Section 1 Introduction to Mitochondrial Medicine

Chapter 1

Mitochondria in Health and Disease

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Mitochondrial Structure and Function

Mitochondria are essential organelles found in nearly all eukaryotic cells [1]. The most prominent role for mitochondria is to supply the cell with spendable energy in the form of adenosine triphosphate (ATP) generated by oxidative phosphorylation (OXPHOS). Besides this fundamental role, mitochondria take part in a number of processes relevant for cellular physiology, including, among others, heat production, apoptosis, generation and detoxification of reactive oxygen species (ROS), intracellular Ca^{2+} regulation, steroid hormone and heme synthesis and lipid metabolism [2] (Figure 1.1).

Mitochondria are organized into four morphologically and functionally distinct compartments (Figure 1.2): (i) the outer membrane (OM), which is freely permeable to ions and small molecules, unlike bigger metabolites and proteins, whose traffic is mediated by specific transporters and channels; (ii) the intermembrane space (IMS), where several important processes take place, such as exchange of proteins, lipids or metal ions between the matrix

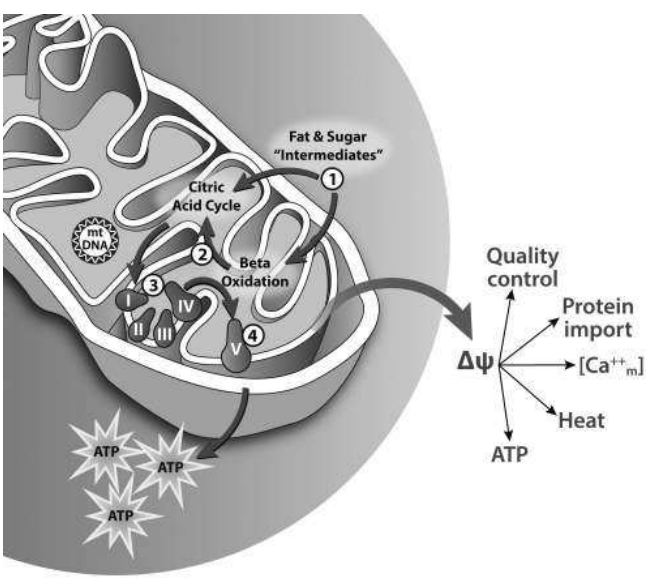

Figure 1.1 Main metabolic pathways within mitochondria. (A black and white version of this figure will appear in some formats. For the color version, please refer to the plate section.)

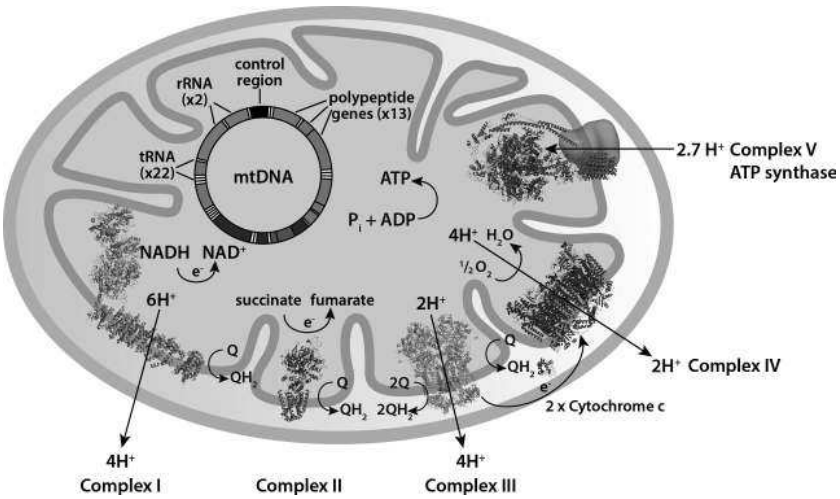

Figure 1.2 Overview of the OxPhos system (complexes I–V) and of the organization of mitochondrial DNA. (A black and white version of this figure will appear in some formats. For the color version, please refer to the plate section.)

and the cytosol, the initiation of apoptotic cascades and insertion of specific proteins, e.g., mitochondrial carriers, into the outer or inner membranes; (iii) the inner membrane (IM), which surrounds the matrix and mediates the transport of ions, metabolites and proteins through specialized transporters and folds into invaginations called cristae, where the respiratory complexes are localized; and (iv) the matrix, which contains the mtDNA and proteins involved in a huge number of biochemical pathways, including the TCA cycle and beta-oxidation of fatty acids (Figure 1.1).

