also deleted, linear mtDNA molecules. One could hypothesize that finding a way to manipulate POLG to be even more accurate than it naturally is, could result in extended longevity by alleviating or even making us resistant to some ageing related diseases. However, it remains unclear if this is indeed possible. Even if it were, replication errors might any way be a minor contributor to mtDNA mutation accumulation in wild type mice, and hence of little if any significance in ageing and longevity.

9.5.4 One functional copy of POLG is enough

The Polg mutator mouse clearly shows that one functional copy of Polg is enough to maintain the wild type phenotype, at least in the case of this loss-of-function Polg mutation. The heterozygous mutator mice live more or less the same as wild type. The heterozygous mice have normal litter sizes in all combinations with each other or wt mice and show no differences in fertility between genders. The finding that many of the infertile CAG heterozygous human males possess a second *POLG* mutation somewhere else in the gene is in

line with all the above mentioned data; one loss-of-function mutation is not harmful enough to show a disease outcome. In the human mutator cell culture model (Spelbrink et al., 2000), the two wild type POLG copies are probably a contributing factor to the seemingly healthy cell phenotype despite the accumulation of point mutations under the influence of a heavily over expressed POLG mutator. Many of the published *POLG* mutations cause a disease phenotype only in the homozygous state or in combination with a second *POLG* mutation, and most of the dominant PEO mutations are situated in the polymerase domain, where they probably exert a dominant negative effect.

Cellular senescence can be regarded as a disruption of cellular integrity in an (ageing) organism and usually results in age-related pathology. Cellular senescence exhibits antagonistic pleiotropy: it suppresses cancer formation in young organisms but facilitates tumorigenesis in old organisms *via* pre-neoplastic mutations (caused by longer exposal to environmental insults) which disrupt the local tissue integrity (Campisi, 2001). One can argue that the homozygous mutator mice do not live long enough to develop tumours, or that the reduced cell proliferation capacity suppresses any tumour formation.

9.5.5 mtDNA point mutations in sperm

Data from my mtDNA mutation screening in human DNA showed that the *POLG* CAG repeat mutation is at best only a weak mutator, but seems to be a contributing factor in the tissue-specific accumulation of mtDNA mutations in sperm. Sperm seems to be more prone to mtDNA point mutations than blood, and sperm of POLG infertile males had more mtDNA mutations than fertile controls. The mutations in sperm mtDNA accumulated in the non-coding control region, whilst the *cytb* gene showed background levels in each case. As discussed earlier, in the mutator mouse the control region, and especially certain parts of it, seem conversely to be protected and the coding regions, such as of the *cytb* gene accumulate high loads of mutations. In ageing humans most mtDNA point mutations are reported in the control region, as in infertile sperm, suggesting similar mechanisms.

Nuclear mitochondrial pseudogenes (NUMTs) could cause false results in experiments where the mitochondrial genome is amplified from total genomic DNA (Woischnik and Moraes, 2002). The role of NUMTs as a contributing factor to the results in both cases, mouse and human, was ruled out by careful ´reconstruction` experiments. Starting from the same concentrations of ρ^0-cell DNA as in actual mtDNA sequencing experiments, no PCR products were visible after the 18 cycles of amplification routinely used in mtDNA amplification. After 30 cycles the nuclear control reactions were visible, together with an amplified pseudogene of the *cytb* region. The sequence of this product was in both human and mouse different enough from the corresponding mitochondrial *cytb*, respectively, to cause no problems in analysis.

Because of the pattern of the unusual mutations in the mtDNA control region in a subset of clones from POLG infertiles, paternal transmission and possible recombination could not be ruled out in some cases, which require further analysis and were not included in data analysis generating *Fig 12*. Only one case of paternal transmission and subsequent mtDNA recombination has been reported in humans thus far (Schwartz and Wissing, 2002; Kraytsberg et al., 2004). On the other hand, nobody has done a systematic analysis on human sperm. The phenomenon of sperm-specific paternal mtDNA transmission has been documented in mussels (Zouros, 2000). Paternal ´leakage`of mtDNA has also been reported in many inter-specific crosses produced by somatic nuclear transfer, including fruit fly, mouse, monkey, sheep and cow, as well as in human ICSI and IVF polyploid embryos at 2-cell stage, 8-cell stage and blastocyst (Kondo et al., 1990; Sato et al., 2005; St John and Schatten, 2004; Zhao et al., 2004; Steinborn et al., 2002; St John et al., 2002, respectively).

The exclusion of paternal mtDNA is suggested to happen through ubiquitination and proteasome degradation in mammals. In the mussel family of Mytilidae the paternal mtDNA transmission is present in the germ-line of male offspring only (Zouros, 2000). The degradation of male mtDNA in female embryos requires a factor, Z, expressed during oogenesis. This factor Z is a nuclear gene that segregates in populations in two alleles, active Z and inactive z, with a ZZ genotype producing the factor that recognizes another factor W on male mtDNA and brings about its destruction. This kind of factor has not been described in mammals, but its existence and species specificity can be predicted from data showing that species-specific exclusion is stringent. In mouse the oocyte cytoplasm can recognize a nuclear encoded factor on sperm of the same species but not sperm *per se* or products encoded by mtDNA. Furthermore, liver mitochondria from the same individual injected into an oocyte are not excluded, suggesting that sperm mitochondria carry some factor that does not exist on somatic mitochondria (Shitara et al., 2000).

Selection acts in living organisms on many different hierarchical levels. In the case of mtDNA, the selection for or against any particular mtDNA molecule, and the segregation of different mtDNA molecules can happen (i) at the level of nucleoids by exchanging material, (ii) from one mitochondrion to another by fusion and fission, (iii) in cell division, (iv) into and inside different tissues during cell migration and differentiation in development. In mammals the germ cells arise, multiply and start differentiation outside the embryo proper on the endodermal layer of the yolk sac. In a four-week old embryo these primordial germ cells are distinguishable and start migration to the posterior body wall, eventually in order to induce the formation of gonads. Any sperm mtDNA acquired during fertilization and not destroyed, could thus end up in various tissues depending on its location during cleavage.

The finding that male homozygous mutator Polg mice are practically infertile and do not have any sperm cells visible in testis at the age of 25 weeks does suggest either that defective proliferation or increased apoptosis is involved. Apoptosis could also play a role in a milder *POLG* genotype (the CAG

homozygous mutant males) resulting in a less severe phenotype, namely oligozoospermia.

The wild type human polymerase gamma however has high fidelity, with a mutation rate of one error in ~3 x 10^5 base pairs. This proof-reading efficiency is severely affected by mutations in the POLG protein, resulting in a 10-100 times higher rate of inserting mutations in the mtDNA (Longley et al., 2005; Kaguni, 2004; this study). The consequences may be visible either only in certain tissues, as in PEO patients, or in the whole organism like the ageing mouse. Some of the mtDNA mutations created by POLG are obviously tolerated because they can be found in seemingly healthy people. Numerous others exhibit a disease phenotype and many are probably diluted out because they are not tolerated. Some of the mutated molecules may also not be replicated efficiently and are thus diluted out at the cellular level. Others may be developmentally lethal, or they are preserved only in tissues where they do not threaten life.

The testis is a highly differentiated tissue, and the demands for proper sperm development are both ´chemical`, *i.e.* the hormonal status, and physical, like the ~2°C lower tissue temperature than in other parts of the body. In addition, environmental factors have an influence on sperm quality and production (De Rosa et al., 2003; Waring and Harris, 2005). Deletions and depletion of mtDNA in sperm have been described and suggested to result in reduced fertility (Kao et al., 1998; Reynier et al., 1998). Holyoake et al., (1999) reported a T8821C mutation in 7 % of immature spermatids but not in mature spermatozoa of one oligoasthenoteratozoospermic male, suggesting that mutated sperm do not develop properly and are eliminated, probably by apoptosis. Although the mutation was not found in mature sperm, the mature spermatozoa had abnormal head morphology indicating, that other factors are also involved in the defective spermatogenesis. One of those factors could be compromised Sertoli cell function because of a *POLG* mutation, resulting in mtDNA mutations and defective mitochondrial function.

9.6 Is there a mitocheckpoint?

From everything discussed in this thesis it is evident that the mitochondrion is a key player in all physiological functions. An intact and fully functional polymerase gamma is essential for maintaining its genome, the mtDNA. Almost anything disturbing the integrity of these key elements is reflected negatively in the organism's well-being.

The eukaryotic cell duplicates through defined phases of DNA replication and cell division. This cell cycle is highly regulated with reversible check points where the process is stopped for corrections if something goes wrong. Cells can, either resume normal cell cycle after corrections, withdraw into senescence, undergo apoptosis or (rarely) escape growth control and transform into malignancy. Mitochondria control energy levels needed in all cell functions, are

key players in apoptosis and have their own genome, mutations in which often result in altered cell phenotype. This raises the question of whether there could be some sort of mitocheckpoint, which would monitor the functional state of mitochondria and interact with the nuclear genome by sending signals to adjust the cell cycle and gene expression accordingly, as suggested by Singh et al., (2005).

This mitocheckpoint or mitochondrial damage checkpoint would help cells to repair damaged mitochondria and restore their function or send apoptotic signals to condemn the cells to destruction. If there was a severe and persistent damage like extensive mtDNA mutation, the checkpoint would fail and cells would either not enter apoptosis or may undergo apoptosis as a default outcome. If apoptosis was suppressed, the result could be a nuclear mutator phenotype with chromosomal aneuploidy, loss of heterozygosity and extensive cell survival with nuclear-mitochondrial cross-talk, which is suggested to play an important role in tumorigenesis (Singh et al., 2005). The nuclear transcription factor and tumour suppressor p53 has been shown to translocate to mitochondria, physically interact with mtTFA, have a role in mitochondrial base excision repair (BER) and have a putative binding site on mtDNA, even though no p53-mtDNA complexes have yet been identified in pull-down assays (Yoshida et al., 2003; Heyne et al., 2004; de Souza-Pinto et al., 2004). Alternatively, the cells could fall into replicative senescence, which would show up as *e.g.* loss of sperm, anaemia and impaired wound healing. Mutator mice do seem to have elevated apoptosis but the ageing phenotypes are probably not due to accelerated senescence (Kujoth et al., 2005).

Pre-apoptotic chromatin condensation is reported to happen before mitochondrial membrane permeabilization (MMP) and caspase activation (Andreau et al., 2004). In foul states of both the innate and adaptive immune responses the potentially autoreactive B and T cells are eliminated by apoptosis, an energy-demanding process. An array of activating signals through the T-cell receptor (TCR) results in mitochondrial hyperpolarization (MHP), associated with transient inhibition of F_0F_1-ATP synthase, ATP depletion and sensitization to necrosis (Leist et al., 1997). MHP would thus function as a checkpoint of T-cell faith.

Do the data reported in this thesis support the idea of the mitocheckpoint or the role of ROS in cellular well being and ageing? The role of mitochondria is clear in regard to energy production and apoptosis. Defects in the mitochondrial genome lead to defects in energy production which in turn is reflected in problems in a plethora of cellular functions. The mitochondrial genome is dependent on the nuclear genome for its function, and there must be some sort of reciprocal signalling to fulfil the needs of mitochondrial protein production by nuclear genes. The suggested role of ROS in mitochondrial mutations and dysfunction, and thereby in ageing, is nevertheless contradicted rather than supported by my results. However, the role of DNA polymerase gamma and thereby of intact mitochondrial DNA in maintaining the homeostasis of cellular functions was conversely strengthened. In that sense, the mitochondria do seem

to constitute an important check point determining the well being of cells and thereby the whole organism, even though the exact mechanisms appear differ from those proposed by Singh et al., (2005).

9.7 Clinical recommendations

The *POLG* mutation is easy to detect from a blood sample or hair roots at any age. Couples with a male specific *POLG* mutation can easily be helped by ART, such as ICSI (Jensen et al., 2004). A simple PCR based analysis would give information about the cause of the problem and save the couples from years of trials and frustration, as well as from emotional and other problems. One ethical question remains however: are we willing to pass this problem to the next generation? Reproductive defects are an ever-increasing problem in the developed countries.

Sperm concentrations have decreased by 1.24 %/year in birth cohorts and are 22 x 10^6 /ml less in males born 1976-79 than males born from 1937-49 (Andersen et al., 2000). There are also regional differences in semen quality in Europe (Jørgensen et al., 2001), with the highest sperm counts and lowest testicular cancer frequency in Turku/Finland compared to Copenhagen/Denmark, Edinburgh/Scotland or Paris/France. This may be a reflection of a cleaner environment in Finland compared to Central Europe. The negative effects of traffic pollutants on semen quality and fertility have been demonstrated in the Spanish population (De Rosa et al., 2003).

Environmental factors as well as the age of the male can have co-operative and cumulative effects on infertility with gene defects like *POLG* mutation over a period of years. The artificial reproductive techniques frequently used today will mean that reproductive fitness is no longer naturally selected in those cases. On the other hand, if young males were tested and found mutant for *POLG*, they should be offered a possibility to preserve their sperm for possible future use in public sperm banks.

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Kangasala, June 2006

Anja Rovio

11. APPENDIX

11.1 Semen analysis protocol

Semen samples were analysed by collaborators at the clinics where the samples were collected under the guidelines of the World Health Organization (WHO 1999). A short overview of the analysis is described here (based on NAFA 2002).

Table7*. Normal and pathological ranges in semen analysis, WHO 1999.*

Semen characteristics	Normal	Borderline	Pathological
Volume (ml)	2.0-6.0	1.5-2.0	<1.5
Sperm concentration (M/ml)	20-250	10-20	<10
Total sperm count (M/ejaculate)	>80	20-80	<20
Motility a and b (%, 0.5-2h after ejaculation)	>50	35-49	<35
Morphology (/100sperm)	≤30		
Head defects	<35	35-59	>60
Middle piece defects	≤20	21-25	>25
Tail defects	≤20	21-25	>25

A period of abstinence of 2-7 days is recommended. The volume of the ejaculate, obtained with masturbation, is measured and the sample is put on an orbital shaker for 20-25 minutes, after which the analysis begins. Liquefaction, visual appearance and smell are documented, as well as semen viscosity. A wet microscopic preparation is examined for sperm aggregation/agglutination and for other cells and debris. Sperm cell concentration is determined at appropriate dilutions, and sperm counts are performed microscopically. Sperm motility assessment is done using 20 μm thick undiluted wet preparations and classifying at least 200 spermatozoa in duplicate into four categories: a; rapid progressive, b; slow progressive, c; non progressive and d; immotile. Morphology is analysed from fixed, Papanicolaou-stained and mounted smears. The morphology seen in the microscope is not the true morphology of a living sperm but the best image that can be created. Other tests include e.g. assessment of vitality, antibody tests and several biochemical analyses.

11.2 GenBank submissions

The following sequences were discovered and submitted to the GenBank during this PhD project.

