Mechanisms of mtDNA segregation and mitochondrial signalling in cells with the pathogenic A3243G mutation

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General introduction

Introduction

Cells consume energy for basic household tasks and specialized functions. Biosynthesis of proteins and nucleic acids, transport of ions across membranes for electric activity, intracellular transport of proteins, RNAs and organelles, locomotive and contractile processes are prominent examples of energy consuming events. Also in the cybernetics of a cell, that is, the control of gene expression and signaling, a small but significant amount of energy is continuously invested.

Carbohydrates and lipids are the main chemical sources of cellular energy. Amino acids in protein may also be used as energy source. Nutrient molecules are taken up by the cell from the environment through dedicated transport systems. Once in the cell they serve in anabolic processes as building blocks for macromolecular constituents or as precursors of intermediate metabolites. The majority of the energy content of especially fatty acids and glucose is, however, transformed in catabolic pathways to a high-energy compound called adenosine 5'-triphosphate, abbreviated as ATP. ATP may be considered as the universal energy currency of cells: virtually all energy demanding processes use ATP directly or indirectly as an energy source by hydrolyzing it to ADP and inorganic phosphate.

Oxidative phosphorylation, often abbreviated as OXPHOS, is the catabolic pathway responsible for most ATP production. Glycolysis also produces ATP but its ATP yield is only about 1/18th of oxidative phosphorylation.

OXPHOS uses energy contained in reduced cofactors called NADH and $FADH_2$ to convert ADP to ATP and in the process reduces dioxygen (O_2) to water. Most of the NADH and FADH2 is produced in the tricarboxylic acid cycle (also called TCA-, Krebs or Citric Acid cycle) and fatty acid ß-oxidation. The doorway to the TCA cycle for all fuel-molecules: sugars, fat and proteins, is acetyl coenzyme A and both the TCA cycle and fatty acid ß-oxidation and OXPHOS itself take place in the mitochondria.

Evidently, mitochondria take a central position in energy metabolism and often they are referred to as the powerhouse of the cell (1). Obviously, failure of the powerhouse is detrimental to the cell. It inevitably leads to loss of cell function and death.

This truism is rightfully used to explain late cellular events in diseases with a mitochondrial etiology. Also in physiological aging, failure of the cell's powerhouse is alleged as etiological. The truism, however, gives no insight in the molecular processes that initiate and amplify mitochondrial dysfunction, nor does it pinpoint leads for therapeutic intervention.

Mitochondria are involved in multiple other processes besides energy production, such as apoptosis through an intricate regulation of cytochrome c release by pro- and anti-apoptopic factors, the regulation of cytosolic calcium concentrations, metabolism of fatty acids and some amino acids, iron homeostasis and cholesterol/steroid biosynthesis (2). The undermining of the various processes involving mitochondrial functions may lead to disease.

In contrast to the other cytoplasmic organelles, mitochondria contain their own DNA in multiple copies. This mitochondrial DNA (mtDNA) is essential for OXPHOS function and mutations in mtDNA can lead to disease. Diseases with mtDNA mutation in their etiology are remarkable in that they display a high phenotypical diversity. This is counter intuitive when realizing that all pathogenic mtDNA mutations lead to respiratory defects at some point, it might therefore be expected that their phenotypical presentation would be similar. A point in case is made by the A3243G mtDNA mutation. This is a mutation in the mitochondrial $tRNA^{(UUR)}$-leucine gene (*MTTL-1*) that is associated with syndromic and non-syndromic phenotypes with as many as 61 clinical manifestations documented (3).

To explain the wide spectrum of clinical expression, mtDNA mutation accumulation by segregation as well as dominant-negative effects of aberrant mtDNA products on the mitochondrial-nuclear crosstalk have been proposed, but the mechanisms remain essentially undisclosed.

This thesis attempts to contribute to disclosing such mechanisms by investigating segregation mechanisms and identifying nuclear genes that alter expression under A3243G mutational mitochondrial dysfunction. Starting off with a brief view on origin of mitochondria, a number of aspects of mitochondrial genetics, disease and cell biology of relevance for the experimental chapters of this thesis are briefly highlighted in the next paragraphs.