Mitochondria harbor the complexes of the respiratory chain (RC), which convert the energy derived from nutrients into ATP (and heat) (Figure 1.2). This function is carried out by two coupled reactions, respiration and phosphorylation. Respiration is performed by four multiheteromeric complexes (CI–IV), which transfer the electrons extracted from the carbon substrates of nutrients along a redox potential to eventually reduce molecular oxygen into water. The energy liberated during these sequential redox reactions is exploited by proton pumps incorporated in RC complexes I, III and IV to translocate protons from the matrix to the IMS, thus forming an electrochemical membrane potential (ΔP) composed of a chemical (ΔpH) and an electrical (ΔΨ) gradient. ΔP is eventually exploited by the mitochondrial, oligomycin-sensitive ATP synthase (or complex V, CV) to convert ADP into ATP through a rotary reaction by which a complete rotation of the CV c-ring leads to the condensation of three ADP+Pi molecules into three molecules of ATP (Figure 1.2). The number of protons necessary for one rotation to complete varies according to the number of c subunits composing the ring rotor, which establishes the gear for the rotation to take place. In humans, the c-ring is composed of eight subunits, consenting the passage of as many protons through the rotor for each complete rotation. The whole process comprising both respiration and ATP biosynthesis is called oxidative phosphorylation. The respiratory complexes are composed of several subunits, require additional assembly factors and chaperons and are organized in supercomplexes, whose functional relevance is a matter of intense debate [3].

Complex I (CI), or NADH:ubiquinone oxidoreductase is the biggest among the RC complexes (molecular mass: 969 kDa) and transfers electrons from NADH to CoQ [4]. It forms an L-shaped structure with a peripheral arm protruding into the matrix and containing the NADH dehydrogenase and electron flux activities, and a membrane arm containing the proton pumps. The "heel" of the L contains a long channel, which accommodates CoQ. The protein backbone of CI is composed of 14 core subunits, seven of which are highly hydrophobic mtDNA-encoded proteins (all part of the membrane arm), and seven hydrophilic nuclear DNA (nDNA)-encoded subunits (part of the peripheral arm). In addition, human CI contains 30 supernumerary nDNA-encoded subunits, which play important, albeit still poorly understood roles for assembly, regulation, stability or protection against oxidative stress. Nonprotein components of CI include a flavin mononucleotide (FMN) moiety acting as the catalytic center for the NADH dehydrogenase activity of the complex, and eight Fe-S clusters which transfer electrons through the peripheral arm to CoQ.

Complex II (CII), or succinate:ubiquinone oxidoreductase, is assembled from four nDNA-encoded polypeptides (SDHA-D, molecular mass: 120 kDa), which transfer electrons from $FADH_2$ to coenzyme Q [5]. In addition to the FAD moiety bound to the largest (70 kDa) subunit, it contains three Fe-S centers carrying out the electron flux through the complex, and a poorly understood heme moiety embedded in the hydrophobic portion anchoring CII to the IM.

Complex III (CIII), or ubiquinol:cytochrome c oxidoreductase, is a homodimeric complex (molecular mass: 480 kDa) transferring electrons from CoQ to cytochrome c, and consists of 11 subunits for each monomer [6]. Cytochrome b is the only mtDNA-encoded subunit, and forms the catalytic core along with the Fe_2S_2 cluster-containing Rieske protein, and cytochrome c_1. Most of the other eight subunits are small proteins that surround the metalloprotein nucleus.