AY377897	*Homo sapiens* POLG-4R
AY376474	*Pan paniscus* POLG-H
AY376475	*Pan paniscus* POLG-I
AY376476	*Pan paniscus* POLG-K
AY376477	*Pan paniscus* POLG-L
AY376478	*Gorilla gorilla* POLG-B
AY376479	*Gorilla gorilla* POLG-C
AY376480	*Pan troglodytes* POLG-U
AY376481	*Pan troglodytes* POLG-V
AY376482	*Pan troglodytes* POLG-Y
AY376483	*Pan troglodytes* POLG-Z
AY376484	*Pongo pygmaeus* POLG-P
AY376485	*Pongo pygmaeus* POLG-Q
AY376486	*Pongo pygmaeus* POLG-R
AY376487	*Pongo pygmaeus* POLG-S
AY376488	*Pongo pygmaeus* POLG-T
AY376489	*Macaca mulatta* POLG
AY376490	*Macaca arctoides* POLG
AY376491	*Papio hamadryas* POLG
AY376492	*Cercopthecus aethiops* POLG
AY376493	*Cercopithecus nictitans* POLG
AY376494	*Callithrix jacchus* POLG
AY376495	*Saguinus oedipus* POLG
AY376496	*Cebus apella* POLG
AF415155	*Gorilla gorilla* POLG-A
AF415156	*Pan troglodytes* POLG-X
AF415157	*Pan troglodytes* POL-W

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ARTICLE

Analysis of the trinucleotide CAG repeat from the human mitochondrial DNA polymerase gene in healthy and diseased individuals

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The human nuclear gene (*POLG*) for the catalytic subunit of mitochondrial DNA polymerase (DNA polymerase γ) contains a trinucleotide CAG microsatellite repeat within the coding sequence. We have investigated the frequency of different repeat-length alleles in populations of diseased and healthy individuals. The predominant allele of 10 CAG repeats was found at a very similar frequency (approximately 88%) in both Finnish and ethnically mixed population samples, with homozygosity close to the equilibrium prediction. Other alleles of between 5 and 13 repeat units were detected, but no larger, expanded alleles were found. A series of 51 British myotonic dystrophy patients showed no significant variation from controls, indicating an absence of generalised CAG repeat instability. Patients with a variety of molecular lesions in mtDNA, including sporadic, clonal deletions, maternally inherited point mutations, autosomally transmitted mtDNA depletion and autosomal dominant multiple deletions showed no differences in *POLG* trinucleotide repeat-length distribution from controls. These findings rule out *POLG* repeat expansion as a common pathogenic mechanism in disorders characterised by mitochondrial genome instability.

Keywords: mitochondrial DNA; mitochondrial myopathy; microsatellite; trinucleotide repeat; polyglutamine tract; DNA polymerase; deletions; progressive external ophthalmoplegia; myotonic dystrophy

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Introduction

Mitochondrial DNA (mtDNA) mutations are associated with a wide spectrum of human diseases[1,2] and exhibit a variety of different types of inheritance. Clonal deletions or partial duplications of mtDNA typically manifest sporadically, whereas point mutations are usually maternally inherited. Two other kinds of mtDNA lesion appear to depend upon nuclear rather than mitochondrial genotype: mtDNA depletion[3–5] and multiple mtDNA deletions with autosomal dominant or recessive inheritance.[6–9] Autosomal dominant progressive external ophthalmoplegia (ad-PEO) associated with multiple deletions of mtDNA is genetically heterogeneous, with mapped loci on chromosomes 3p14.1–21.2 and 10q24, and other affected families unlinked to either of these.[7,8]

One candidate gene for involvement in mtDNA disorders showing nuclear inheritance is *POLG*, encoding the catalytic subunit of mitochondrial DNA polymerase (DNA polymerase γ). The gene has been mapped to the region of chromosome 15q24–15q26,[10–12] and exhibits an unusual feature not shared with its orthologues in mouse, *Drosophila* or yeast, namely the presence of a trinucleotide (CAG) microsatellite repeat within the N-terminal region of the coding sequence.[11,13] The sequenced gene (Figure 1) contains 10 consecutive, glutamine-encoding CAG codons, followed by a single CAA and two further CAGs. Instability at trinucleotide repeats is associated with various human disorders, including Huntington's disease (HD), myotonic dystrophy (DM), and several forms of spinocerebellar atrophy (eg SCA1). For review see La Spada[14] and Koshy *et al*.[15] Such disorders may be conveniently sub-classified according to where the repeat is found in relation to the coding sequence of the affected gene. Where the repeat is found in coding DNA, as here, such disorders are usually dominant, reflecting a gain of function associated with expanded repeat number. Expansions to 35 or more repeats are usually sufficient to create a pathological phenotype in such cases, eg in HD (see Wellington *et al*[16] for review). Where the repeat is found outside coding DNA (eg in Friedreich ataxia), inheritance is usually recessive, reflecting loss of function, and repeat expansions can be, and usually are, much larger. Different repeat-length alleles of a single gene can be associated with distinct pathological phenotypes, for example in the case of the CAG repeat in the androgen receptor gene, where short 'normal' alleles are associated with enhanced predisposition to prostate cancer,[17] long 'normal' alleles with androgen insensitivity, and expanded alleles ($\geq$ 40 repeats) with Kennedy's disease. Dominant disorders characterised by repeat expansion also exhibit the phenomenon of anticipation: the progressive increase in repeat length and in disease severity from generation to generation.

Expansion of the *POLG* CAG repeat would be a plausible disease mechanism in disorders characterised by mtDNA instability inherited as an autosomal trait, since expansion of the polyglutamine tract might impair the function of the mitochondrial DNA polymerase in a dominant-negative manner. Interestingly, anticipation has been reported in one family with ad-PEO unlinked to the mapped ad-PEO loci on chromosomes 3p or 10q.[18] A modest polyglutamine expansion might also be associated with apparently sporadic cases of mtDNA rearrangement, or might even promote the mutational events that manifest subsequently as diseases associated with inherited point mutations of mtDNA. In addition, dysfunction of the mitochondrial DNA polymerase not associated with repeat size might be of pathological significance. Repeat-length variation in the *POLG* gene might therefore provide a useful linkage marker for evaluating the involvement of the gene in diverse human disorders.

In order to investigate the possible role of the *POLG* gene in human disease, we have studied repeat-length variation at the locus, both in healthy populations and in various groups of patients. Our aim was to look both for gross expansions associated with disease, as well as to examine possibly more subtle relationships between *POLG* repeat-length and pathological phenotype. The results indicate that *POLG* repeat expansion cannot be a frequent cause of mitochondrial disease. Diversity at the *POLG* locus is also lower than optimal for use generally as a linkage marker, but sufficient that the gene can be excluded in specific cases.

Results and Discussion

Nested primers flanking the *POLG* CAG repeat were designed, based on a region of the coding sequence[11,13] contained within a single exon (see Figure 1). The primers amplified the predicted fragments from genomic DNA (Figure 1), as verified by direct sequencing. Testing against the monochromosomal inter-species hybrid and Genebridge 4 radiation hybrid panels[19] confirmed that they detected only the expected gene sequence from chromosome 15q, and not a pseudogene

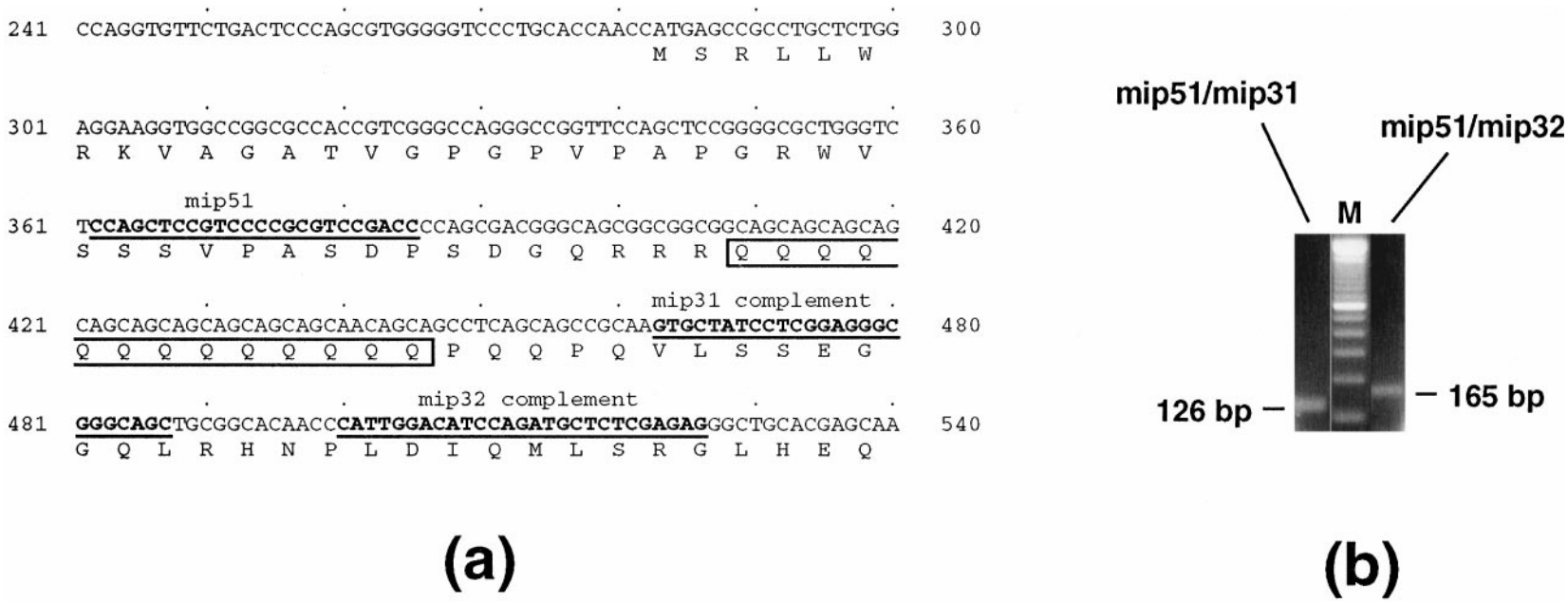

Figure 1 *(a) Partial sequence of the human gene for the catalytic subunit of the mitochondrial DNA polymerase (POLG), from Genbank data entry MIHSDNAPL (accession number X98093), numbered as in the database. The N-terminus of the coding region is shown in the one-letter amino acid code, with the polyglutamine tract boxed. The sequences corresponding to the PCR primers used here (or their complement) are bold, underlined. (b) 2.5% agarose gel of PCR products made using primer pairs mip51/mip31 and mip51/mip32, on human genomic DNA template.*

(data not shown). The 5' primer (mip51) was also tested with other, more downstream 3' primers from the same exon of the *POLG* gene, successfully and routinely amplifying genomic DNA fragments up to 0.6 kb in size (data not shown). This indicates that even grossly expanded CAG repeats (eg of the order of 150) would be detectable.

POLG trinucleotide repeat-length was then evaluated in two groups of healthy controls, plus a number of patient groups, as shown in Table 1, using a fluorescence-based PCR method.[20] Genotype was also confirmed in many cases by direct sequencing of PCR products or by cloning. The previously reported variant of 10 repeats[11,13] was found much more frequently than any other allele. In an ethnically diverse sample of 73 unrelated, healthy persons, it was found at a frequency of 88%. In a sample of 61 unrelated, healthy Finns, a genetically isolated and bottlenecked population that has been much exploited in disease linkage studies, the frequency of the common allele was almost the same (89%). Several groups of patients were also studied, including a series of 51 unrelated DM patients from the UK, and a group of 12 unrelated Chinese patients with acute aminoglycoside ototoxicity (AAO), one of whom had the np 1555 mtDNA mutation. The frequency of the common (10-repeat) allele was not significantly different (z-test, $P > 0.05$) in any group studied, nor was any evidence found for an association between a specific, unusual allele and any of the patient groups investigated. Amongst DM patients, there was furthermore no correlation between disease severity and *POLG* genotype. The overall frequency of the 10-repeat allele in 214 ethnically diverse, unrelated individuals included in the study was 88%. The next commonest allele was of 11 repeats (7%), with other, rarer alleles ranging from 5 to 13 repeats comprising the remainder. Observed homozygosity values for the common allele are close to equilibrium predictions.

No expanded alleles were observed in healthy controls or in any of the patient groups studied, including the 51 DM subjects. This rules out *POLG* repeat expansion as a contributory factor in DM, and is consistent with repeat instability being locus-specific in this disorder. Expanded alleles were also absent from diverse patients with mtDNA disorders, as summarised in Table 2. These results do not exclude the possibility of very large expansions undetectable by conventional PCR, but coding region expansions of the type found in other disorders (i.e. up to several hundred copies) would have been easily observed. Expansions in the CAG repeat of the DMPK gene of over 500 repeat units were easily detected in DM patient DNAs, using primers that amplify a similarly sized (165 bp) fragment spanning the repeat, and otherwise identical materials and methods (data not shown). Most patients we studied were homozygous for the common *POLG* allele, (except where stated in Table 2), but heterozygotes were found amongst several patient groups, in which cases it is possible to exclude even very large expansions undetectable by PCR.

Table 1 Allele frequencies[a] at the POLG trinucleotide repeat in different populations

Allele (no. of repeats)	*Healthy controls[b] (non-Finns)*	*Healthy controls[b] (Finns)*	*Healthy controls (all)*	*DM patients (UK)*	*AAO patients[c] (China)*	*Total[d]*
5	0	0	0	0.01	0	<0.01
7	0	0	0	0	0.04	<0.01
8	0	0	0	0.01	0	<0.01
9	0.01	0.02	0.01	0.01	0.04	0.01
10	0.88	0.89	0.89	0.80	0.92	0.88
11	0.09	0.06	0.07	0.15	0	0.07
12	0.04	0.02	0.03	0.02	0	0.02
13	0	0.01	<0.01	0	0	<0.01
No. of persons	73	61	134	51	12	214
10/10 homozygotes	57 (78%)	48 (79%)	105 (78%)	33 (65%)	10 (83%)	164 (77%)

[a]All allele frequencies quoted to 2 decimal places, except where rounding would give 0.00, which is quoted as <0.01. Therefore, not all columns total 1.00.
[b]Healthy controls had no hearing impairment and reported no manifestations of systemic disease.
[c]Only 12 Chinese patients with acute aminoglycoside ototoxicity (AAO) were included, hence the reported allele frequencies of 0.04 represent single alleles in two heterozygotes.
[d]The final column includes 17 individuals not listed under any other category. Hence the total of 214 persons is greater than the sum of controls, UK DM patients and chinese AAO patients. The additional persons were one hearing-impaired individual, plus 16 English patients with various mitochondrial and non-mitochondrial disorders.

Table 2 Exclusion of *POLG* CAG repeat expansion in cases of mtDNA disorders

Clinical phenotype	*Molecular phenotype*	*Literature reference*	*No. of patients studied*	*Comment*
ad-PEO	multiple mtDNA deletions	8	3	no linkage to ad-PEO loci on chromosomes 3p or 10q
ad-PEO	multiple mtDNA deletions	7	2	Finnish family linked to chromosome 10q24
ad-PEO with anticipation	multiple mtDNA deletions	18	2	Swedish family, no linkage to ad-PEO loci on chromosomes 3p or 10q
PEO	single mtDNA macrodeletion	24	10	sporadic cases
infantile mt myopathy with COX deficiency	mtDNA depletion	25, 26	3, plus unaffected relatives	mtDNA depletion verified in muscle and/or liver: one Italian, two English patients
infantile mt myopathy with COX deficiency	suspected mtDNA depletion	26	3	English patients described in Holt *et al*[24]
fatal neonatal hepatic failure	mtDNA depletion in liver	5	2, plus unaffected relatives	consanguineous family described in Bakker *et al*,[5] heterozygous
MELAS	np 3243 point mutation	27, 28	2	plus 36 unaffected maternal relatives from Finnish and English pedigrees with the mutation
MERRF	np 8344 point mutation	29	2	
Non-syndromic deafness	np 1555 mutation	30	16	Hispanic pedigrees with the mutation; total includes 8 unaffected maternal relatives; 7 heterozygotes
NARP	np 8993	31	1	from UK

Genotyping of the *POLG* repeat furthermore enabled us to exclude the locus from further consideration in the case of the family with hepatic mtDNA depletion, assuming autosomal recessive inheritance.[21] Linkage studies have also excluded the *POLG* region of chromosome 15q from involvement in ad-PEO in Finnish, Italian and Swedish families in which it is unlinked to the previously mapped loci on chromosomes 3 or 10 (A Suomalainen 1998, unpublished data).