Origin of mitochondria

It is a well accepted evolutionary view that mitochondria result from a prokaryotic symbiosis of a fermenting cell producing ATP only by substrate level phosphorylation as in glycolysis and a cell capable of much more efficient ATP production by coupling respiration to ADP phosphorylation. The former is envisioned to have engulfed the latter by an endocytotic process. The double membrane appearance of mitochondria upon electron microscopical examination, presence of DNA, sensitivity of mitochondrial protein synthesis to prokaryotic translation inhibitors, similarities of the mitochondrial inner membrane lipid composition and bacterial membranes are among the popular arguments for the endosymbiotic view (4), which is confirmed by modern comparative genomics (5;6). Comparative mtDNA genomics across eukaryotes presents the view of mtDNA being structurally very diverse, but well-conserved and limited in genetic function. It contributes invariably to mitochondrial protein synthesis and oxidative phosphorylation and occasionally to transcription and protein import (7).
The origin and evolution of the mitochondrial protein content is under active study. The proteome of the ancestral endosymbiont appears to have undergone major changes in protein content by extensive losses and gains as assessed by comparative, mass-spectrometry based proteomics (8). It is remarkable that in the evolutionary process of shaping eukaryotic mitochondria a tiny fraction of the ancestral genome with very limited and universally conserved functions resisted elimination.

Mitochondrial DNA

The double stranded, circular mitochondrial human mtDNA of 16.569 basepair is densely packed with genetic information (figure 1) and occurs in 100s to 1000s of copies per cell. The only non-coding part of ~ 1 kb (the D-loop) is highly polymorphic and the target of demographic and forensic mtDNA studies. mtDNA encodes 13 proteins of the ~90 that make up the 5 protein Complexes of the oxidative phosphorylation system (figure 2).

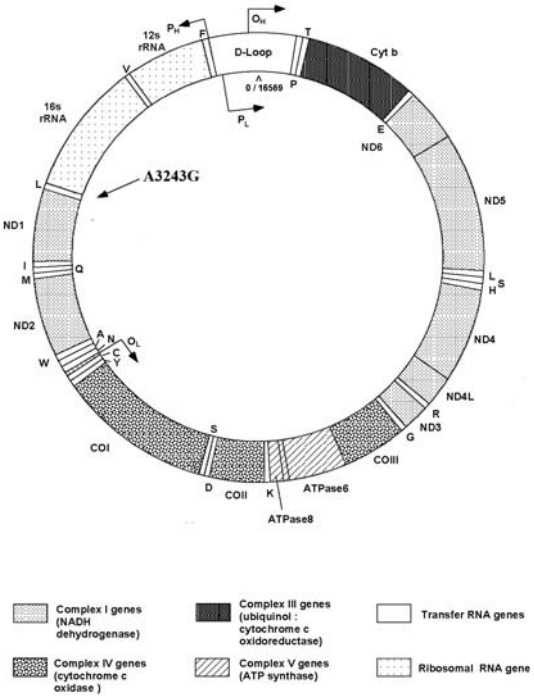


Figure 1: Mitochondrial DNA
The mitochondrial DNA is a circular, double stranded molecule of 16.569 basepairs, most of which are coding. Next to the 13 OXPHOS protein genes it contains the sequences for 2 rRNAs and 22 tRNAs as indicated. The A3243G mutation is located in the tRNA leucine (UUR) gene, note that there is a second tRNA leucine gene present (CUN). The D-loop is the only non-coding part of the mtDNA, which is highly variable and contains the center of origin for replication of the heavy chain (O_H) and the transcription start sites of both the heavy and light chain (P_H and P_L).

Noteworthy, only Complex II is fully encoded by the nuclear genome. mtDNA also codes 22 tRNAs and 2 rRNAs which function in translation of the mtDNA encoded proteins. All other OXPHOS proteins and factors needed for mitochondrial protein synthesis are nuclear encoded and imported from the cytoplasm. The same holds true for factors involved in mtDNA replication and transcription. It is beyond the scope of this thesis to review mtDNA transcription (9), translation (10), repair (11) and replication (12). However it is of importance to note that mtDNA replication is relaxed, that is, it occurs independent of the nuclear DNA synthesis phase and that it is in constant turnover (13). In post-mitotic cells also extensive mtDNA turnover occurs and based on available half life data of 2 – 10 days (14;15), it can be calculated that in a life-time of a neuron or cardiomyocyte its mtDNA content is refreshed approximately 1500 to 15.000 times.

Due to inefficient mtDNA repair mechanisms (11) and a mutagenic oxidative environment constituted by the nearby location of the respiratory chain, mtDNA experiences high mutation rates. mtDNA mutation rates are on average 5-10 times higher compared to nuclear DNA, but hotspots exist. Today there are hundreds of point mutations, deletions and rearrangements in mtDNA known, many of them associated with disease (see www.mitomap.org).