Complex IV (CIV), or cytochrome c oxidase (COX), is the terminal enzyme of the electron transfer chain, and catalyzes the electron transfer from reduced cytochrome c to molecular oxygen [7]. Mammalian CIV is a heteromeric complex composed of 14 subunits. The three largest, Cox1, Cox2 and Cox3, are highly hydrophobic transmembrane proteins encoded by mtDNA and form the catalytic core, while the 11 nDNA-encoded, smaller subunits are involved in the regulation of COX activity, its stability and dimerization of the catalytically active enzyme. Two copper centers, CuA and CuB, are contained in Cox2 and Cox1, respectively, whereas Cox1 also contains two heme a moieties, which are also part of the CIV catalytic core.

Complex V (CV), or ATP synthase, catalyzes the synthesis of ATP from ADP and inorganic phosphate (P_i) using the energy provided by the proton electrochemical gradient [8]. ATP synthase consists of the F_1 portion, a soluble portion situated in the mitochondrial matrix, composed of three copies of subunits α and β, and one copy of subunits γ, δ and ε, and the F_o portion, bound to the inner mitochondrial membrane consisting of eight c-ring subunits and one copy each of subunits a, b, d, F6 and the oligomycin sensitivity-conferring protein (OSCP). Subunits b, d, F6 and OSCP form the peripheral stalk that lies to one side of the complex. A number of additional subunits (e, f, g and A6 L), all spanning the membrane, are

associated with F_o. Two of the F_o subunits, subunit *a* and A6 L, are encoded by the mtDNA ATP6 and ATP8 genes, respectively.

Mitochondrial Biogenesis and Quality Control

Mitochondria are under the double genetic control of mtDNA and nDNA, and a finely tuned genetic network has evolved in order to functionally connect the two genomes [9]. The pathways controlling mitochondrial biogenesis are centered on the activity of the PPARγ coactivators (PGC)1-α and -β, which interact and drive the activity of several OXPHOS-related transcription factors, including the Nuclear Respiratory Factors (NRF1 and 2), and the Peroxisomal Proliferator Activator receptors (PPAR, β, and γ) among others. NRFs and PPARs in turn increase the transcription of OXPHOS and fatty acid oxidation (FAO)–related genes. They also regulate the expression of mitochondrial transcription factor A (TFAM), which is an indispensable component of mitochondrial transcription and replication systems. PGC1α is the best-characterized member of this family, its activity being repressed by acetylation, operated by acetylase GCN5 and increased by deacetylation, mainly through the nuclear deacetylase SIRT1; and by phosphorylation, regulated by several kinases, including p38 MAPK, glycogen synthase kinase 3b (GSK3b) and AMP-dependent kinase (AMPK).

Mitochondria are highly dynamic organelles whose shape and network is regulated by the opposing processes of fusion and fission [10]. Mitodynamics transactions provide an important quality-control mechanism, since fusion contributes to mitochondrial maintenance, and fission allows the segregation and eventually disposal of dysfunctional mitochondria [11]. Dynamin-related GTPases on the OM (Mitofusins, MFN 1 and 2) and IM (OPA1) control the fission process, while the cytosolic soluble dynamin-related protein 1 (DRP1) regulates fission. DRP1 interacts with docking adaptors (FIS1, MFF and MiD49/51), forming spiral filaments that drive mitochondrial constriction and fragmentation upon translocation to the OM. This process is particularly important for the elimination of dysfunctional mitochondria that are then targeted for autophagic degradation, a process called mitophagy. Mitophagy can also be triggered by mitochondrial dysfunction through a decrease in membrane-potential-driven protein import. In normal conditions, the kinase PINK1 is imported into mitochondria and rapidly degraded; a decrease in import caused by ΔP drop causes PINK1 to accumulate on the outer membrane, where it recruits the E3 ligase Parkin, which in turn ubiquitinates a specific subset of OM proteins including Mitofusins, and promotes their proteasomal degradation. The Parkin-dependent degradation of factors involved in mitochondrial motility and fusion enhances the selectivity for the removal of defective mitochondria by mitophagy. Contrariwise, mitochondrial hyperfusion observed during nutrient starvation is supposed to protect mitochondria from mitophagy through steric hindrance [12].