The amount of heterozygosity at the *POLG* repeat is probably too low to enable it to be employed as a useful marker in disease linkage studies generally, although it will have utility in specific cases. The fact that a similar pattern of variation is evident in widely scattered populations suggests the possibility of selection, acting to drive the repeat length down (or up) to the modal value of ten CAG repeats. However, all other alleles found amongst patients were also found in controls, hence there is no evidence for association of particular repeat-length variants with any given pathological phenotype.

Methods

DNA Samples

DNA was obtained from blood or muscle biopsy of healthy controls and patients, as described previously,[22] but with proteinase K inactivated by incubation at 92°C for 10 min. All the samples were taken with informed consent. DNA from Chinese patients with acute aminoglycoside ototoxicity was kindly donated by Dr W-Q Qiu (Tiedao Medical College, Shanghai, China), from UK myotonic dystrophy patients by Dr Helen Harley (University of Wales, Cardiff), from Spanish subjects with the np 1555 mtDNA mutation by Dr Ignacio del Castillo (Hospital Ramón y Cajal, Madrid, Spain), and from members of Finnish pedigrees carrying the

np 3243 mtDNA mutation by Dr Kari Majamaa (University of Oulu, Finland). DNA samples for the Genebridge 4 radiation hybrid panel[19] and the monochromosomal human-rodent hybrid panel were supplied by the UK MRC HGMP Resource Centre, Cambridge.

Oligonucleotide primers

Nested oligonucleotides corresponding to regions of the N-terminal coding sequence of *POLG*, as indicated in Figure 1, were purchased from Life Technologies (Paisley, Scotland) or DNA Technology (Aarhus, Denmark). For fluorescent PCR[20] one of the primers (mip51) was 5' pre-labelled with ROX (Perkin-Elmer dye) by the manufacturer.

PCR

Unlabelled PCR reactions used primer pairs mip51/mip31 (126 bp product from reference sequence) or mip51/mip32 (165 bp product) at 0.8 μM each, 200 μM dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden), plus 0.25 units of Dynazyme thermostable DNA polymerase (Finnzymes, Espoo, Finland) in the manufacturer's buffer. Reactions comprised 30 cycles of denaturation for 1 min at 95°C, annealing for 45 s at 62°C, and extension for 2 min at 72°C (5 min extension in final cycle). Products were analysed by agarose or polyacrylamide gel electrophoresis. For fluorescent PCR, 12.5 μl reactions contained 0.2 μM each primer, 200 μM dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.5 μl of DNA template[22] and 0.16 units of Dynazyme thermostable DNA polymerase (Finnzymes, Espoo, Finland) in the manufacturer's buffer, and used the same cycle conditions, except the extension step which was for 1 min only. Reaction products were diluted 1:10 in water, and samples containing 1 μl of diluted PCR product, 12 μl of deionised formanide and 0.15 μl of Tamra Genescan-350 DNA size standards (Perkin-Elmer, Norwalk, CT, USA) were analysed on the ABI 310 Genetic Analyzer (Perkin-Elmer, Norwalk, CT, USA), using the manufacturer's data collection and Genescan analysis software.[20,23]

Cloning and cycle sequencing

Unlabelled PCR products were purified on QIAquick spin columns (Qiagen). Cloning used the TA-cloning kit (Invitrogen), and insert-containing plasmids were identified by miniprepping and *Eco*RI digestion. Cycle sequencing employing the ABI 310 Genetic Analyzer used the Terminator Ready Reaction Kit (Perkin-Elmer), together with unlabelled mip51 primer (direct sequencing of PCR products) or M13 reverse primer (plasmid sequencing).

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Mutations at the mitochondrial DNA polymerase (*POLG*) locus associated with male infertility

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Human mitochondrial DNA polymerase, encoded by *POLG*, contains a polyglutamine tract encoded by a CAG microsatellite repeat[1,2]. Analysis of *POLG* genotypes in different populations identified an association between absence of the common, ten-repeat allele and male infertility typified by a range of sperm quality defects but excluding azoospermia.

Previous studies[3] indicated that the common *POLG* allele is found in different ethnic groups at a uniformly high frequency (0.88) and is absent in only approximately 1% of individuals. This indicates that the common allele may be maintained by selection. We used fluorescent PCR with custom primers[3] to genotype infertile and control individuals for *POLG* CAG-repeat length (see Web Notes for methods). Using sperm DNA from individuals in whom azoospermia was excluded, we found 9 of 99 infertile males (9%) from Finland (6/59) or England (3/40) to be homozygous for the absence of the common allele (Fig. 1*a* and Web Table A). In contrast, the common allele was present in sperm DNA from all 98 fertile males studied, as well as in all but 6 of 522 healthy controls whose blood DNA was analyzed in parallel (see Web Table A).

Based on standard Hardy–Weinberg predictions (see Web Table A), the 'homozygous mutant' genotype (absence of the common allele, whether or not this reflected homozygosity for a particular mutant allele) should be found in approximately 1.7% of individuals. We found it at a frequency slightly below expectation (1.2%) in the general population, although this deviation is not statistically significant. In contrast, our finding that the 'homozygous mutant' genotype occurs in 9/99 infertile but 0/98 fertile males is highly significant, based on two different statistical tests (see Web Notes). We also found a higher frequency of heterozygosity in infertile men (35%) than in fertile men (18%) or in the general population (23%): some infertile men may be compound heterozygotes, with a second mutation elsewhere in the gene. Clearly, further study of the entire *POLG* coding region in these individuals is warranted.

We found many different repeat-length alleles, singly and in combination, in individuals lacking the common allele, with a frequency distribution otherwise similar to that in the general population (Fig. 1*b*). This indicates that it is the absence of the common allele, rather than the presence of a particular alternate allele, that is associated with infertility.

To establish the type of infertility associated with *POLG*, we studied blood DNA (Table 1) collected from three groups of infertile men in Scotland, Ireland and Germany, as well as sperm DNA collected from men in Taiwan and Australia for whom extensive phenotypic data, including clinical profiles and sperm analysis, were available. Varying criteria had been applied in their selection (see Web Notes).

The main conclusions that emerge are the following:

(i) The *POLG* mutant genotype (absence of the common *POLG* allele) is found at an elevated frequency, specifically in infertile men with moderate oligozoospermia, in all populations studied.

(ii) Infertile males homozygous for the *POLG* mutant genotype are below the commonly accepted thresholds for at least two sperm quality parameters among the following three: sperm number ($<20\times0^6$ sperm/ml), motility (<50% motile) or morphology (<10% of spermatozoa morphologically normal according to strict criteria).

(iii) The *POLG* genotype in blood and sperm is similar in each individual, excluding any effect of *de novo* tissue-specific mutation.

(iv) Homozygosity with respect to the *POLG* mutant genotype is not associated with azoospermia or severe oligozoospermia, based on three different populations studied (Scottish, Irish and German). The overall frequency of mutant homozygotes in this group (3/318, or 0.9%) is similar to that in the general population.

(v) *POLG* mutations are also not associated with isolated asthenozoospermia, at least in the Chinese population in Taiwan from which a small set of such individuals were obtained.

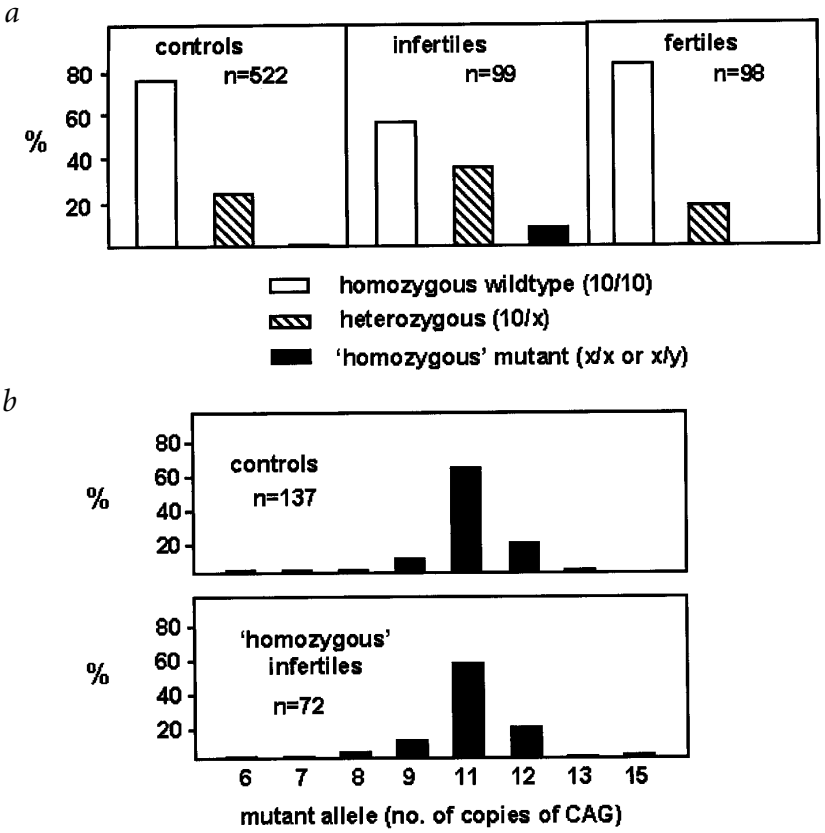

Fig. 1 *POLG* genotype in infertile and fertile males, plus control individuals (see Web Table A for details). ***a***, Genotype frequencies in the three groups: 10/10, homozygous for wildtype allele (allele with 10 CAG repeats); 10/x, heterozygous for wildtype and one mutant allele; x/x, homozygous for one mutant allele; x/y, 'homozygous' as to mutant status but bearing two different mutant alleles. ***b***, Frequencies of different mutant alleles (alleles with other than 10 CAG repeats) in unselected controls (top panel, 125 heterozygotes plus 6 homozygotes) and in infertile men lacking the common allele (bottom panel, all clinical sources combined).

Table 1 • *POLG* genotype in different categories of infertile males

Population	Phenotype	Total	Homozygous mutants[a]	% (1 d.p.[f])
Finnish & English[b]	excluding azoospermia and extreme oligozoospermia	99	9	9.1
Scottish & German[c]	azoospermia or severe oligozoospermia (<5×10^6 sperm/ml)[d]	135	0	0
Scottish & German[c]	moderate oligozoospermia (>5×10^6 sperm/ml) with or without other defects	113	8	7.1
Chinese[b]	asthenozoospermia	56	0	0
Australian[e]	combined oligo- and asthenozoospermia	13	1	7.7
Australian	unspecified (testicular biopsies)	17	2	11.8
Irish[c]	<5×10^6 sperm/ml	183	3	1.6
Irish[c]	>5×10^6 sperm/ml or no data	287	10	3.5

[a]Individuals lacking the common, ten-repeat allele. [b]Sperm DNA. Combined data from Web Table A. [c]Blood DNA. Data on Scottish and German patients are combined, but data on Irish patients are presented separately because information on other causes of infertility was not available on a case-by-case basis. The proportion of *POLG* mutants detected in this group is thus about half that seen in other, comparable groups (see Web Notes). [d]Includes 62 men with azoospermia plus 73 with severe oligozoospermia (<5×10^6 sperm/ml). [e]Sperm DNA. Excludes one additional mutant homozygote with a sperm count in the low normal range. [f]Decimal place.

Our findings indicate that variant *POLG* alleles are somehow deleterious to sperm function or differentiation but have no obvious, phenotypic effects in other tissues. Spermatozoa are heavily dependent on respiratory energy for motility, and impaired energy metabolism has long been hypothesized to contribute to infertility[4]. Mitochondrial defects[5], sometimes associated with mtDNA lesions[5–7], have been reported in sperm samples from infertile males. In addition, sperm motility[8] and other parameters of sperm quality[9] appear to be correlated with mitochondrial respiratory activity and membrane potential[10]. Recently, an association was reported between mtDNA haplogroup and asthenozoospermia[11].

The POLG polyglutamine tract, located within the amnio-terminal portion of the mature polypeptide[12], is absent from the ortholog in other metazoans and yeast[2]. Its deletion has no detectable effect on enzymatic activity in cultured HEK293T cells[12]. Polyglutamine tracts are commonly regarded as interfaces for protein–protein interactions; thus, a sperm-specific protein could interact with this region of POLG. Given the many rounds of cell division during spermatogenesis and the functional necessity of mtDNA for sperm function, it seems plausible that a suboptimal mtDNA polymerase could result in the accumulation of mtDNA mutations and in failure to complete differentiation. Changes in polyglutamine tract length in another gene product, the androgen receptor, also affect male fertility[13].

Male infertility is genetically heterogeneous[14,15]. Our findings indicate that, at least within European populations and when there is no other obvious etiology, *POLG* contributes to at least 5–10% of cases. If the prediction is substantiated, early detection via screening would potentially allow appropriate counseling or assisted reproduction to a significant, 'at risk' population. Genes for other components of the mtDNA maintenance machinery should also be regarded as candidates for involvement in male infertility.

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EIAV–LacZ control ($n = 7$). Each mouse was injected with a total dosage of 90 μl of viral solution. Six sites per hindlimb muscle were injected with 5 μl per site.

Behavioural analysis

We analysed a rotarod task of the SOD1 mice by an Economex Rotarod instrument (Colombus Instruments) every ten days during the light phase of the 12 h light/12 h dark cycle. We performed three trials, and recorded the longest duration on the rod for every mouse. The timer is stopped when the mice fall from the rod or after an arbitrary limit of 180 s. Footprint analysis was also performed. Mouse hind paws were covered with ink to record walking patterns during continuous locomotion, and stride length was measured.

Histology and immunohistochemistry

Animals were perfused transcardially with 0.9% NaCl solution followed by ice-cold 4% paraformaldehyde. Spinal cord, brain and muscle tissues were dissected out and post-fixed overnight in the same solution and then transferred to 30% sucrose. Tissues were analysed by immunohistochemistry and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) reaction. Spinal cord and brain were cut on a sliding cryostat microtome (Leica) at a thickness of 20 μm and collected as free-floating sections in PBS containing sodium azide. Primary antibodies were used as follows: rabbit anti-β-gal (1:1,000, Europe BioProducts); rabbit anti-CGRP (1:3,000, Sigma); mouse anti-ChAT (1:1,000, gift from B. K. Hartman and C. Cozari); CD3-T cells (1:50, Dako); P7/7-MHCII (1:100, Dako); goat anti-Glut-1 (1:20, Santa Cruz Biotechnology); and rabbit anti-albumin (1:250, ICN/Cappel).

VEGF ELISA

Dog osteosarcoma D17 cells were transduced in the presence of 8 mg ml^{-1} polybrene as described previously[24]. Cells were transduced with either EIAV–VEGF or EIAV–LacZ vectors. Transduced cells were passaged three times before analysis of transgene expression. One week post-transduction supernatants were collected and the VEGF levels measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). The protein amino-acid sequences of dog VEGF are highly homologous to the human VEGF protein used in this kit, which has been used previously to determine dog VEGF levels[27]. To determine plasma VEGF levels, we collected blood in 10-ml vacuum tubes containing 100 μl of a 4% tri-sodium citrate solution, quickly centrifuged it and stored plasma fractions at −80 °C until analysis. VEGF ELISA assay measurements were also carried out using tissue samples from spinal cord and brain stem.