As said a cell may contain hundreds to thousands copies of mtDNA and a sequence variant (or mutant form) can therefore co-exist with the original sequence. The occurrence of both wild type and mutant mtDNA in one cell is called heteroplasmy as opposed to homoplasmy. The (pathogenic) mutation load can reach percentages up to 80 percent or more without any perceptible malfunction of the cell (16;17), although the actual threshold varies per cell type and mutation. Thus wild type mtDNAs can compensate mutants to a large extent. The importance of understanding mechanisms that lead to mtDNA mutation accumulation resulting in failure of oxidative phosphorylation will be evident.

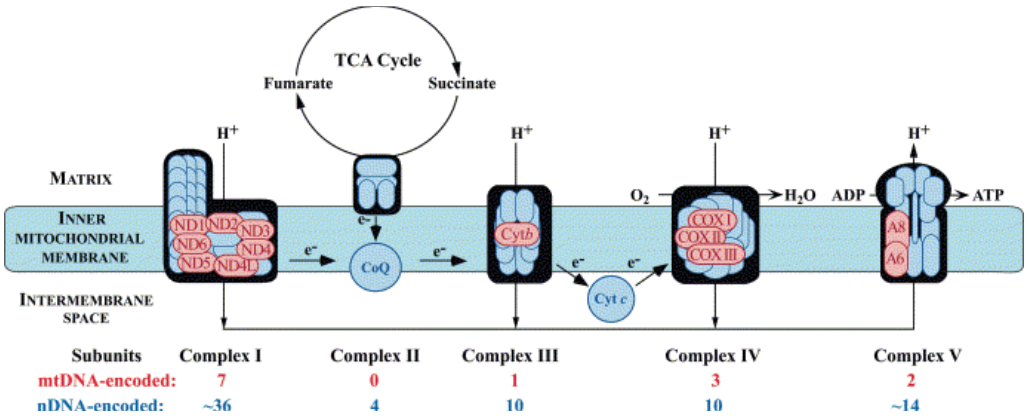


Figure 2: Scheme of oxidative phosphorylation
Oxidative phosphorylation can be divided in two parts, electron transport through the respiratory chain and parallel proton pumping, and the formation of a high-energy bond by phosphorylation of ADP to ATP employing the energy contained in the electrochemical proton gradient. The respiratory chain consists of 4 complexes which contain multiple subunits, most of which are nuclear encoded. Note that complex II does not contain any mitochondrial DNA encoded subunits. The mitochondrial encoded proteins are indicated in the figure as is the flow of electrons and hydrogen ions through the different complexes. The generation of ATP takes place in complex V.

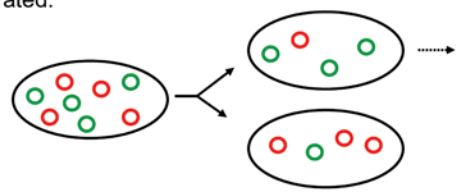
Maternal inheritance and random segregation in germ line and soma

Sperm mtDNA is rapidly eliminated after fertilization (19). Consequently, mtDNA inheritance is indeed maternal in virtually all conceptions (20). Because of the absence of a paternal partner recombination of mtDNA does not occur in the germ line. When a female transmits two mtDNA variants to the zygote, the cells descending from the heteroplasmic primordial germ cell that differentiate to primary oocytes undergo random segregation of the maternal mtDNA alleles. Along with a reduction in mtDNA copy number this is assumed to lead to a large increase in the variance of heteroplasmy in the mature oocytes and consequently offspring (21). This mtDNA genetic bottleneck elegantly explains why in the mammalian population homoplasmy is the norm and heteroplasmy rare. It leads to fixation of either of the mtDNA alleles in only a few or even one organismal generation, contributing to characteristic demographic distribution of mtDNA haplogroups and exposing new non-neutral sequence variants homogenously to evolutionary forces (22).

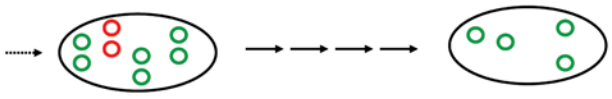
Non-neutral mtDNA mutations are under positive or negative selection. Positive selection may contribute to climatic adaptation. Conversely, negative selection eliminates deleterious mutations from the population. It is thus random mitotic mtDNA segregation (see box) and negative selection that is assumed to purge deleterious mtDNA, with rate of random segregation (and purging) being primarily determined by mtDNA copy number in the female germ line. Recently it has become clear however, that in the germ line of mice mtDNA segregation may not go hand in hand with strong copy number reduction (23). Thus, other mechanisms than random mtDNA segregation may underlie rapid generational mtDNA variant changes.