Genetic Basis of Mitochondria (Including Inheritance)

The human mitochondrial genome is a double-stranded 16.6 kb circular DNA molecule (mtDNA) encoding 13 proteins, which are all part of four (CI, CIII, CIV, CV) of the five canonical multiheteromeric enzyme complexes constituting the OXPHOS system [13]. In addition, mtDNA contains genes encoding 22 tRNAs, and 12S and 16S rRNAs that are required for mitochondrial protein synthesis. There are no introns in the mitochondrial genome, and all 37 genes are adjacent to each other with few exceptions. The distribution of

genes in the two mtDNA strands (called the heavy (H) and light (L) strands because of the different content in G-T versus C-A nucleotide residues) is asymmetrical. Both genes encoding the 12S and 16S ribosomal RNA and most of the tRNA- and protein-encoding genes are contained in the H strand, whereas the L strand contains only the ND6 gene, in addition to some tRNA-encoding genes. An untranslated region of approximately 1 Kb harbors the replication origin of the heavy strand and the promoters for transcription of both strands. Nuclear DNA (nDNA) genes encode all the other subunits which take part in the OXPHOS complexes, and also all the proteins required for their assembly, those carrying out the maintenance and expression of mtDNA, the biosynthesis of the respiratory cofactors and prosthetic groups etc. Thus, the mitochondrial proteome includes approximately 1,500 nDNA-encoded mitochondrial genes, in addition to the 37 mtDNA genes [14].

The intricate molecular machinery involved in the maintenance, transcription and translation of mtDNA includes several proteins encoded by nDNA [15]. These proteins are essential for mtDNA replication (POLγA and B, TWINKLE), for transcription/translation (TFAM, TFB1 M, mTERFs, LRPPRC) or for the balanced maintenance of the mitochondrial dNTPs pool (TP, TK, ANT1 and RRM2B).

Each human cell has hundreds to several thousand mitochondria and every mitochondrion can carry as many as 10 copies of mtDNA packed in nucleoprotein structures called nucleoids. The main protein component of nucleoids is TFAM, which acts as a DNA-packaging molecule by thoroughly binding the mtDNA molecule with a stoichiometry of 35–37 base-pairs per TFAM dimer. Cells and tissues with higher ATP demand typically have more mtDNA.

Usually, all mtDNA copies are identical, a condition known as homoplasmy. However, errors occurring during mtDNA replication or repair can lead to the formation of a mutant mtDNA molecule, which can clonally expand through unknown mechanisms, and eventually fixate in a metastable condition referred to as heteroplasmy, where mutant and wild-type genomes coexist in the same organelles/cells/tissues, in different proportions. Low levels of heteroplasmy have been shown to be present in normal cells, particularly in post-mitotic tissues such as skeletal muscle, or in stem cells of the colonic crypts, and increase over time in parallel with the aging of the individual. However, only when the mutation load of mtDNAs offsets a minimum critical threshold, usually ranging between 70 percent and 90 percent, does mitochondrial dysfunction become manifest in a particular tissue, leading to organ failure and development of a mitochondrial disease. Different tissues exhibit variation in their mutant threshold, with germ cells, for example, having minimal tolerance for the accumulation of mtDNA mutations.

