Clinical and Molecular Aspects of Diseases of Mitochondrial DNA Instability

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Mitochondria within human cells contain vast numbers of mitochondrial DNA (mtDNA), which are small, circular, and double-stranded. The proper functions of mtDNA depend totally on specific proteins that are encoded by the nucleus and then imported into mitochondria. Thus instability of mtDNA can stem from the mtDNA itself, or secondarily from abnormalities in nuclear DNA. In this review, we will first introduce mtDNA, including its characteristics, replication, transcription, translation, and the proteins involved in its metabolism, in particular DNA polymerase γ (POLG), DNA helicase Twinkle (Twinkle), and mitochondrial transcription factor A (TFAM). Secondly, we will stress the importance of mitochondrial nucleoid structures in the protection and facilitation of mtDNA metabolism, and report on the few known protein components of nucleoid, especially Twinkle, TFAM, and the recently discovered ATAD3. Based on this information, mtDNA instabilities will be categorized in accordance with their molecular etiologies, those that are caused by primary defects of mtDNA, and those by secondary effects from abnormalities in nuclear DNA. The former includes large defects or point mutations of mtDNA. The latter involves the nuclear genes of POLG1, Twinkle, ANT1, TK2, dGK, and TP. With the comprehensive categorization in this review, links are provided between the molecular and clinical aspects of mitochondrial DNA diseases. This report should help medical staff understand the complexity of these diseases and encourage them in further investigations. (Chang Gung Med J 2009;32:354-69)

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1. General features of mitochondria

Mitochondria are subcellular organelles within the cytoplasm of nearly all eukaryotic cells (a rare exception is the erythrocyte), which usually constitute ~25% of the volume of cytoplasm. They are not static entities. Instead they move along the cytoskeletal network, undergoing frequent fusion and fission. When cells divide, mitochondria are shared almost equally among the daughter cells. Mitochondria have an outer membrane and an inner membrane separated by a cavity, the intermembrane space. The outer membrane is highly permeable, containing many pores for small molecules to pass through. In contrast, the inner mitochondrial membrane (IMM) is impermeable to most small molecules and ions, a property required for maintenance of an electrochemical gradient. The IMM folds into lamellar structures called cristae, which increase its surface area and mass. It is estimated that three quarters of the mass of the IMM is protein, and many of these proteins are components of the oxidative phosphorylation system (OXPHOS). The interior com-

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partment of mitochondria is composed of a gel-like substance, the matrix, which contains enzymes of the tricarboxylic acid (TCA) cycle, and \( \beta \)-oxidation. These oxidation-reduction reactions and energy transfer processes make mitochondria the power-houses of eukaryotic cells.

The above chemical reactions (TCA cycle, \( \beta \)-oxidation, and OXPHOS) involve a large number of proteins in the mitochondria. It is estimated that the mitochondrion contains approximately 1000 proteins, of which only 3-32, depending on the organism, are encoded by mtDNA.\(^{(8)}\) The remainder are encoded by nuclear DNA, translated in the cytosol and imported into mitochondria via translocases of the outer membrane (TOM complex) and inner membrane (TIM22, TIM23 complexes).

2. Mitochondrial DNA

Mitochondria, like chloroplasts, have their own DNA, mitochondrial DNA (mtDNA). However, mtDNA is itself dependent on nuclear genes for its replication, expression, and maintenance (see sections 3, 4 & 5 of this report). In mammals, mtDNA are covalently closed circular molecules of double-stranded DNA interspersed with a handful of ribonucleotides on each strand.\(^{(9)}\) In a single cell the copy number of mtDNA is typically over 1,000. This huge number of mtDNA molecules is organized into discrete foci called mitochondrial nucleoids (mt-nucleoids; see sections 5 and 6), which are believed to be tethered to the IMM. Mt-nucleoids comprise multiple copies of mtDNA and an unknown number of proteins. Nuclear DNA encodes all the proteins involved in mt-nucleoid metabolism. Most of the little that is known about mammalian mt-nucleoids has come from studies of human diseases caused by mtDNA perturbation (section 10).

There is little variation in the size of mtDNA in animals; the range is approximately 16-19 kb. The genes are tightly packed; there are no introns, many genes are contiguous, and some even overlap.