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Premature ageing in mice expressing defective mitochondrial DNA polymerase

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Point mutations and deletions of mitochondrial DNA (mtDNA) accumulate in a variety of tissues during ageing in humans[1], monkeys[2] and rodents[3]. These mutations are unevenly distributed and can accumulate clonally in certain cells, causing a mosaic pattern of respiratory chain deficiency in tissues such as heart[4], skeletal muscle[5] and brain[6]. In terms of the ageing process, their possible causative effects have been intensely debated because of their low abundance and purely correlative connection with ageing[7,8]. We have now addressed this question experimentally by creating homozygous knock-in mice that express a proof-reading-deficient version of PolgA, the nucleus-encoded catalytic subunit of mtDNA polymerase. Here we show that the knock-in mice develop an mtDNA mutator phenotype with a threefold to fivefold increase in the levels of point mutations, as well as increased amounts of deleted mtDNA. This increase in somatic mtDNA mutations is associated with reduced lifespan and premature onset of ageing-related phenotypes such as weight loss, reduced subcutaneous fat, alopecia (hair loss), kyphosis

(curvature of the spine), osteoporosis, anaemia, reduced fertility and heart enlargement. Our results thus provide a causative link between mtDNA mutations and ageing phenotypes in mammals.

The mtDNA polymerases of yeast and animal cells have an inherent 3′-5′ exonuclease activity necessary for proof-reading newly synthesized mtDNA[9–11]. The exonuclease activity of the *Saccharomyces cerevisiae* mtDNA polymerase, Mip1p, is critically dependent on specific, conserved aspartate residues located in three 'exonuclease' domains[10,11]. We created knock-in mice (Fig. 1a–d) expressing mtDNA polymerase with an altered catalytic PolgA subunit that contained an alanine instead of the critical aspartate residue of the second exonuclease domain (D257A). We first generated the *PolgA*^{mutNeo} allele (Fig. 1a) in embryonic stem cells (Fig. 1b) and obtained germline transmission of this allele (Fig. 1c, d). Next, we excised the *Frt*-site-flanked neomycin-resistance gene by mating heterozygous *PolgA*^{mutNeo} mice with a line ubiquitously expressing *Flp*-recombinase[12], thus generating heterozygous *PolgA*^{mut} (+/*PolgA*^{mut}) mice (Fig. 1a, c, d). We chose to perform this *in vivo* excision procedure because others have shown that the presence of the neomycin resistance gene in a targeted locus can result in a hypomorphic allele[13]. Finally, we performed intercrosses of +/*PolgA*^{mut} mice to generate homozygous knock-in mice (*PolgA*^{mut}/*PolgA*^{mut}), hereafter denoted 'mtDNA-mutator' mice.

We analysed recombinant mtDNA polymerase holoenzyme complexes *in vitro* and found that mtDNA polymerase containing a PolgA subunit bearing the Asp → Ala exonuclease domain mutation had a profound reduction of exonuclease activity (Fig. 1e). Others have shown that certain exonuclease-deficient versions of yeast mtDNA polymerase have reduced capacity to synthesize DNA[10,11]. However, no decrease in DNA polymerase activity was observed in biochemical assays with recombinant exonuclease-deficient mtDNA polymerase (Fig. 1f) or in mitochondrial extracts from heterozygous knock-in (+/*PolgA*^{mut}) or mtDNA-mutator mice (Fig. 1g).

The mtDNA-mutator mice had a normal appearance until the age of ~25 weeks, when we first noted slight kyphosis and alopecia. As the animals got older the kyphosis became marked and varying degrees of alopecia appeared (Fig. 2a). The median lifespan of the mtDNA-mutator mice was ~48 weeks and all of them died before the age of 61 weeks (Fig. 2e). Weight gain of the mtDNA-mutator mice started to decelerate from ~15–20 weeks of age and weight loss was noted from ~24 weeks of age (Fig. 2f, g). Alopecia is common in human ageing[14]. In addition, a decrease in body weight occurs in humans older than 60 years[15] and is also well documented in mice older than ~1.5 years[16].

We performed quantitative assessments of body composition with X-ray densitometry of the whole mouse (Fig. 2c) or dissected

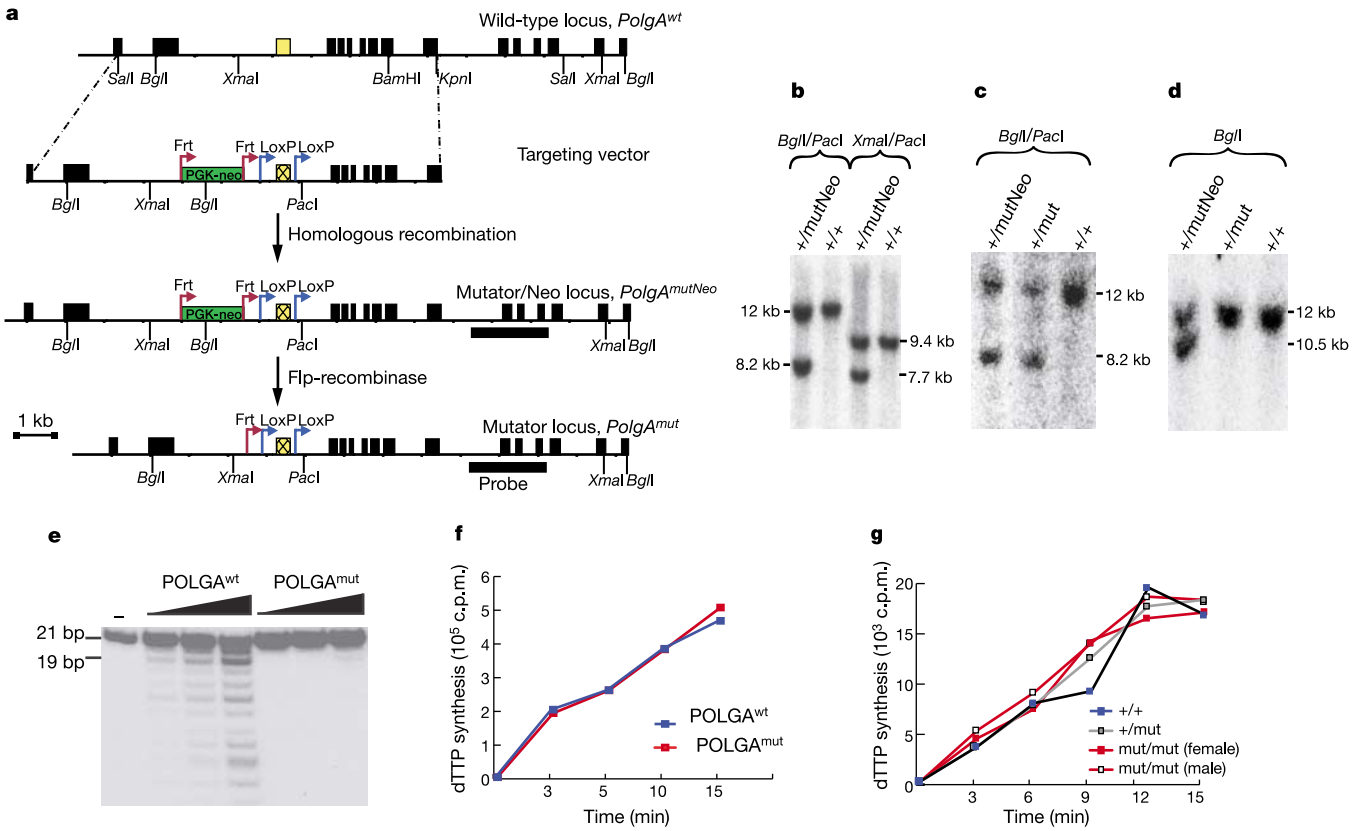

Figure 1 Creation of mtDNA-mutator mice and biochemical assays of DNA polymerase and exonuclease activities. **a**, Restriction map of the 5′ region of the wild-type *PolgA* locus (*PolgA*^{wt}), the targeting vector, the *PolgA* locus after homologous recombination (*PolgA*^{mutNeo}) and the mutator locus obtained after *Flp*-recombinase-mediated excision of the *PGK-neo* gene (*PolgA*^{mut}). The sequence of exon 3 (yellow box) of the targeting vector was changed to encode an alanine instead of the highly conserved aspartate residue. The thick black line indicates the probe used to screen for homologous recombination. **b**, Digestion of ES cell DNA with the restriction endonucleases *BglI*/*PacI* or *XmaI*/*PacI* generates a novel fragment of ~8.2 kb and ~7.7 kb, respectively, in clones that are heterozygous for *PolgA*^{mutNeo}. **c**, **d**, Mice heterozygous for *PolgA*^{mutNeo} (+/mutNeo) were mated to *Flp*-mice to excise the *Frt*-flanked *PGK-neo* cassette to obtain heterozygous *PolgA*^{mut} mice (+/mut). **e**, Biochemical assays of exonuclease activity. Recombinant human POLGA proteins were co-expressed with the human accessory subunit, POLGB, to obtain recombinant mtDNA polymerase holoenzyme. The enzyme containing the POLGA^{wt} polypeptide exhibits exonuclease activity, whereas the POLGA^{mut}-containing enzyme lacks this activity. **f**, DNA synthesis activities with recombinant holoenzymes consisting of human POLGA^{wt} or POLGA^{mut} and POLGB. **g**, DNA-synthesis activities in mitochondrial extracts isolated from liver of wild-type (+/+), heterozygous *PolgA*^{mut} mice (+/mut) and mtDNA-mutator mice (*PolgA*^{mut}/*PolgA*^{mut}; mut/mut).

femur bones (Fig. 2d). The ratio of lean body mass to length was normal, whereas the fat content was reduced in mtDNA-mutator mice (Fig. 2c), consistent with their overall appearance (Fig. 2a) and histological analyses of subcutaneous fat (Fig. 2b). In humans, body fat decreases with age after 65 years and a reduction in subcutaneous fat is common in the ageing skin[14]. Measurements of whole-body bone mineral density (BMD) showed a clear reduction at the age of 40 weeks in mtDNA-mutator mice (Fig. 2c), consistent with the clinical features of osteoporosis (marked kyphosis; Fig. 2a). There was a tendency to reduction in the ratio of whole-body bone mineral content (BMC) to length at 40 weeks of age (Fig. 2c). We also analysed dissected femur, to improve the precision of the BMD and BMC measurements, and found no difference between mtDNA-mutator and wild-type mice at 20 weeks of age. However, there was a clear reduction in BMD (Fig. 2d), BMC (not shown) and BMC/length (Fig. 2d) in femur of mtDNA-mutator mice at the age of 40 weeks. In conclusion, the X-ray densitometry revealed a change in body composition, with reduced fat content and development of osteoporosis in the mtDNA-mutator mice. Human ageing is similarly accompanied by kyphosis and osteoporosis[14]. Osteoporosis is also found in ageing rodents as demonstrated by decreased bone densities in aged wild-type mice[17].

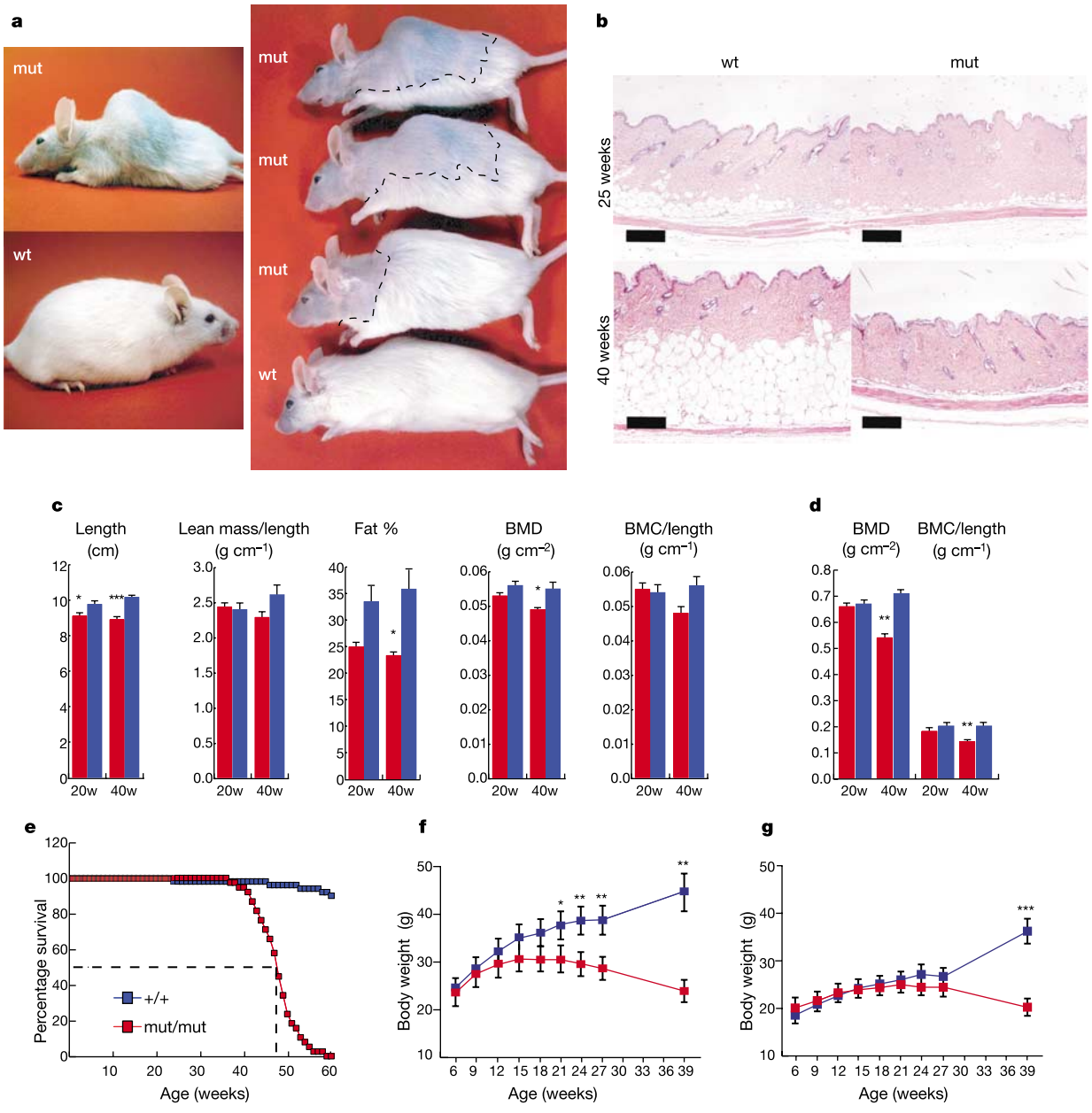

Figure 2 Ageing-related phenotypes observed in mtDNA-mutator mice. **a**, The mtDNA-mutator mice show reduced body size, kyphosis, reduced hair density and different stages of alopecia (delineated by dashed line) in comparison with littermate wild-type (wt) mice at the age of ~40–45 weeks. **b**, Haematoxylin- and eosin-stained cross-section of dorsal skin from 25- and 40-week-old wild-type (wt; $n = 3$ animals at each time point) and mtDNA-mutator (mut; $n = 3$) mice. Scale bar, 100 μm. **c**, Quantitative assessment of body length, lean mass, fat content, BMD and BMC by X-ray densitometry of mtDNA-mutator mice (red bars) and wild-type littermates (blue bars). **d**, Quantitative assessment of BMD and BMC by X-ray densitometry of dissected femur from mtDNA-mutator mice (red bars) and wild-type littermates (blue bars). 20w, 20 weeks; 40w, 40 weeks. **e**, Survival curves for wild-type ($n = 50$; blue line) and mtDNA-mutator ($n = 38$; red line) mice. **f**, Body weight curves for male wild-type ($n = 5$–13 mice at the different time points; blue line) and mtDNA-mutator ($n = 8$–10; red line) mice. **g**, Body weight curves for female wild-type ($n = 5$–7; blue line) and mtDNA-mutator ($n = 9$–11; red line) mice. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$, Student's *t*-test. All error bars indicate s.e.m.