Genetic Basis and Mechanisms of Mitochondrial Diseases

As a result of the dual genetic control of mitochondrial OXPHOS, genetic defects affecting mtDNA or OXPHOS-related nuclear DNA (nDNA) genes can compromise ATP synthesis, determine mitochondrial dysfunction and cause human disease [16]. Mitochondrial disorders are in fact defined as clinical entities associated with defects of mitochondrial OXPHOS, which are ultimately genetically determined. They can exhibit any kind of transmission, including maternal, autosomal dominant, autosomal recessive and X-linked modes of inheritance. While different gene mutations can give rise to a similar range of phenotypes, mutations in the same gene can often lead to a variety of different clinical entities. In addition, different levels of heteroplasmy of the same mtDNA mutation can

result in a wide spectrum of phenotypes. Hundreds of pathogenic mtDNA mutations have been documented (MITO-MAP 2012) that can affect virtually every tissue in the body, leading to different phenotypes depending on their intrinsic severity, targeted gene and heteroplasmy levels. Tissue and organ functions critically depend on adequate ATP production, especially when energy demand is high, like in neurons and muscle fibers [17]. This explains why primary disorders of mitochondrial bioenergetics usually cause neurodegeneration and/or muscle weakness, leading to neuromuscular disease in children and adults. However, specific mitochondrial syndromes can involve any other organ, either individually or in combination with brain and muscle dysfunction. Many mitochondrial disorders also involve multiple organs causing cardiomyopathy and heart conduction defects, liver dysfunction, diabetes mellitus, sensorineural deafness, ophthalmoparesis (weak eye muscles), ptosis (drooping eyelids) and optic neuropathy causing blindness due to degeneration of the optic nerve. Mutations in mtDNA can affect specific proteins of the respiratory chain or the synthesis of mitochondrial proteins as a whole (when mutations or deletions involve tRNA or rRNA genes) and can be in turn divided into large-scale rearrangements (i.e. partial deletions or duplications) and inherited point mutations. Both groups have been associated with well-defined clinical syndromes. While large-scale rearrangements are usually sporadic, point mutations are typically maternally inherited. Large-scale rearrangements include several genes and are invariably heteroplasmic. In contrast, point mutations may be heteroplasmic or homoplasmic, the latter characterized by incomplete penetrance (e.g. Leber's Hereditary Optic Neuropathy).

An increasing number of mutations in nuclear genes encoding mitochondrial proteins have been described, leading to a range of different syndromes.

A genetic classification of mitochondrial disorders is presented in Table 1.1.

Table 1.1 Genetic classification of nDNA-related mitochondrial diseases

mtDNA mutations	Large-scale rearrangements of mtDNA
	Point mutations of mtDNA
nDNA mutations	Genes encoding structural subunits of the OXPHOS complexes
	Genes encoding factors affecting mtDNA maintenance, transcription and translation
	Genes encoding factors involved in the biosynthesis of lipids and cofactors
	Genes encoding proteins involved in mitochondrial protein import and dynamics
	Genes encoding assembly factors of the OXPHOS complexes
	Genes encoding enzymes involved in detoxification pathways
	Genes encoding factors involved in protein quality control
	Genes encoding factors involved in mitodynamics
	Genes encoding factors involved in apoptosis
	Genes encoding factors involved in ion transport
	Genes encoding factors involved in protein import

Mutations in mtDNA

Large-scale rearrangements of mtDNA: mtDNA-rearrangement syndromes are invariably heteroplasmic (homoplasmic large deletions being incompatible with life), and can result in a range of clinical manifestations and a wide spectrum of severity. The size of deletions can vary from a few hundred bases to several kilobases and several genes are usually involved. The syndromes associated with rearrangement of mtDNA range from maternally inherited type 2 diabetes and deafness due to an mtDNA-duplication mutation, to adult-onset chronic progressive external ophthalmoplegia (PEO), childhood or juvenile onset multisystem Kearns-Sayre syndrome (KSS) or a perinatal, life-threatening condition, Pearson's syndrome (PS). PEO is characterized by a progressive paralysis of the eye muscles, leading to impaired eye movement and bilateral drooping eyelids (ptosis). CPEO is typically caused by sporadic large-scale single deletions or multiple mtDNA deletions. KSS is characterized by early onset (childhood or young adulthood) of progressive external ophthalmoplegia, ptosis, mitochondrial myopathy with ragged red fibers, CNS involvement (progressive ataxia and cognitive decay) and potentially life-threatening abnormalities of the cardiac rhythm. PS is characterized by severe, usually fatal pancytopenia and insufficiency of exocrine pancreas. Interestingly, children surviving the pancytopenic phase of PS show a rapid decrease of deleted mtDNA species in bone marrow cells, their accumulation in skeletal muscle (and brain) and the evolution of the clinical features into early-onset KSS. The majority of single large-scale rearrangements of mtDNA is sporadic and therefore believed to be the result of the clonal amplification of a single mutational event, occurring in the maternal oocyte or early during the development of the embryo.