Mammalian mtDNA differs from nuclear DNA in several ways. It is small, circular, has a high copy number per cell, and is inherited through the maternal line. The two strands of mtDNA are separable on alkaline caesium chloride gradients, owing to a bias in the nucleotide composition. The so-called heavy strand (H-strand) is rich in guanines; while the light strand (L-strand) is rich in cytosines. The human mitochondrial genome is 16,569 base pairs (Fig. 1). Despite its small size, mtDNA encodes 22 transfer RNAs, 2 ribosomal RNAs and 13 polypeptides which are required for OXPHOS. Although the number 1000 is widely quoted as the mtDNA complement of a typical cell, there is in fact considerable variation in the mtDNA copy number among different cell types and tissues. The key determinant of the copy number is energy demand; thus, cardiac muscle has a considerably higher mtDNA copy number than skin cells. A high mtDNA copy number appears to protect against defects in mtDNA because OXPHOS can still be maintained without a significant cellular phenotype, until or unless a large proportion of mtDNA molecules is defective.

Animal oocytes contain an extraordinary number of mtDNA molecules, about 100,000 in frogs and humans. In contrast to oocytes, spermatozoa contain few mitochondria, even less than a typical epithelial cell, although there is no doubt that their function is required for sperm motility. Mitochondria of sperm are located in the mid-piece, which is actively degraded upon entry into the egg.\(^{(10)}\) Immediately following fertilization, mtDNA replication is quiescent, and is only activated at the 2-cell stage in mouse embryos.\(^{(11)}\) The degradation of sperm mitochondria and the preponderance of oocyte mtDNAs ensure strict maternal inheritance of mtDNA.

Human mtDNA is compact and economical, managing to compress 37 genes into fewer than 20 kilobases of DNA. There is, however, one substantial non-coding region (NCR), of \( \approx 900 \) bp in mammals (Fig. 1). Within the NCR, many molecules of mtDNA contain a triple-stranded region, or displacement loop (D-loop).\(^{(12-14)}\) The third strand, or 7S DNA, is synthesised via a transcription event initiating at the light strand promoter (LSP), and then a transition to DNA synthesis at the origin of heavy strand replication (OH). In humans, the size of 7S DNA is about 650 bp, spanning nucleotide numbers 16,111 to nucleotide 191. The purpose of the D-loop is a matter of conjecture.

3. Mitochondrial DNA replication

The mitochondrial DNA copy number is related to energy demand: tissues with the highest respiratory demand, such as muscle, liver, brain, and pancreatic islets, have the greatest number of copies of mtDNA. Therefore replication of mtDNA not only is
fundamental to cell biogenesis, but should also be regulated by the metabolic status of a given tissue at different stages of development or diseases.

3.1. Mechanisms of mtDNA replication

There are two competing models of mtDNA replication in mammals, strand-asynchronous replication, and coupled leading and lagging strand replication. In the strand-asynchronous model, the 2 strands of mtDNA initiate DNA synthesis in a temporally asynchronous way from 2 spatially separate points on the mtDNA, termed heavy (O_\text{H}) and light (O_\text{L}) strand origins. H strand replication is primed from a processed transcript initiated at the LSP with-
in the NCR. The transcript is terminated at another region within the NCR known as the conserved sequences blocks (CSBs) and then processed, thereby creating a primer for DNA synthesis; the RNA-DNA transition point defines the origin of heavy strand replication (OH, typically nucleotide 191 of human mtDNA). H-strand DNA synthesis proceeds until it has traversed two-thirds of the genome, whereupon it exposes a short spacer region which is theoretically capable of forming a stem loop structure; separation of the two strands allows initiation of DNA synthesis of the second strand to commence. This mechanism predicts extensive single-stranded regions in replicating molecules. Recently the model has been revised and alternative light strand ‘origins’ have been proposed.

Over the past few years an alternative view of mtDNA replication has been elaborated. Mitochondrial DNA molecules separated by two-dimensional agarose gel electrophoresis have the appearance and properties of duplex replication intermediates. A fraction of these have all the hallmarks of products of conventional coupled leading and lagging strand synthesis. However the majority are sensitive to RNase H and this has led recently to the proposal that RNA is incorporated throughout the lagging strand at high frequency during mtDNA replication, the RITOLS mechanism.

### 3.2. Proteins involved in mtDNA replication

The machinery of mtDNA replication in mammals involves a host of proteins. DNA polymerase γ, POLG, is believed to be the replicative polymerase for mtDNA. It forms a heterotrimer composed of one molecule of POLG1, which is the catalytic subunit, and two molecules of POLG2, which are accessory subunits required for synthesizing long stretches of DNA in vitro. The catalytic subunit, POLG1, has one conserved domain for polymerase activity located towards the C-terminus; at the other end is a domain for 3′→5′ exonuclease ‘proofreading’ activity.