We found significantly lower haemoglobin concentrations in peripheral blood ($P < 0.001$, Student's t-test) of mtDNA-mutator mice (81 ± 3 g per litre, mean ± s.e.m; $n = 9$ analysed animals) in comparison with wild-type littermates (106 ± 3 g per litre; $n = 8$) at the age of 25 weeks. The anaemia in mtDNA-mutator mice was macrocytic and hypochromic (Supplementary Table 1). In addition, we observed extramedullary haematopoiesis in the liver (Fig. 3b) and spleen enlargement (Supplementary Tables 2 and 3), with increased relative contribution of red pulp (Fig. 3a) in mtDNA-mutator mice. Increased haematopoiesis in the spleen and foci of haematopoiesis in the liver are common findings in ageing mice[16]. It should also be noted that anaemia of unknown aetiology is a frequent clinical problem in elderly humans[18].

The heart weight in relation to body weight was increased in mtDNA-mutator mice (Supplementary Table 3). The 40-week-old mtDNA-mutator mice also had an enlarged lumen of the left ventricle of the heart (Fig. 3c). Increases of heart weight and left ventricle hypertrophy are normally found in the ageing human heart[14,19]. Cardiomyopathy is common with increasing age in wild-type mice[16]. We performed enzyme histochemical staining of heart muscle tissue sections from mtDNA-mutator mice and found a mosaic pattern, with cytochrome c oxidase deficiency in some cardiomyocytes (Fig. 3d), similar to findings in ageing human hearts[4]. In accordance with this finding, light microscopy of thin sections of heart muscle showed that some cardiomyocytes had a vacuolated appearance because of accumulations of enlarged mitochondria (Fig. 3e). Electron microscopy studies of tissue sections from heart confirmed that there was an accumulation of abnormal mitochondria in a subset of the cardiomyocytes (Fig. 3f) in mtDNA-mutator mice.

A profound reduction in fertility of mtDNA-mutator mice of both sexes was found. In matings between 15 mtDNA-mutator females and 15 wild-type males, 14 of the females did become pregnant, each giving birth to one or two litters of normal size. However, the females did not become pregnant again after the age of 20 weeks, despite being continuously exposed to males for several months. We also set up matings over several months between 8 mtDNA-mutator males and 16 wild-type females, but obtained only a single, small litter. Testes of the mtDNA-mutator males were considerably smaller than wild-type testes from 12 weeks of age onwards (Fig. 3g; Supplementary Tables 2 and 3). Inspection of testes from mtDNA-mutator mice revealed reduced sperm content

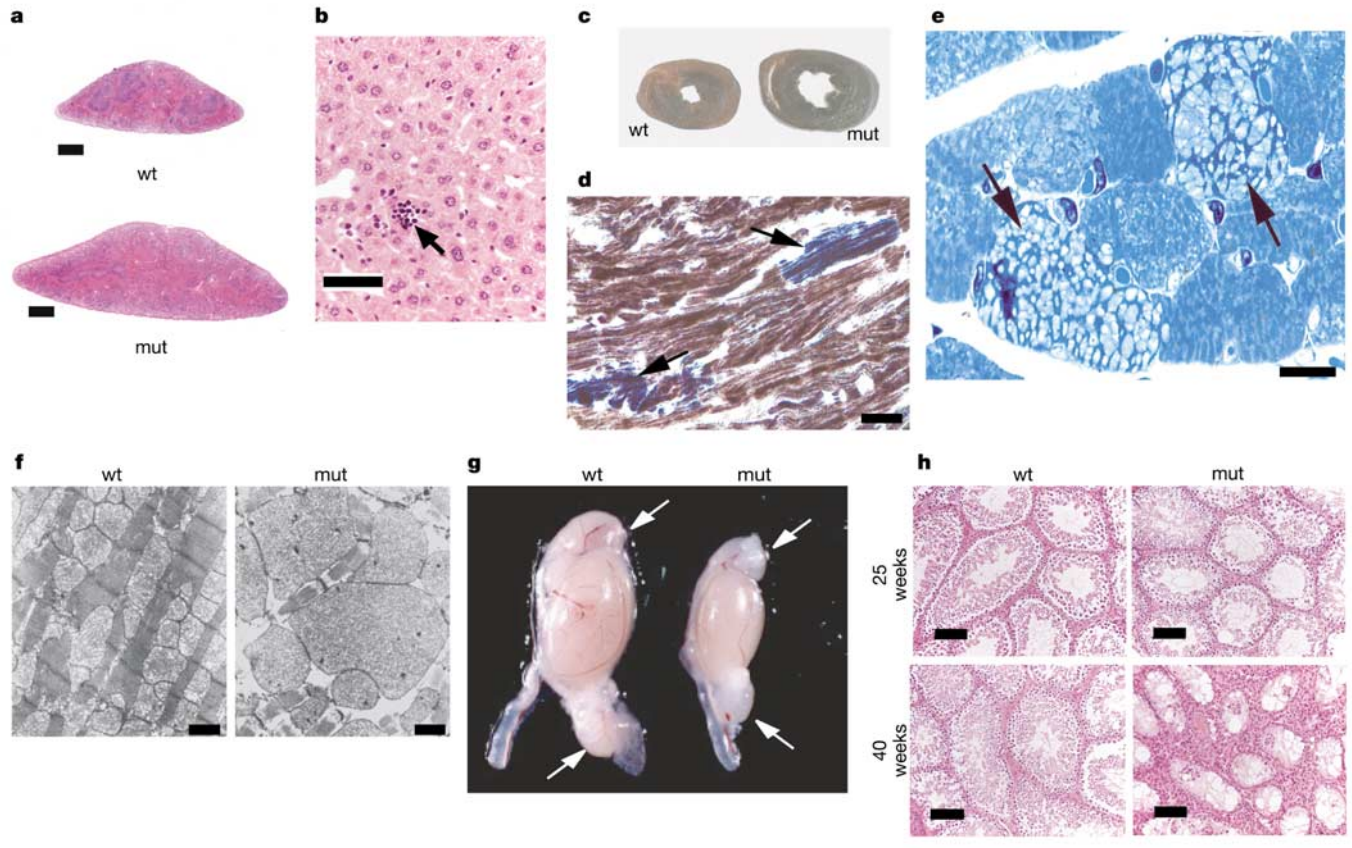

Figure 3 Histological analyses of different tissues from mtDNA-mutator mice. **a**, Haematoxylin- and eosin-stained sections of spleen of wild-type (wt; $n = 8$ analysed animals) and mtDNA-mutator mice (mut; $n = 8$) at age 25 weeks. Scale bar, 0.5 mm. **b**, Haematoxylin- and eosin-stained section from the liver of mtDNA-mutator ($n = 8$) and wild-type ($n = 8$, not shown) mice at age 25 weeks. Extramedullary haematopoiesis (arrow) is present in mtDNA mutator mice. Scale bar, 50 μm. **c**, Unstained thick sections of heart from wild-type ($n = 3$) and mtDNA-mutator mice ($n = 3$) at age 40 weeks. **d**, Enzyme histochemical double staining for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activities in heart of an mtDNA-mutator mouse at age 40 weeks. The presence of both COX and SDH activity results in a brown colour. A mosaic pattern is present in the mtDNA-mutator mouse heart and some cardiomyocytes appear blue (arrows) because of a lack of COX activity (catalytic subunits are mtDNA encoded) and preserved SDH activity (all subunits are nucleus encoded). Scale bar, 20 μm. A total of three mtDNA-mutator and three wild-type mice were analysed. **e**, Light microscopic image of a thin section of Epon-embedded heart muscle from an mtDNA-mutator mouse at age 40 weeks shows cardiomyocytes with accumulation of enlarged mitochondria (arrows). Scale bar, 10 μm. **f**, Electron micrographs of cardiomyocytes from wild-type ($n = 3$) and an mtDNA-mutator ($n = 3$) mice. Some cardiomyocytes of the mtDNA-mutator mouse contains enlarged abnormally shaped mitochondria. Scale bar, 1 μm. **g**, Testis from wild-type ($n = 13$) and mtDNA-mutator ($n = 13$) mice at the age of 25 weeks. The size of the testis is reduced and no sperm is visible on macroscopic inspection of the epididymis (arrows) of the mtDNA-mutator mouse. **h**, Cross-sections of testis of wild-type ($n = 4$–6) and mtDNA-mutator ($n = 4$–6) mice at 25 and 40 weeks of age. Scale bar, 100 μm.

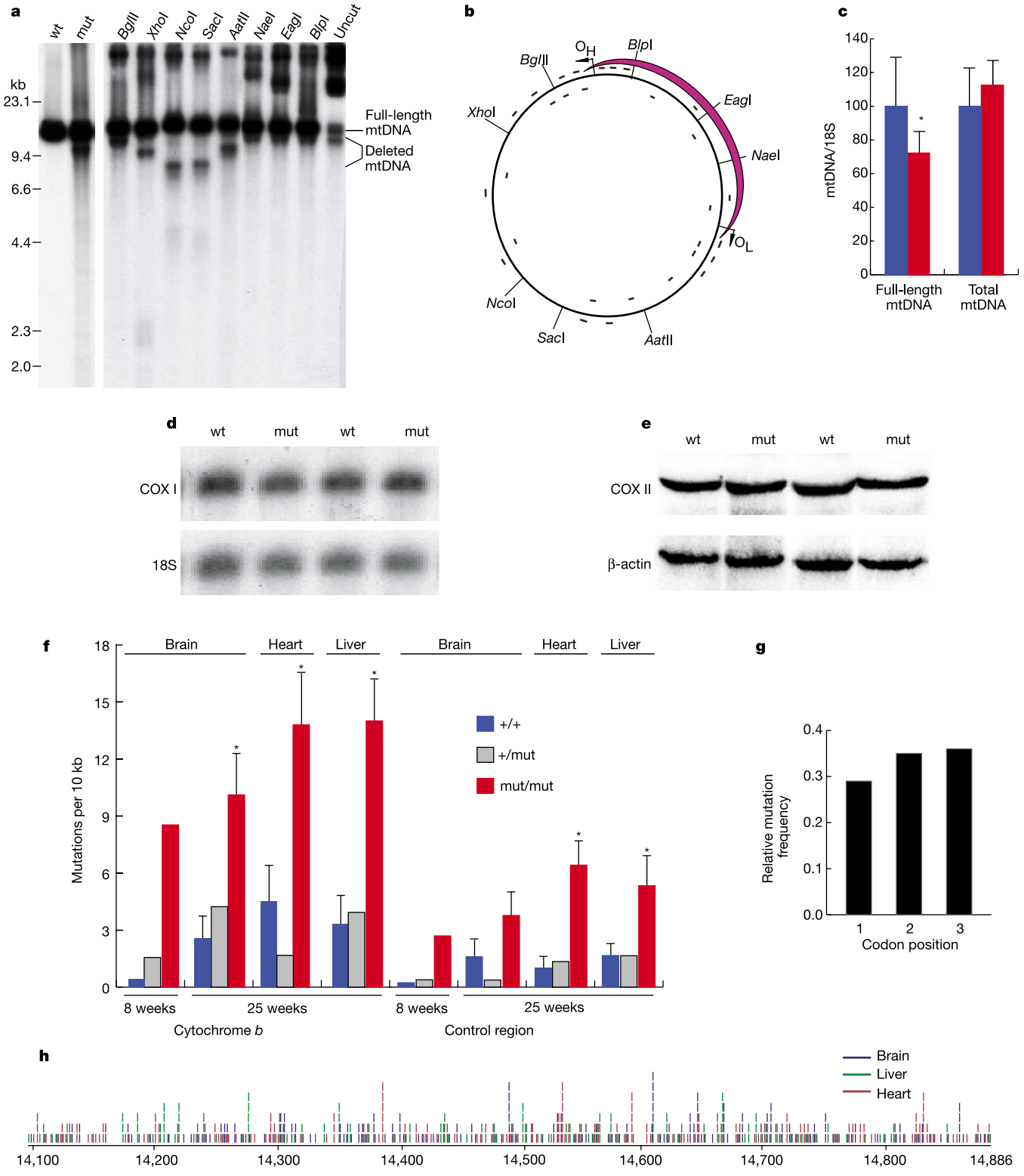

Figure 4 Analysis of mtDNA. **a**, Southern blot analysis of *Bgl*II-digested liver mtDNA from a wild-type and an mtDNA-mutator mouse at age 25 weeks (lane 1–2). Southern blot analysis of restriction-enzyme-digested mtDNA from an mtDNA-mutator mouse at age 25 weeks (lanes 3–11). **b**, The deleted mtDNA region is indicated with a purple arc. The black bars represent 29 strand-specific oligonucleotide probes used to map the mtDNA deletions. Oligonucleotides identical to the L-strand of mtDNA are inside and those identical to the H-strand are outside the circle. **c**, Quantification of mtDNA levels (mean ± s.e.m; asterisk, $P < 0.01$, Student's *t*-test) in liver from mtDNA-mutator (red bars; $n = 6$) and wild-type (blue bars; $n = 6$) mice at age 12–40 weeks. **d**, Northern blot analysis of cytochrome *c* oxidase subunit I (COX I) transcript levels in heart of wild-type and mtDNA-mutator mice at age 40 weeks. **e**, Western blot analysis of cytochrome *c* oxidase subunit II polypeptide (COX II) levels in heart of wild-type and mtDNA-mutator mice at age 25 weeks. **f**, mtDNA mutation loads in mtDNA-mutator (mut/mut; red bars), heterozygous *PolgA* ^{mut} (+/mut; grey bars) and wild-type (+/+; blue bars) mice. The bars indicate mean values. The standard deviation (indicated by error bars) was calculated in groups where DNA from three or four mice of the same genotype was sequenced. Absence of error bars indicates cases where only one or two animals were analysed. Asterisk, $P < 0.005$, Student's *t*-test. **g**, Relative mutation frequencies at all codon positions in the cytochrome *b* gene. **h**, Positions of individual mutations in the cytochrome *b* gene of brain (blue), heart (red) and liver (green) from 25-week-old mtDNA-mutator mice.

of the epididymis (Fig. 3g) and histological analyses of testis sections confirmed reduced sperm content in epididymis of mtDNA-mutator mice (data not shown). We observed severe testicular tubular degeneration with complete absence of sperm in 40-week-old mtDNA-mutator mice (Fig. 3h). In humans, female fertility decreases with age and males develop an age-associated decline in sperm count[20]. Degenerated tubules may occupy as much as 60% of the testis in aged, wild-type mice[21].