Also in the soma, random (mitotic) segregation is thought to be the norm. However, a multitude of clinical investigation and heteroplasmy mouse model studies with naturally occurring neutral mtDNA mutations, unambiguously show that there are notable exceptions to the presumed rule of random segregation in somatic cells (24;25).

The process of random mitotic segregation of mtDNAs from a cell can be compared to drawing half of the balls from a box containing a finite number of red and green balls, one by one, without replacement. If a founding cell has equal numbers of wild type (green) and mutant (red) molecules (balls), there is a chance of the daughter cells not containing equal numbers of both types, as illustrated:



Replication of mtDNA is illustrated by duplication of the balls. Several succeeding duplication-and-drawing experiments will lead to a situation where the resulting boxes contain only balls of one uniform color. Such cells are said to be genetically fixed.



The number of draws it takes to reach a fixed situation is dependent on the number and ratio of the balls. The final population will have the same ratio of red and green cells as the ratio of red and green molecules in the founding cell.

Box: Illustration of random segregation; the hypergeometric distribution

mtDNA is organized in nucleoids

In recent years it has become evident that mtDNA does not occur in mitochondria as naked molecules (26). Several mtDNA molecules form a complex with a set of proteins, of which mitochondrial transcription factor A (TFAM) is the most abundant and, next to its role as a transcription factor, seems to function in a similar way as the histones do for nuclear DNA in that it packages mtDNA (27). Other proteins in the mtDNA-protein complexes known as nucleoids are identified and include mitochondrial single-stranded DNA-binding protein (mtSSB), polymerase γ and the DNA helicase Twinkle (28). The number of mtDNA molecules and the actual shape and molecular composition of the nucleoids remain uncertain. Two publications give an indication for the number of mtDNAs per nucleoid: it should be around 2 to 10 in human cells (29;30).

At the next higher level of mtDNA genome organization may reside the mitochondrial compartment. Its morphological appearance as many fragmented mitochondria, as a single tubular reticulum or intermediates, is highly dynamic and dictated by the balance of the activities of nuclear encoded mitochondrial fusion and fission genes (see page 14).

In conclusion, it is not a collection of freely diffusing single mtDNA molecules that constitute the mtDNA segregation unit. Rather, there appear two levels of organizational complexity of mtDNA genome segregation, the nucleoid and the dynamic mitochondrial compartment.

mtDNA and disease

Mitochondrial diseases afflict ~1 in 5000 of the human population (31), part of which are caused by inherited mitochondrial DNA mutations. In addition, healthy individuals acquire mtDNA mutations during life, contributing to such common ageing phenomena as muscle weakness and neuro-degeneration.

Since all pathogenic mtDNA mutations lead to respiratory defects at some point, it might be expected that their phenotypical representation would be similar. On the contrary, however, there is great clinical variance among mtDNA diseases, with many characterized by tissue-specific defects (32). An important feature of mitochondrial DNA disease is the threshold effect: a biochemical defect e.g. as measured by oxygen consumption becomes overt only after the percentage of mutation exceeds a given threshold. Typically, the consumption of oxygen, which is an indication for the amount of ATP produced by the mitochondria, decreases tenfold if the cells carry over approximately 80 percent mutated mtDNA (16), though actual thresholds may vary .

Understanding the molecular mechanisms which control the level of mutant mtDNA and the ensuing pathobiochemistry obviously is an important aspect to understanding mitochondrial and ageing diseases, as far as mtDNA is involved in the latter. Spreading and accumulation of mtDNA mutations by segregation in cells and tissues may underlie mtDNA disease variability, but mechanisms are elusive.