Mitochondrial single-stranded DNA-binding protein (mtSSB) has an N-terminal domain similar in sequence to prokaryotic SSB. Like its counterparts elsewhere, it binds single-stranded DNA cooperatively as tetramers. In flies and frogs, mtSSB enhances the functions of POLG in various assays of primer-template binding, 3′→5′ exonuclease activity, and initiation and elongation of DNA replication.

Mitochondria also contain a DNA helicase called Twinkle, which is related to the primase-helicase of T7 phage. The helicase domain is conserved; however, the primase domain of T7 primase-helicase has no obvious similarity to Twinkle. Recombinant Twinkle has a DNA helicase activity with 5′→3′ directionality in vitro, and DNA unwinding by Twinkle is specifically stimulated by mtSSB. In vitro, Twinkle, POLG, and mtSSB form processive replication machinery, which can use double-stranded DNA as a template to synthesize single-stranded DNA molecules of about 16 kb.

Mitochondrial DNA is supercoiled in vivo and its replication, as in other DNA systems, requires topoisomerases to relax and wind up DNA molecules. Mitochondrial topoisomerase 1 (TOP1MT) is homologous to nuclear topoisomerase 1, except that it has an N-terminal mitochondrial targeting signal in place of a nuclear localization sequence. TOP1MT releases the tension generated in the circular mitochondrial genome during replication and transcription.

### 4. Transcription, RNA processing, post-transcriptional modification, and translation

#### 4.1. Transcription of human mtDNA and processing of primary transcripts

Genetic information is contained in both strands of mammalian mtDNA (Fig. 1). Transcription initiates at two promoters in the NCR, one for each strand, hence heavy strand promoter (HSP), and light strand promoter (LSP). These give rise to polycistronic transcripts. H-strand transcription starts at nucleotide position 561 located within HSP, whereas L-strand transcription starts at nucleotide position 407 within LSP. The initiation of transcription is facilitated by mitochondrial transcription factor A (TFAM), and mitochondrial transcription factor B (TFBM). RNA synthesis in mitochondria is mediated by MTRPOL, in which the conserved C-domain specifies promoter selectivity and polymerase activity. A second HSP is located immediately outside the NCR within the tRNA gene. A ‘transcription termination factor’ (mTERF) simultaneously binds to this site and another position immediately downstream of the two rRNA genes, thereby forming a DNA loop, and then generating truncated H-strand
transcripts containing the rRNAs with little else.\(^{33}\)

In the absence of mTERF, full-length H-strand transcripts are produced, which specify 10 mRNAs and 14 tRNAs. Since tRNA genes abut mRNAs in the polycistronic transcripts, cleavage of tRNAs by RNase P and RNase Z yields mature mRNAs.\(^{34}\)

Thus there is no requirement for a complex spliceosome in mitochondria.

### 4.2. Post-transcriptional modifications

Post-transcriptional modifications of several bases in excised tRNAs are important for folding, aminoacylation, and codon recognition. Maturation of tRNAs requires addition of CCA to the 3′ end for the attachment of amino acids. The 3′ ends of mRNA undergo polyadenylation, which is believed to stabilize transcripts.

### 4.3. Translation

Mitochondrial ribosomes are broadly prokaryotic-like. They are sensitive to aminoglycosides and chloramphenicol, and resistant to cyclohexamide.\(^{35}\)

Mammalian mitochondrial ribosomes contain relatively little RNA, but have a high protein content of 29 and 48 polypeptides in the small and large ribosomal subunits, respectively. The result is a low sedimentation coefficient of 55S, compared with the 80S of nuclear ribosomes and 70S of bacterial ribosomes.

Mitochondrial mRNAs have neither upstream untranslated regions to facilitate ribosomal binding, nor a cap structure at the 5′-end to direct ribosomes to the initiation codon. Instead, the small subunit of mitochondrial ribosomes binds mRNA in a sequence-independent way, and moves to the initiation codon. There the small subunit is bound by mitochondrial translation initiation factor 2 (mtIF2) and N-formylmethionyl-tRNA, facilitating recruitment of the large ribosomal subunit. Elongation of translation is carried out by mitochondrial elongation factors (mtEFs) that are homologous to EF-Tu, EF-Ts, and EF-G of \(E. coli\). Likewise, termination of translation is believed to involve the recognition of stop codons by mitochondrial release factors (mtRFs) homologous to those in \(E. coli\).

### 5. Nucleoids

In vivo DNA is rarely, if ever, naked. Rather, it adopts an ordered condensed structure in association with a number of proteins. In bacteria, such nucleic acid-protein complexes are termed nucleoids.\(^{36}\)

Packaging of DNA with protein offers a means of protection from extraneous damage, facilitating orchestrated copying and segregation of DNA molecules. Both are processes essential to the faithful transmission of genetic material.