Point mutations of mtDNA: In contrast to large-scale rearrangements, mtDNA point mutations are usually maternally inherited. Mutations are considered pathogenic if they affect highly conserved nucleotide/amino acid, segregate with phenotype, show quantitative correlation between heteroplasmy and phenotype severity and are present in affected families from ethnically distinct human populations.

Mutations have been found in all mtDNA-encoded genes. The most common syndromes include: (i) mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS), mainly due to mutations in the $tRNA^{Leu(UUR)}$ gene; (ii) myoclonus epilepsy and ragged red fibers (MERRF) due to mutations in the $tRNA^{Lys}$ gene; (iii) Leber's hereditary optic neuropathy (LHON) due to mutations in the CI encoding genes ND1, ND4 and ND6; and (iv) neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP) due to mutations in the ATP6 gene. Different clinical presentations are also associated with mutations of CIII and CIV mtDNA-encoded subunits. In particular, *CYTB* mutations may lead to isolated myopathy, but also to a multisystem disorder characterized by encephalomyopathy, cardiomyopathy and septo-optic dysplasia; whereas mutations in *COX1, COX2* and *COX3* are associated with several manifestations, including MELAS, encephalomyopathy and motor neuron disease-like presentation.

Mutations in Nuclear Genes

Mutations in nDNA-encoded OXPHOS-related genes have also been linked to a variety of multisystem disorders [18]. Importantly, most of nuclear genes responsible for mitochondrial disease are not encoding structural subunits of the OXPHOS system, but rather ancillary factors involved in their assembly, activity and turnover, components of the

mtDNA replication and expression machineries, enzymes controlling the intramitochondrial supply of deoxynucleotides, proteins involved in mitodynamics and quality control, in the biosynthesis of the lipid milieu or in the formation of prosthetic groups and cofactors (Table 1.1).

In spite of the substantial progress made in the past two decades in the molecular definition of numerous mitochondrial disorders and in the understanding of their pathophysiology, we still face major limitations for the development of new treatments and preventing these disorders in the near future [19].

First, in a large proportion of cases it is still not possible to reach a molecular genetic diagnosis. In fact, more than 50 percent of adult patients, and an even greater percentage of pediatric cases, remain undefined genetically [20], and the diagnosis is based on biochemical and/or morphological finding in muscle or, more rarely, in cultured fibroblasts. The lack of a genetic diagnosis prevents the patients from receiving reliable family counseling, prevents reliable prenatal diagnosis and necessitates referrals for biochemical methods that vary from lab to lab and are often incomplete. While the analysis of mtDNA is a well-standardized procedure in most Centers, the list of known disease genes associated with mitochondrial dysfunction is constantly increasing, and the identification of new genes has largely relied on the availability of large and/or multi-consanguineous families. New re-sequencing technologies offer the possibility of rapid and relatively low-cost characterization of whole genomes in individual patients or families, and is especially powerful when parent-child trios are sequenced in parallel. This enables the molecular dissection of mitochondrial disorders in humans at an unprecedented level.

Second, once a new mitochondrial disease gene has been identified, the process of validation of the mutant variants, and, even more importantly, the characterization of the function of the corresponding gene product, are essential steps for a complete understanding of the disease process. This often takes considerably longer than the initial genetic studies, and can limit progress, particularly when only one family has been identified with a possible pathogenic mutation in a new disease gene.

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