Segregation leading to mutation accumulation above threshold and loss of OXPHOS may as such be able to explain mtDNA diseases with a heteroplasmy degree exceeding the 80-90% threshold, as for instance in patients with the phenotype of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS; OMIM 540000) in which the A3243G mutation studied in this thesis was first discovered (33;34). Segregation however fails to explain pathogenesis of the maternally inherited diabetes and deafness phenotype of the A3243G mutation (MIDD; OMIM 520000). MIDD is a type of diabetes (contributing ~5%) which resembles mostly type II diabetes without the obese appearance. It is a maternally transmitted disease with an average age-of-onset of 38 years and the glucose intolerance is caused by impaired insulin secretion of the pancreas, but the relevant tissue, pancreatic ß-cells, contains only moderate levels of mutation load (35), far from the ~80 % threshold levels required for mitochondrial OXPHOS dysfunction. A dominant negative gain-of-function would explain the lack of threshold

effect seen in this case. The $tRNA^{(UUR)}$-leucine mutation could lead to dominantly acting, qualitative translation defects like amino acid mis-incorporation or premature translation termination products directing synthesis of a set of aberrant translation products (36). Such dominant acting factors could by e.g. reprogramming the nuclear transcriptional program, lead to ß-cell dysfunction.

mtDNA and aging

The phenotypes of mitochondrial DNA diseases often resemble diseases of the elderly (deafness, muscle weakness, diabetes, neuro-degeneration, cardiomyopathy), implying mtDNA mutation in aging. For many decades the vicious circle theory of somatic accumulation of mtDNAs has dominated the mtDNA aging literature. It states that a first somatically acquired mtDNA mutation ignites a self-amplifying process of mitochondrial ROS generation, oxidative mtDNA mutation and deleterious oxidative protein modification. It predicts that in a single cell, random mtDNA mutations will accumulate exponentially with age. Formally never proven and lingering in scientific literature, recent experimental evidence now strongly defy this view.

First, single cell analyses of mitotic and post-mitotic tissues of healthy, aged individuals show that in individual (Cytochrome c oxidase negative) cells of a given tissue (brain (37;38), muscle (39), colon mucosa (40), kidney (41), cardiomyocytes (42), accumulation of unique, not random, mtDNA mutations occurs. Second, model system studies with the mtDNA mutator mice (mice with proofreading-deficient polymerase γ), which dramatically and prematurely replicates physiological aging, do not show exponential mtDNA accumulation (43;44). They do show increased apoptosis (45), in line with notion of reduction of organ function with aging. What transpires is that clonal accumulation of unique mtDNA mutations acquired early in development through mtDNA replication errors or mitochondrial ROS, appears to be a significant contributor to physiological aging.

Morphological dynamics of mitochondria

By light microscopy mitochondria can be observed in unstained living cells as filamentous or grain like structures. These appearances underlie their etymology. The word mitochondrion is derived from the Greek words for thread (mito) and grain (chondro). Major developments in fluorescence microscopy and in vivo staining nowadays enables a much more detailed view on organelle dynamics and motility of molecular constituents. Particularly the use of lipophilic, cationic fluorescent dyes (which accumulate in well functioning mitochondria due to the electrochemical proton gradient) and expression of mitochondrially targeted auto-fluorescent proteins in combination with sophisticated fluorescence microscopy techniques, led to the contemporary view that the mitochondrial compartment is dynamic, not only in terms of shape, size, motion and number of mitochondria, but also in terms of fusion and fission and mobility of intra-mitochondrial molecules. It is overtly clear now that the balance between the activity of fusion and fission proteins determines the actual morphology of the mitochondrial compartment. Depending on cell type and stage, many distinct, individual mitochondria, a single continuous mitochondrion of filamentous shape and any intermediate state can thus be found (46). The mitochondrial GTPases Mitofusin 1 and 2 and Opa1 are now well established as mediators of mitochondrial fusion (47). Mitochondrial fission is mediated by Drp1, another mitochondrial GTPase of the dynamin family

Microscopic Fluorescence Recovery After Photobleaching (FRAP) experiments permit to analyze diffusion parameters of macromolecules in living cells. Such experiments with mitochondrially targeted auto-fluorescent proteins showed their extensive intra-mitochondrial diffusion (48).

It follows naturally from the above that cells have the potential to homogenize their mitochondrial compartments among which is mtDNA.

Indeed, cells deprived of mitochondrial fusion genes do not mix their reporter contents as shown by elegant cell fusion experiments with mfn-/- and mfn+/+ embryonic mouse cells differentially labeled with permanent mitochondrial markers (49) By sampling synchronized cells in time, the morphology of mitochondria has been found to oscillate between the reticular and fragmented state in a cell cycle dependent manner (50). Mitochondria of cells in mid-interphase appear as a tubular network (figure 3), whereas shortly before, during and after mitosis the mitochondrial mass is fragmented. It is this fragmentation that ensures that daughter cells get their share of mitochondria, including mtDNA.