We performed Southern blot analyses and found a widespread tissue distribution of a class of shorter mtDNA molecules, up to ~12 kilobases (kb) in length, in mtDNA-mutator mice (Fig. 4a and Supplementary Fig. 1). Further analyses were performed with restriction enzymes that have single recognition sites in normal mouse mtDNA (Fig. 4a). The restriction enzymes *Nae*I, *Eag*I and *Blp*I did not digest the shorter mtDNA molecules, whereas the restriction enzymes *Bgl*II, *Xho*I, *Nco*I, *Sac*I and *Aat*II did digest the shorter molecules and gave a DNA fragment pattern showing that the shorter species behaved as a linear, subgenomic—that is, deleted—mtDNA molecule (Fig. 4a). The amount of deleted mtDNA did not change over time and the levels of deleted mtDNA were comparable in all investigated tissues (Supplementary Fig. 1). Southern blot analyses with radiolabelled oligonucleotides or short DNA fragments (Supplementary Fig. 1) localized the commonly deleted region to the small arc between O_H and O_L (Fig. 4b), which is consistent with the results from the restriction enzyme analysis (Fig. 4a). The mean level of full-length mtDNA was ~70% of the level in wild-type mice (Fig. 4c). The reduced copy number of full-length mtDNA did not affect overall mtDNA expression; the steady-state levels of the mtDNA-encoded cytochrome *c* oxidase subunit 1 messenger RNA (Fig. 4d) and the mtDNA-encoded cytochrome *c* oxidase subunit 2 polypeptide (Fig. 4e) were normal. These findings are consistent with our previous observation that ~35–40% general reduction of mtDNA copy number in heterozygous mitochondrial transcription factor A knockout ($+/Tfam^{-}$) mice has minimal effects on mtDNA expression and respiratory chain function[22].

We cloned and sequenced segments of mtDNA from brain, heart and liver, and found that mtDNA-mutator mice contain an increased load (~3–5 times) of somatic mtDNA point mutations (Fig. 4f). The mutation background from PCR (polymerase chain reaction) was subtracted when the mtDNA mutation load was calculated. We also discarded the few multiple occurrences of a single mutation because they could reflect clonal expansion, which includes selection and/or genetic drift in addition to *de novo* mutagenesis. The presented frequency of somatic mtDNA mutations (Fig. 4f) thus represents a conservative minimal estimate. We combined the cytochrome *b* gene sequencing results from all tissues and time points of mtDNA-mutator mice (>600 individual mutations) and found that all codon positions were equally affected (Fig. 4g). In addition, the mutations were evenly distributed along the cytochrome *b* gene (Fig. 4h). There were thus no obvious mutational hot spots in mtDNA-mutator mice, although mutation load in the non-coding control region was lower than in the cytochrome *b* gene. The mutation load in heterozygous mice (Fig. 4f) was indistinguishable from that of wild-type littermates, and these mice displayed no phenotype, showing that the $PolgA^{mut}$ mutation is recessive.

We further characterized respiratory chain function in the hearts of mtDNA-mutator mice (Supplementary Fig. 2) because of the findings of focal cytochrome *c* oxidase deficiency (Fig. 3d) and increased mitochondrial mass in some cardiomyocytes (Fig. 3e, f). We found slightly increased citrate synthase activities, consistent with a moderate increase in mitochondrial mass. There was a progressive reduction of respiratory chain enzyme activities and of mitochondrial ATP production rates (MAPR) in the hearts of mtDNA-mutator mice. The observed decline of respiratory chain enzyme activities and MAPR had profiles consistent with the suggestion that the deficiencies were induced by mutations of mtDNA (Supplementary Fig. 2).

Our results establish a direct, experimental link between increased levels of somatic mtDNA mutations, respiratory chain dysfunction and phenotypes present in ageing mammals. The enhanced mtDNA mutation load of the mtDNA-mutator mouse is clearly generated somatically, because it is not shared with heterozygous or wild-type littermates. However, the detailed kinetics of accumulation of somatic mtDNA mutations remains to be elucidated. The mutation load is already substantial by 2 months, and is also rather uniform between tissues, suggesting that much of the mutation accumulation may occur during embryonic and/or fetal development. The onset of premature ageing is not accompanied, temporally, by a large *de novo* accumulation of mtDNA mutations around 6 months. Rather, it seems more plausible to ascribe it to cumulative physiological damage caused by the high mutation load during adult life, and/or to segregation or clonal expansion of specific mutations, as supported by the observed mosaicism for respiratory chain deficiency in the heart. Loss of vital cells in which mtDNA mutations have accumulated beyond a critical threshold (for example, through apoptosis or replicative senescence associated with telomere shortening brought on by oxidative stress) may be the critical process that manifests as premature ageing and reduced lifespan. We have previously generated a series of tissue-specific *Tfam* knockout mouse strains with reduced mtDNA expression and severe respiratory chain deficiency in different organs, accompanied by increased cell death and/or apoptosis[23–26]. However, we found only minor or no induction of defence mechanisms against reactive oxygen species (ROS) in these knockouts[24,27]. The possible involvement of increased ROS production as a mechanism of physiological damage leading to premature ageing in the mtDNA-mutator mouse should nevertheless not be discounted. Unlike tissue-specific *Tfam* knockouts, in which the effects on respiration and oxidative phosphorylation are essentially quantitative, the mtDNA-mutator creates an apparently random set of point mutations in genes for respiratory chain subunits. These may be expected to exhibit qualitative, as well as quantitative, abnormalities, of which increased ROS production and proton leak are likely consequences. The mtDNA-mutator mice will thus be a valuable tool for future experiments to determine whether the consequences of increased somatic mtDNA mutation can be counteracted by genetic, pharmacological or dietary interventions that affect ROS formation, bioenergetic homeostasis, cell death, apoptosis or other pathophysiological effects of respiratory chain dysfunction. Such approaches may allow us to design strategies to antagonize or delay deleterious consequences of naturally occurring somatic mtDNA mutations in human ageing. □

Methods

Creation of mtDNA-mutator mice

A genomic clone containing exons 1–16 of the mouse *PolgA* gene was isolated from a 129/SvJ λ Fix II phage library (Stratagene). A *Sal*I–*Kpn*I *PolgA* fragment of 8.1 kb (Fig. 1a) was cloned into pBluescript II SK+ (pBS, Stratagene) to generate plasmid pST1. A *Bam*HI–*Sal*I fragment of 3.6 kb (Fig. 1a) was cloned into pBS to generate plasmid pST4. The inserts of plasmids pST1 and pST4 were completely sequenced. The plasmid pST8 was constructed by cloning a 1.4-kb *Sac*I fragment of pST1 containing exon 3 (which encodes exonuclease domain 2) into pBS and used for site-directed mutagenesis with the oligonucleotides E3-1 (5′-gttagtggtggggcacaatgtttcctttgcccgagccc-3′) and E3-2 (5′-gggctcgggcaaaggaaacattgtgccccaccactaac-3′) to change the aspartic acid codon (GAC) at position 257 to an alanine codon (GCC). The pDelboy plasmid (provided by D. Rossi) contains an *Frt*-site-flanked neomycin gene expressed from the phosphoglycerate kinase promoter (*PGK-neo*), two *loxP* sites and a cassette containing the herpes simplex virus thymidine kinase gene expressed from the phosphoglycerate kinase promoter (*PGK-HSV-TK*). The plasmid pST7 was constructed by exchanging one *loxP* site and the *PGK-HSV-TK* cassette in pDelboy with a DNA fragment containing a *Sac*I–*lox*P–*Pac*I sequence. The insert of pST8 was introduced into the *Sac*I-site of pST7 to generate pST9. Plasmid pST6 was generated by replacing the 1.4-kb *Sac*I fragment of pST1 with a DNA fragment containing a *Kpn*I/*Eco*RI/*Pac*I polylinker sequence. The final targeting vector for introducing the $PolgA^{mutNeo}$ allele was obtained by ligating a 3.6-kb *Xho*I/*Pac*I fragment from pST9 into pST6. The composition of DNA constructs was confirmed by restriction-

enzyme mapping and partial sequencing after each cloning step. The targeting vector was linearized with *Sal*I and electroporated into 129 R1 embryonic stem (ES) cells. A total of 61 ES cell clones were subjected to Southern blot analysis and nine specifically targeted clones were found (Fig. 1b). We generated chimaeras by blastocyst injection of ES cells and obtained germline transmission of the $PolgA^{mutNeo}$ allele from two clones. Mice heterozygous for the $PolgA^{mutNeo}$ allele were mated with transgenic C57BL/6 mice ubiquitously expressing *Flp*-recombinase[12] to remove the *PGK-Neo* gene, thus generating heterozygous $PolgA^{mut}$mice (Fig. 1a, c, d). Heterozygous $PolgA^{mut}$ mice ($+/PolgA^{mut}$) were intercrossed to generate mtDNA-mutator mice ($PolgA^{mut}/PolgA^{mut}$). This cross resulted in normal litter sizes (mean litter size 8.9 pups) and mendelian distributions of genotypes ($n = 500$ genotyped animals; wild type 25.8%, $+/PolgA^{mut}$ 50.1% and $PolgA^{mut}/PolgA^{mut}$ 24.1%) showing that homozygosity for $PolgA^{mut}$ allele does not result in embryonic or early postnatal lethality. We followed the survival of wild-type ($n = 50$) and mtDNA-mutator ($n = 38$) mice (Fig. 2e). A total of 18 mtDNA-mutator mice died spontaneously, whereas the remaining 20 mice were killed in accordance with the ethical permit requirements because of moribund appearance. Standard Southern blot protocols were used for genotyping.

Analyses of DNA polymerase and exonuclease activities

A recombinant mtDNA polymerase holoenzyme was obtained by co-expression of human POLGA and POLGB subunits in the baculovirus system and biochemical purification. The DNA polymerase activities of recombinant proteins and mitochondrial extracts were determined by measuring incorporation of [α-^{32}P]dTTP into the artificial (rA).p(dT)$_{12-18}$ template. The exonuclease activities of recombinant proteins were determined by adding a substrate obtained by annealing a 5′ ^{32}P-labelled 21-base oligonucleotide (5′-TGCATGCCTGCAGGTCGACTG-3′) to M13mp18 single-stranded DNA (Biolab). This substrate consists of a 20-base-pair double-stranded region with a one-nucleotide 3′ mismatch. The exonuclease reactions were analysed by electrophoresis in 3 M urea/25% polyacrylamide gels.

Quantification of lean body mass, body fat, BMD and BMC

Mice were killed by CO_2 inhalation and densitometry was performed by using a PIXImus imager (GE Lunar). A total of three wild-type and three mtDNA-mutator mice were analysed at the age of 20 weeks, and five wild-type and five mtDNA-mutator mice were analysed at the age of 40 weeks. Body fat (percentage), lean body mass, BMD (g cm^{-2}) and BMC (g) were determined in the whole mouse. BMD and BMC were also determined in the dissected femur bone. Field calibration and calibration versus the quality control phantom were performed each day before mouse or femur imaging.

Analysis of mtDNA mutations

Total DNA was extracted from the brains of 2-month-old wild-type, heterozygous and mtDNA-mutator mice. Enriched mtDNA was extracted from the isolated mitochondria (prepared by differential centrifugation) of different tissues of 6-month-old mice. The somatic mtDNA mutation load was determined by PCR, cloning, and sequencing, as described earlier[28], using primers that specifically amplified the cytochrome *b* gene (nucleotide pair 14,073–14,906) and non-coding control region (15,357–138) of mouse mtDNA[29] (GenBank NC_005089) together with flanking sequences.

Histology and biochemical analyses

Different tissues were fixed in buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Enzyme histochemical and ultrastructural analyses of myocardium were performed as described[25,26]. The measurement of respiratory chain enzyme complex activities, citrate synthase activity and MAPR was performed as previously described[25,26,30].

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An autonomous molecular computer for logical control of gene expression

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Early biomolecular computer research focused on laboratory-scale, human-operated computers for complex computational problems[1–7]. Recently, simple molecular-scale autonomous programmable computers were demonstrated[8–15] allowing both input and output information to be in molecular form. Such computers, using biological molecules as input data and biologically active molecules as outputs, could produce a system for 'logical' control of biological processes. Here we describe an autonomous biomolecular computer that, at least *in vitro*, logically analyses the

Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production

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The mitochondrial theory of aging proposes that reactive oxygen species (ROS) generated inside the cell will lead, with time, to increasing amounts of oxidative damage to various cell components. The main site for ROS production is the respiratory chain inside the mitochondria and accumulation of mtDNA mutations, and impaired respiratory chain function have been associated with degenerative diseases and aging. The theory predicts that impaired respiratory chain function will augment ROS production and thereby increase the rate of mtDNA mutation accumulation, which, in turn, will further compromise respiratory chain function. Previously, we reported that mice expressing an error-prone version of the catalytic subunit of mtDNA polymerase accumulate a substantial burden of somatic mtDNA mutations, associated with premature aging phenotypes and reduced lifespan. Here we show that these mtDNA mutator mice accumulate mtDNA mutations in an approximately linear manner. The amount of ROS produced was normal, and no increased sensitivity to oxidative stress-induced cell death was observed in mouse embryonic fibroblasts from mtDNA mutator mice, despite the presence of a severe respiratory chain dysfunction. Expression levels of antioxidant defense enzymes, protein carbonylation levels, and aconitase enzyme activity measurements indicated no or only minor oxidative stress in tissues from mtDNA mutator mice. The premature aging phenotypes in mtDNA mutator mice are thus not generated by a vicious cycle of massively increased oxidative stress accompanied by exponential accumulation of mtDNA mutations. We propose instead that respiratory chain dysfunction *per se* is the primary inducer of premature aging in mtDNA mutator mice.

mitochondria | mtDNA mutator mice

The free radical theory of aging, formulated 50 years ago by Harman (1), proposes that aging and associated degenerative diseases can be attributed to deleterious effects of reactive oxygen species (ROS). Intracellular ROS are primarily generated by the mitochondrial electron transport chain, making the mitochondrial network a prime target of oxidative damage. Building on this, the mitochondrial theory of aging predicts that a vicious cycle contributes to the aging process. (*i*) Normal metabolism causes ROS production by the electron transport chain. (*ii*) ROS production induces damage to lipids, proteins, and nucleic acids in mitochondria. (*iii*) ROS-induced mtDNA mutations lead to the synthesis of functionally impaired respiratory chain subunits, causing respiratory chain dysfunction and augmented ROS production (2). (*iv*) This vicious cycle is proposed to cause an exponential increase of mtDNA mutations over time, resulting in aging and associated degenerative diseases. A substantial amount of correlative data from morphological, bioenergetic, biochemical, and genetic studies of mammalian tissues supports this theory (3). Mitochondria are larger and fewer in older individuals, and mitochondrial abnormalities such as vacuoles, abnormal cristae, and paracrystalline inclusions are more often found (4). In addition, mammalian aging has been associated with increased oxidative damage to proteins (5) and accumulation of somatic mtDNA mutations (6–8), and there is also an emerging consensus that there is an age-associated decline of respiratory chain function with increasing age (9). Furthermore, several studies have demonstrated a correlation between the rate of ROS formation and maximal lifespan in various animal species (10). Caloric restriction, shown to prolong lifespan in all studied organisms, reduces ROS production and induces antioxidant defenses (11). However, most of the available data are merely correlative and therefore do not exclude the possibility that mitochondrial damage and ROS production are consequences rather than driving forces of aging. We have recently developed a mouse model that provides experimental evidence for a causative link between mtDNA mutations and aging phenotypes in mammals (12). We created homozygous knock-in mice expressing an error-prone version of mtDNA polymerase γ ($PolgA^{mut}$) (12). These mtDNA mutator mice accumulate a substantial burden of somatic mtDNA mutations, associated with premature aging and reduced lifespan. The mtDNA mutator mice display an apparently normal phenotype at birth and during early adolescence. Aging phenotypes such as weight loss, reduced s.c. fat, alopecia, kyphosis, osteoporosis, anemia, reduced fertility, and heart hypertrophy develop from 25 weeks of age (12).

We have now further characterized the patterns of mutation accumulation and cellular metabolism in mtDNA mutator mice and surprisingly found normal ROS production and only a very minor increase in oxidative damage, despite profound respiratory chain deficiency. Mutations were found to accumulate in an approximately linear manner over the mouse lifetime. The profound aging phenotypes generated by accumulation of somatic mtDNA mutations are thus not mediated by dramatically increased ROS production.

Materials and Methods

Genotyping. Standard Southern blot protocols were used for *PolgA* genotyping (12). Genotyping was also performed by PCR with primers (forward, 5′-gcc atc tca cca gcc cgt at-3′; reverse, 5′-gtg agg aga gtg gcc gct aa-3′) as described (12). Animal studies were approved by the animal welfare ethics committee and performed in compliance with Swedish law.

Analysis of mtDNA Mutations. Total DNA was extracted from upper parts of heads of embryonic day 13.5 (E13.5) embryos and brains of 10- to 11-month-old wild-type and mtDNA mutator mice. The somatic mtDNA mutation load was determined by PCR, cloning, and sequencing, by using primers that specifically amplified the cytochrome *b* gene (nucleotide position 14093–14880) and noncoding control region (nucleotide position 15378–117) of mouse mtDNA as described (12).

Conflict of interest statement: No conflicts declared.

Abbreviations: E*n*, embryonic day *n*; GPX1, glutathione peroxidase; H^2-DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MEF, mouse embryonic fibroblasts; mut/10 kb, mutations/10 kilobase pairs; oxo^8-dG, 8-oxo-2′-deoxyguanosine; ROS, reactive oxygen species; SOD, superoxide dismutase; Tfam, transcription factor A.

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Isolation and Culturing of Mouse Embryonic Fibroblasts (MEFs). Primary MEF cultures were isolated from individual E13.5 embryos obtained from an intercross of heterozygous mtDNA mutator mice. The MEFs were expanded for two passages and genotyped by PCR analysis with total DNA. MEFs were cultured in complete DMEM with high glucose containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg of streptomycin. We used three wild-type and four mtDNA mutator MEF primary cultures to establish immortalized cell lines according to the 3T3 protocol (13). We transferred 3×10^5 cells into new 60-mm dishes every 3 days. All cell lines were continuously cultured without cloning, and immortalized MEF cell lines were obtained.

Measurements of Intracellular ROS. MEFs were harvested by centrifugation, washed twice with ice-cold phosphate-buffered saline (pH 7.4), and suspended in 1 ml of phosphate-buffered saline. The cells were then loaded with either 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H^2-DCFDA) (Invitrogen) or 10 μM dihydroethidium (Invitrogen) and incubated at 37°C for 30 or 120 min.

We used an FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ) to measure ROS generation by the fluorescence intensity of 10,000 cells. The results were analyzed with the CELL QUEST program (BD Biosciences).

Oxygen Consumption Studies. Polarographic analyses were adapted from Rustin *et al.* (14). In summary, 5×10^6 to 2×10^7 cells (80% confluent) were harvested and transferred to 1 ml of respiratory buffer A (10 mM Hepes, pH 7.4/225 mM mannitol/75 mM sucrose/10 mM KCl/10 mM KH_2PO_4/5 mM $MgCl_2$/1 mg/ml BSA) in a polarography cuvette. Baseline respiration was recorded for 2 min before 0.5-μl aliquots of 0.1 mM rotenone were added until total inhibition of complex I was obtained. Cell membrane integrity was controlled by measuring succinate-driven respiration (10 μl of 0.1 M ADP plus 20 μl of 1 M succinate, pH 7.4) before and after digitonin permeabilization. Cells were permeabilized by using 1.1 μl of 5 mg/ml digitonin per 1×10^6 cells. Maximum uncoupled respiration rate was obtained by the addition of 1 μl of 2.5 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Finally, inhibited respiration was measured after addition of 2.5 μl of 200 mM KCN. A two-way ANOVA was used to calculate statistical significance.

Quantification of Protein Carbonyls. Liver mitochondria were isolated by differential centrifugation as described (15). Total protein extracts and mitochondrial extracts were isolated as described (16). Protein carbonyl groups were quantified with the OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA). In brief, proteins (20 μg) were incubated with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenyl hydrazone derivatives. 2,4-dinitrophenyl-derivatized proteins were blotted to nitrocellulose membranes (Hybond-C+; Amersham Biosciences, Arlington Heights, IL) in serial dilutions by using the Hybri.dot 96-well filtration manifold system (Thistle Scientific, Glasgow, U.K.). Proteins were then immunostained by using rabbit anti-2,4-dinitrophenyl antiserum (1:250) and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1,000; Amersham Biosciences). The oxidized proteins were detected by chemiluminescence (Amersham Biosciences), followed by exposure on x-ray films (Eastman Kodak, Rochester, NY). The films were scanned and then analyzed with IMAGE MASTER 1D ELITE software (Amersham Biosciences). The blots were subsequently immunostained with an anti-actin antibody (Sigma) for standardization of total protein levels. The total amount of protein in all samples was also controlled by SDS/PAGE. An aliquot of 20 μg of each protein sample was separated on a 12% polyacrylamide gel and stained by Coomassie blue. The gels were scanned, and the signal in each lane was quantified with IMAGE MASTER 1D ELITE software.

Aconitase Activity. Isolated mitochondria were diluted in a solution consisting of 0.1% Triton X-100, 50 mM KH_2PO_4, and 1 mM EDTA at pH 7.5, and aconitase activity was determined as described (17). The aconitase activity was expressed as units per unit of citrate synthase activity in the isolated mitochondria (18).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from heart by using Trizol Reagent (Invitrogen) following the instructions of the manufacturer. RT-PCR products were separated on gels, purified with the QIAEX II gel extraction kit (Qiagen, Hilden, Germany), radiolabeled with [α-^{32}P]dCTP, and used as probes to detect glutathione peroxidase 1 (*GPX1*) and superoxide dismutase 2 (*SOD2*) transcripts (19). The signal intensities were recorded with a FUJIX Bio-Imaging Analyzer BAS 1000 (Fuji), and data were analyzed with the IMAGE GAUGE 3.3 program (Fuji). The RNA loading was normalized to 18S rRNA.

Western Blot Analysis. Total liver and heart homogenates and liver mitochondrial preparations were suspended in protein buffer [equal parts of suspension buffer [100 mM NaCl/10 mM Tris·HCl (pH 7.6)/1 mM EDTA (pH 8.0)/1% aprotinin/1% phenylmethylsulfonyl fluoride] and loading buffer [100 mM Tris·HCl (pH 6.8)/4% SDS/20% glycerol/200 mM DTT]. Each sample was boiled for 10 min, followed by sonication and differential centrifugation for 10 min at $10{,}000 \times g$. The supernatants containing the proteins were collected and analyzed by Western blot to determine protein levels of mouse SOD2 as described (20). We used 1:1,000 dilutions of a polyclonal antibody raised against the human recombinant SOD2 protein that also recognized mouse, bovine, and rat protein (Upstate Biotechnology, Lake Placid, NY).

H_2O_2 Treatments and Analysis of Apoptosis. Cells growing in flasks (70–80% confluent) were incubated for 1 h in DMEM with high glucose and with the addition of 0, 0.5, 2, and 10 mM H_2O_2. Treated cells were harvested and stained with annexin V and propidium iodide included in the Vybrant apoptosis assay kit 2 (Invitrogen), according to the supplied protocol. Flow cytometric analyses were performed on a BD Biosciences flow cytometer (FACScan), and results were analyzed with the CELL QUEST program (BD Biosciences).

Results

Lifetime Increase of mtDNA Mutation Load in Mutator Mice. We measured the accumulation of somatic mtDNA mutations by sequencing multiple cloned mtDNA fragments from embryo heads and/or adult mouse brain. We found 1.5 ± 0.9 mutations/10 kilobase pairs (mut/10 kb) in wild-type embryos ($n = 3$) and 7.8 ± 0.4 mut/10 kb in mtDNA mutator embryos ($n = 3$) at E13.5 (Fig. 1*A*). The mutation levels were 2.3 ± 1.1 mut/10 kb in wild-type and 10.1 ± 1.6 mut/10 kb in mtDNA mutator mice at 25 weeks of age (12), whereas mutation levels were 5.3 ± 1.7 mut/10 kb in wild-type and 15.7 ± 1.8/10 kb in mtDNA mutator mice at 40 weeks of age (Fig. 1*A*). The load of somatic mtDNA mutations is thus substantial in midgestation mtDNA mutator embryos, and the levels increased further in an approximately linear manner during postnatal life. The same trend was evident in the noncoding control region of mtDNA (data not shown), although mutation levels were lower than in the cytochrome *b* gene, as seen in ref. 12.

Normal Superoxide and Hydrogen Peroxide Levels in mtDNA Mutator MEFs. We quantified $O_2^{\bullet -}$ and H_2O_2 production in E13.5 MEFs by using intracellular probes and FACS analysis. The generation of $O_2^{\bullet -}$ was monitored with dihydroethidium, which is oxidized by $O_2^{\bullet -}$ and/or OH^- to yield fluorescent ethidium. We found no significant difference in $O_2^{\bullet -}$ levels between wild-type and mtDNA mutator MEFs (Fig. 1*B*). It has been observed that very long incubation with dihydroethidium may be required to detect small alterations in ROS production (21). Despite extending the incubation time from 30 to

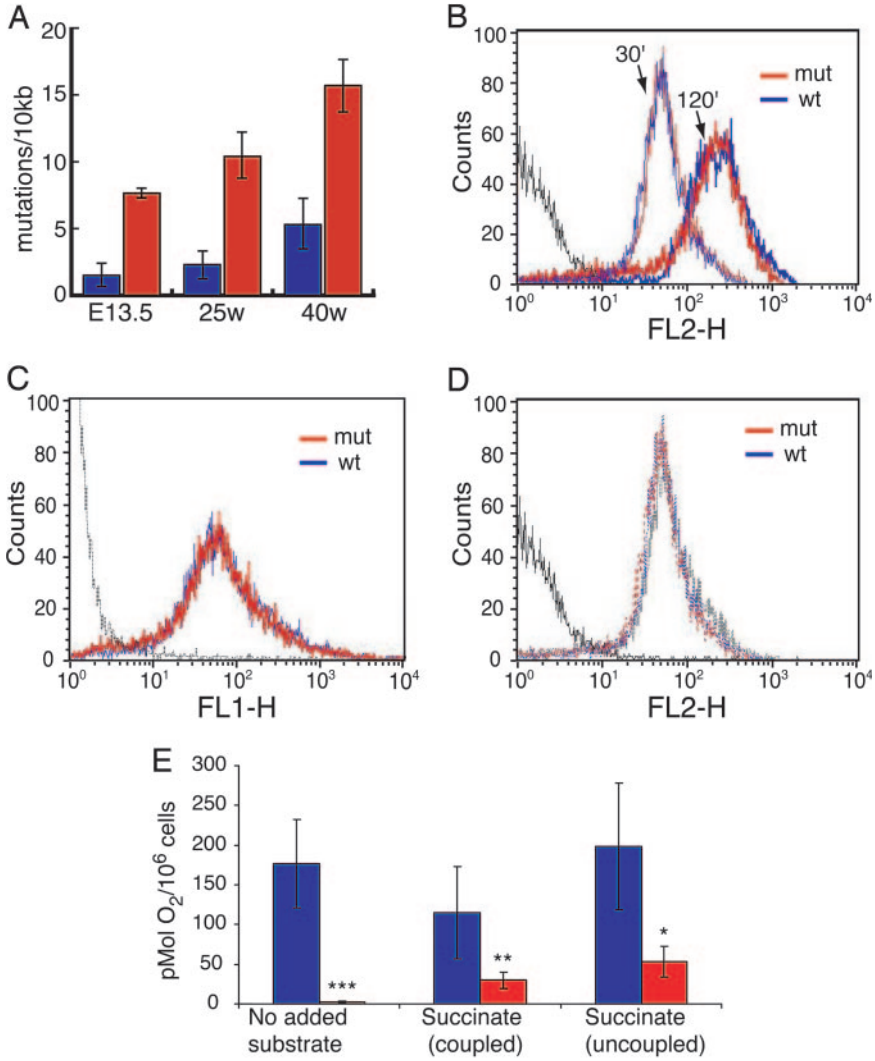

Fig. 1. Analysis of levels of mtDNA mutations in tissues and characterization of ROS production and respiratory chain function in MEFs. (*A*) The levels of mtDNA mutations (mut; cytochrome *b* region) in wild-type (wt; blue bars) and mtDNA mutator (red bars) embryo heads and mouse brains at different time points. Bars show mean values, and error bars indicate the SD. The low background level of PCR-induced mutations has been subtracted. (*B*) FACS analysis of $O_2^{\bullet-}$ production after 30 min (thin lines) and 120 min (thick lines) incubation with dihydroethidium in primary cultures of MEFs from wild-type (blue lines) and mtDNA mutator (red lines) embryos. (*C*) FACS analysis of H_2O_2 production after 30 min incubation with carboxy-H_2DCFDA in wild-type (blue line) and mtDNA mutator (red line) primary MEF cultures. (*D*) FACS analyses of $O_2^{\bullet-}$ production after 30 min incubation with dihydroethidium in wild-type (blue line) and mtDNA mutator (red line) immortalized MEFs and primary MEFs from mtDNA mutator embryos (yellow line). The black line in all FACS analyses indicate negative control, i.e., wild-type cells analyzed without previous treatment with dihydroethidium or H_2DCFDA. FL1-H and FL2-H, fluorescence intensity in log scale (green and red fluorescence, respectively). (*E*) Polarographic investigation of respiratory chain function in immortalized MEFs from wild-type (blue bars) and mtDNA mutator (red bars) embryos. Measurements (mean values ± SD) were performed without addition of exogenous substrate (rotenone-sensitive respiration) and with addition of succinate, with or without uncoupler. Asterisks indicate level of statistical significance: *, $P < 0.05$; **, $P < 0.01$.

120 min, the $O_2^{\bullet-}$ levels remained similar in wild-type and mtDNA mutator MEFs (Fig. 1*B*). We assessed H_2O_2 production with the carboxy-H_2DCFDA probe. The acetate group of this probe is cleaved by esterases upon cell entry, leading to intracellular trapping of the nonfluorescent 2′,7′-dichlorofluorescin. Subsequent oxidation by ROS, particularly H_2O_2 and hydroxyl radical, yields the fluorescent product DCF. We found no difference in DCF formation between mtDNA mutator and wild-type MEFs when incubated with carboxy-H_2DCFDA for 30 min (Fig. 1*C*) or 120 min (data not shown).

We used the standard 3T3 protocol (13) to immortalize MEFs. The mtDNA mutator and wild-type MEFs were subject to 30 passages, corresponding to approximately 40–50 cell divisions, during the immortalization procedure. We hypothesized that this large number of cell divisions might lead to enhanced ROS production in mtDNA mutator cells. However, the $O_2^{\bullet-}$ (Fig. 1*D*) and H_2O_2 (data not shown) production was similar in immortalized wild-type and mtDNA mutator MEFs.