Electron microscopy and conventional electron microscopical contrast techniques provide a view that is quite familiar to many researchers. Healthy mitochondria viewed through an electron microscope look at first sight like oval-shaped forms, grain-like indeed, which appear covered with stripes. On detailed inspection, a two membrane system becomes usually obvious. What is well known as the outer membrane lines the oval shape, whereas the heavily folded inner membrane is responsible for the stripes, known as cristae. The two membranes clearly define sub-mitochondrial compartments: the inter membrane space and the mitochondrial matrix. Of note in normally functioning mitochondria, linear cristae are seen while aberrant patterns such as circular or concentric ring pattern are an indication of dysfunction. The popularity of electron microscopic images of mitochondria may have contributed to the long held, but incorrect view that they occur as distinct and individual organelles each containing their own mtDNA, instead of the dynamic network which the mitochondria actually form.

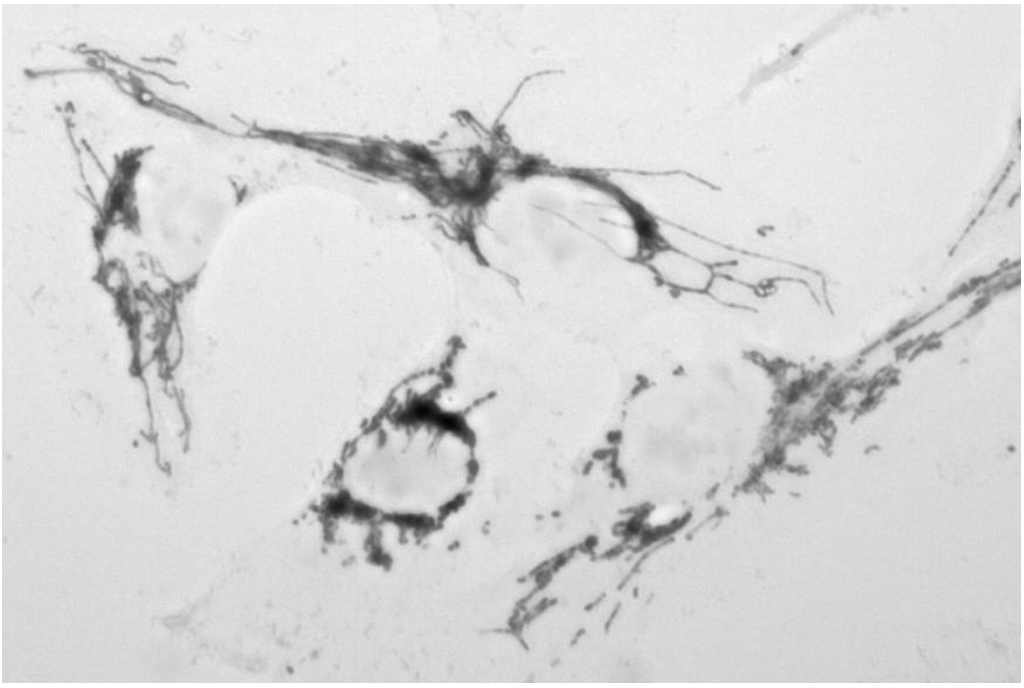


Figure 3: Mitochondrial morphology assessed by enzyme-cytochemical staining
Using exogenous cytochrome c as substrate Cytochrome c oxidase (COX) in these fixed cells has converted diaminobenzidine tetrahydrochloride (DAB) into a brown reaction product which is visible. The shape of the functional mitochondria becomes visible, either as fragments or a tubular network.

Scope of this thesis

In the Introduction of this thesis it is argued that tissue–specific mtDNA segregation and dominant negative effects of mtDNA mutations may underlie variable disease expression of A3243G mutant mtDNA. These two aspects have been investigated in the experimental chapters of this thesis. Chapters 2 and 3 deal with mtDNA segregation analysis. Specifically, Chapter 2 describes two methodologies that were key to the experiments of Chapter 3, where the role of nucleoids in segregation has been analyzed.

In Chapter 4 and 5, genome wide gene expression experiments are presented, analyzing the effect of mitochondria with an A3243G mutation or total DNA depletion (ρ^0 cells) on nuclear gene expression, with the aim to uncover mutation-specific nuclear transcriptional responses. In Chapter 5 additional experiments are described to dissect the signaling of mitochondrial dysfunction to the cytosolic protein synthesis machinery. Chapter 6 discusses experimental results.

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