Respiration Is Severely Impaired in mtDNA Mutator MEFs. Next, we investigated whether the absence of ROS accumulation could be explained by mitochondrial respiratory function remaining normal despite the accumulation of mtDNA mutations. We cultivated MEFs in high glucose media (4,500 mg per liter) to 70–100% confluency and observed that the pH became acidic after 1–3 days when mtDNA mutator MEFs were cultured. The medium of wild-type MEFs remained neutral even after 1 week of culturing under the same conditions. This finding is highly suggestive of increased dependence on glycolysis and correspondingly decreased oxidative phosphorylation in the mtDNA mutator MEFs. We therefore evaluated respiratory chain function by polarographic

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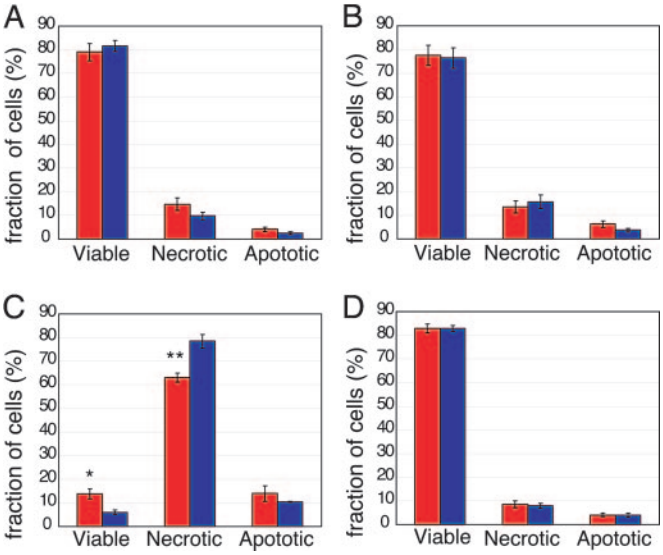

Fig. 2. H_2O_2 induced necrosis and apoptosis in wild-type (blue bars) and mtDNA mutator (red bars) primary MEF cultures. Cells were stained with propidium iodide and annexin V and assayed for viability (mean values ± SD) by FACS analysis after 1 h incubation in culture media containing 0.5 mM (*A*), 2 mM (*B*), and 10 mM (*C*) H_2O_2 or in medium without H_2O_2 (*D*). Asterisks indicate level of statistical significance: *, $P < 0.05$; **, $P < 0.01$.

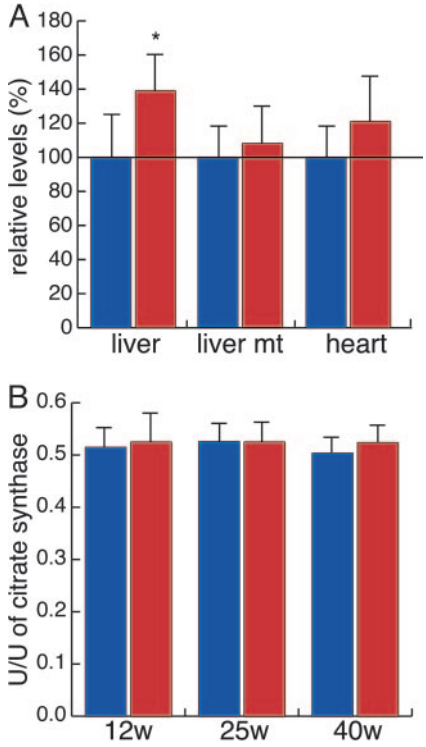

Fig. 3. Oxidative damage to proteins in tissues of wild-type (blue bars) and mtDNA mutator (red bars) mice. (*A*) Relative amounts of carbonyl groups in liver total protein extracts, liver mitochondrial protein extracts, and heart total protein extracts at 40 weeks of age. (*B*) Aconitase enzyme activity in hearts of 12-, 25-, and 40-week-old mice. Bars show mean values, and error bars indicate SD. Asterisks indicate level of statistical significance: *, $P < 0.05$.

measurements of O_2 consumption. The respiration of the mtDNA mutator MEFs was <5% that of wild-type MEFs in the absence of exogenously added respiratory substrate (Fig. 1*E*). We assessed the function of the respiratory chain by permeabilization of the MEFs and subsequent addition of succinate. We found markedly reduced respiration in the presence of succinate and rotenone in both coupled and uncoupled conditions in mtDNA mutator MEFs (Fig. 1*E*). The finding of impaired respiratory chain function in mtDNA mutator MEFs is in agreement with our previous report that high levels of somatic mtDNA mutations in the heart impair the activities of individual respiratory chain enzyme complexes as well as the mitochondrial ATP production rate (12).

No Increase in Hydrogen Peroxide-Induced Cell Death in mtDNA Mutator MEFs. We tested whether mtDNA mutator MEFs were prone to undergo apoptosis in response to oxidative stress by adding increasing levels of H_2O_2 to the culture medium. Treated cells were incubated with fluorescently labeled annexin V, which binds to phosphatidyl serine exposed on the cell surface during apoptosis, and propidium iodide, which accumulates in necrotic cells. This approach allows assessment of cell viability by FACS analysis because live cells do not retain propidium iodide and do not bind annexin V on the cell surface. We treated wild-type and mtDNA mutator MEFs with 0.5 mM H_2O_2 (Fig. 2*A*) or 2 mM H_2O_2 for 1 h (Fig. 2*B*) and observed no difference in the fraction of necrotic or apoptotic cells. We also incubated cells with 10 mM H_2O_2 for 1 h (Fig. 2*C*) and observed no difference in the fraction of apoptotic cells, whereas the fraction of necrotic cells was lower in treated mtDNA mutator MEFs (63.0 ± 3.9%) compared with wild-type MEFs (78.5 ± 4.9%). We also performed similar analyses in immortalized MEFs and found no difference in the proportion of necrotic and apoptotic cells when comparing wild-type and mtDNA mutator cells (data not shown). We also extended the H_2O_2 treatment to 24 h in primary culture MEFs and observed similar fractions of necrotic and apoptotic cells irrespective of the genotype (data not shown). The mtDNA mutator MEFs are thus no more prone to undergo necrosis and apoptosis in response to oxidative stress than wild-type MEFs.

No Difference in Oxidative Damage to Proteins. To determine the level of oxidative damage to proteins, we measured the amount of carbonyl groups and found no difference in total protein extracts from hearts of 40-week-old mtDNA mutator and wild-type mice (Fig. 3*A*). However, a small increase in oxidative damage was found in total liver protein extracts from mtDNA mutator mice (relative levels, 139 ± 21%) compared with wild-type mice (100 ± 23%). Surprisingly, carbonyl levels in protein extracts from liver mitochondria were not significantly different between mtDNA mutator (107 ± 19%) and wild-type mice (100 ± 18%), suggesting that the increase in total liver extracts was because of extra mitochondrial protein carbonylation in mtDNA mutator mice.

Iron–sulfur clusters are particularly sensitive to ROS damage, and proteins harboring such clusters may lose activity in response to oxidative stress (22). The activity of mitochondrial aconitase is critically dependent on an iron–sulfur cluster, and this enzyme activity is, therefore, often used as a marker of oxidative damage. We found normal aconitase activities in heart mitochondria from 12-, 25-, and 40-week-old mtDNA mutator mice (Fig. 3*B*). Hence, no evidence of increased protein oxidation was found in mitochondria of the prematurely aging mtDNA mutator mice.

No Increased Expression of Antioxidant Defense Enzymes. Increased ROS levels will induce the expression of antioxidant defense enzymes, and increased ROS scavenging could potentially explain the normal ROS levels and absence of oxidative damage in cells and tissues from mtDNA mutator mice. We performed Northern blot analyses and found normal levels of manganese-dependent SOD (or SOD2) transcripts (Fig. 4*A*) and only a minor (1.5-fold, non-significant) increase in glutathione peroxidase 1 (GPX1) transcript levels in heart of 25- and 40-week-old mtDNA mutator mice (Fig. 4*A*). We also analyzed SOD2 protein levels in total protein extracts

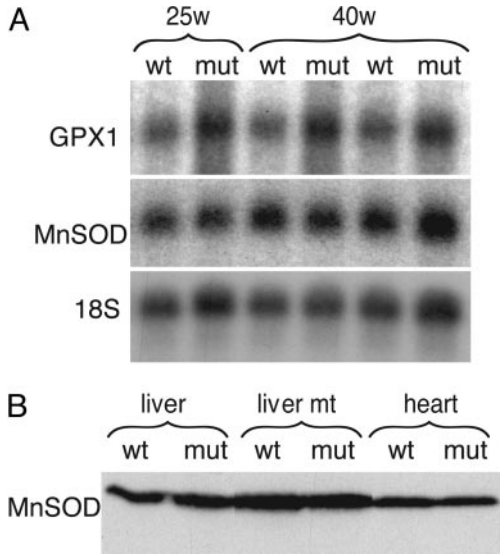

Fig. 4. Expression of antioxidant defense enzymes. (*A*) Northern blot analyses showing transcript levels of GPX1 and SOD2 in hearts of 25- and 40-week-old mice. The levels of 18S ribosomal RNA was used as loading control. (*B*) Western blot analysis showing SOD2 protein levels in total protein extracts from heart, liver, and purified liver mitochondria at 40 weeks of age. Mut, mutants; wt, wild types.

from heart and liver and liver mitochondrial protein extracts (Fig. 4*B*) and found no difference between wild-type and mtDNA mutator mice at age 40 weeks. The minimal induction of expression of SOD2 and GPX1, two ROS scavenging enzymes located in mitochondria, suggests that there is no major increase in oxidative stress in mtDNA mutator mice.

Discussion

We demonstrate that increased ROS formation and oxidative damage are unlikely to be major pathophysiological events in the premature aging process induced by elevated levels of mtDNA mutations. These results are in agreement with findings from a similar mtDNA mutator mouse model that was described recently (23). We have previously studied mitochondrial transcription factor A (*Tfam*) conditional knockout mice and demonstrated that loss of this protein causes severe mtDNA depletion and a profound respiratory chain deficiency in affected tissues (19, 20, 24–26). Loss of *Tfam* leads to considerable cell death by apoptotic and/or necrotic pathways in embryos (19, 20) and differentiated tissues such as heart (19, 24), neurons (25), and insulin-secreting β-cells (26). However, in *Tfam* knockout mice, no or minor induction in the expression of enzymes involved in ROS scavenging was found, suggesting that oxidative stress is not an important pathophysiological mechanism in these animals. Loss of TFAM protein should lead to a global decrease of all 13 mtDNA-encoded respiratory chain subunits, resulting in an essentially quantitative defect of respiratory chain function. In contrast, the mtDNA mutator mice acquire an apparently random set of mtDNA point mutations, which would be expected to result in both quantitative and qualitative effects on respiratory chain function. It can be anticipated that at least some of the point mutations should result in increased ROS production in mtDNA mutator mice. It is therefore surprising that we found no increase of ROS production and no or minor increase of oxidative damage in cultured cells and tissues from mtDNA mutator mice at different ages.

The mitochondrial theory of aging predicts that levels of mtDNA mutations should increase exponentially as a consequence of a vicious cycle by accelerating oxidative stress. However, we found an approximately linear increase of mtDNA mutation levels from midgestation to late adult life in mtDNA mutator mice, implying that there is no vicious cycle. It should be noted that our measurements of mtDNA mutation load might be an underestimate because cells with the highest levels of deleterious mutations may be lost because of cell death and/or replicative disadvantage. Point mutations in protein coding genes affecting codon positions 1 and 2 are more likely to result in amino acid replacements and respiratory chain dysfunction than mutations affecting codon position 3. It can therefore be anticipated that point mutations resulting in a large amount of cell death would lead to preferential loss of cells with mutations at codon positions 1 and 2. In contrast to this prediction, the spectrum of mtDNA mutations in protein-coding genes in mtDNA mutator mice is apparently random with respect to codon position (12), even in the oldest animals studied (data not shown).

Formation of 8-oxo-2′-deoxyguanosine (oxo^8-dG) is the most common consequence of oxidative damage to DNA in a cell (6). It has been reported that aging human brain exhibits an increase of oxo^8-dG in mtDNA and that this increase correlates with mitochondrial dysfunction (27). It has since been suggested that accumulation of oxo^8-dG to high levels leads to increased levels of mtDNA mutations, triggering mtDNA instability, reduced rates of mitochondrial protein synthesis, and production of mutant polypeptides that compromise mitochondrial respiration (for review, see ref. 28). However, a recent study failed to find evidence of mitochondrial respiratory dysfunction even when levels of oxo^8-dG are highly elevated (29). Mice deficient for oxoguanine DNA glycosylase (OGG1), an enzyme responsible for oxo^8-dG removal, have a 20-fold increase in oxo^8-dG levels compared with wild-type mice, yet their mitochondria are functionally normal. The authors found no differences in ATP synthesis rate or maximal activities of complexes I and IV and no indication of increased oxidative stress in mitochondria from *OGG1*$^{-/-}$ mice, as measured by protein carbonyl content (29). Such findings support the idea that oxidative stress may not be a major inducer of mtDNA mutations in mammals.

Numerous investigators have reported a positive correlation between increased ROS production/oxidative damage and age (3, 30–33). Furthermore, various manipulations that increase lifespan also diminish the age-related increase in oxidatively damaged molecules (3, 34–36). Support for ROS involvement in aging has been obtained from experiments on caloric restricted rodents (37–41) and on different genetically modified flies and worms (36, 42–45). However, most of the evidence that is consistent with the free radical theory of aging is indirect, and some results contradict the theory. Overexpression of cytosolic Cu–Zn SOD (or SOD1) and catalase in long-lived strains of *Drosophila melanogaster* produced no beneficial effects on survival in the mutant flies (46). The introduction of transgenes encoding SOD2 or thioredoxin reductase in the same genetic background also failed to extend lifespan (46). However, others have shown that overexpression of Cu–Zn SOD in the fly resulted in an increased lifespan of up to 48%, with no additional benefit of overexpressing catalase in the same model (47). Experimental results obtained from genetic mouse models, in which expression levels of different antioxidant enzymes have been altered, are even more confusing. Overexpression of Cu–Zn SOD in mice resulted in animals that were more resistant to cerebral ischemia but had the same lifespan as wild-type mice (48). Conversely, overexpression of human catalase targeted to the mitochondria resulted in extension of median and maximum lifespan in transgenic mice (39). In these animals, H_2O_2 production and H_2O_2-induced aconitase inactivation were attenuated, the oxidative damage and the development of mitochondrial deletions were decreased, and the development of cataract and cardiac pathology was delayed. In contrast, targeting human catalase to peroxisomes or nucleus did not extend the lifespan of transgenic mice (39). A mouse heterozygous for a null mutation of *SOD2* displayed 30–80% reduced activity of this enzyme in a variety of tissues (37, 40). This resulted in increased oxidative damage in all examined tissues

of $SOD2^{+/-}$ mice compared with wild-type mice and higher incidence of cancer (37, 41). However, no difference in either mean or maximal lifespan was evident (37). Furthermore, mice deficient for the both of the major mitochondrial antioxidant enzymes SOD2 and GPX1 ($SOD2^{+/-}/GPX1^{-/-}$) are extremely sensitive to oxidative stress, yet appear phenotypically normal, and can reproduce and have normal lifespan (38). It should be emphasized that the interpretation of the above-mentioned experiments in model organisms is complicated by the fact that ROS do not only cause oxidative damage but also have important roles in cell signaling (49).

There is clearly an intense, ongoing debate about the role of ROS and oxidative stress in the aging process. The present study shows that there is no direct connection between increased levels of mtDNA mutations and elevated ROS production and argues against any direct role of oxidative stress in the aging process. Because mtDNA mutator mice develop aging-related phenotypes without evidence of increased oxidative stress, but nevertheless do exhibit respiratory chain deficiency, the latter would seem to be a better candidate for the primary inducer of premature aging. Respiratory chain dysfunction may accelerate aging by provoking bioenergy deficit in physiologically crucial cells, by decreasing the signal threshold for cell death, by causing replicative senescence in stem cells, or by some other mechanism. The mtDNA mutator mouse will be an invaluable resource to dissect these pathways further.

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