Mitochondrial DNA is no exception; it also forms nucleoprotein complexes, which have become the subject of intense study in recent years.\(^{37}\)

Mitochondrial nucleoprotein complexes, or mitochondrial nucleoids (mt-nucleoids), are believed to contain multi-copy mitochondrial genomes based on microscopic studies in which the number of mtDNA foci in a cell, indicated by a fluorescent dye specific to DNA, was significantly less than the mtDNA copy number. In humans, mt-nucleoids are about 0.068 µm in diameter as judged from immunocytochemical staining, and the mitochondrial genome per nucleoid is estimated to be between 2 and 10 copies.\(^{38,39}\) In \(S. cerevisiae\), mt-nucleoids are larger than humans. In yeast growing aerobically, mt-nucleoids are 0.2 to 0.4 µm in diameter, and the mitochondrial genome per nucleoid is estimated to be 1 to 2 copies. Under anaerobic conditions, yeast mt-nucleoids grow to 0.6 to 0.9 µm and each contains the equivalent of ~20 copies of the mitochondrial genome.\(^{37}\)

Most progress in understanding mitochondrial nucleoids has been made in yeast. The concept of mitochondrial nucleoids is based on in vivo and ex vivo observations that yeast mtDNA forms stable complexes which are the units of segregation within the mitochondrial network. Yeast studies have led the field, largely because yeast genetics allows the rapid creation and screening of mutants. Caron et al.\(^{40}\) first identified a histone-like protein from yeast mitochondria. This protein is encoded by the ABF2 gene. It has two high-mobility-group (HMG) domains capable of binding DNA and is required for maintenance of mtDNA.\(^{41}\)

The bacterial DNA packaging protein HU can restore mtDNA stability to \(abf2\) mutant cells when targeted to mitochondria.\(^{42}\) Thus Abf2 is likely to function, at least in part, as a DNA-packaging protein. However, it is not clearly understood how Abf2 is involved in the maintenance of the mitochondrial genome because Yhm2, which is an inner membrane protein with in vitro DNA binding activity, andaconitase (Aco1), which is required for TCA cycle, can both complement \(abf2\) mutant cells.\(^{43,44}\)
In organello formaldehyde cross-linking experiments identified a number of candidate mitochondrial nucleoid proteins of yeast;\(^{(45)}\) subsequently several of these have been shown to associate closely with mtDNA and to contribute to mtDNA stability.\(^{(46,47)}\) These include Abf2, Aco1, Rim1, Mgm101, Hsp60, and Ilv5. Rim1 is a single-stranded binding protein and by analogy with other systems is presumed to be involved in yeast mtDNA replication.\(^{(48)}\) Mgm101 binds DNA and is required for the repair of damaged mtDNA.\(^{(49)}\) Further investigation revealed that in vivo, Mgm101 colocalized with actively replicating mtDNA.\(^{(50)}\) Even in the absence of mtDNA, Mgm101 forms a self-replicating complex with an outer membrane protein Mmm1, which itself is required for mtDNA stability.\(^{(51)}\) Hsp60, a well known mitochondrial chaperone and analogue of E. coli GroEL, was found in yeast mitochondrial nucleoprotein preparations; when tested in vitro it was shown to bind to mtDNA ‘ori-like sequences’ preferentially.\(^{(45)}\) Moreover, mtDNA is unstable in a hsp60 mutant and further studies showed that this instability was caused by a defect in mtDNA transmission to daughter cells,\(^{(47)}\) suggesting the nucleoid dynamics underlying mtDNA transmission are regulated by the interaction between Hsp60 and mtDNA ori sequences. Ilv5 participates in branched-chain amino acid biosynthesis and plays a direct role in the partitioning of mtDNA into nucleoids.\(^{(46)}\) Ilv5 is essentially two proteins glued together, as the two functions are quite separable.\(^{(52)}\) These findings provide convincing evidence of highly organized mitochondrial nucleoids whose maintenance and transmission is presumably dependent on mechanisms similar to bacteria. However, yeast mitochondria have few obvious direct homologues of bacterial DNA maintenance and segregation proteins.\(^{(36,37)}\)

Few protein components of yeast mitochondrial nucleoids have sequence-conserved homologues in mammals. In addition, targeted mutation of mammalian cells is cumbersome and laborious. Therefore less is known about mammalian mitochondrial nucleoids; they contain Twinkle, a DNA helicase, which is localized with mtDNA when over-expressed in cultured cells,\(^{(58)}\) and TFAM,\(^{(53)}\) which is believed to be the major packaging protein of mammalian mtDNA.\(^{(50)}\) TFAM is a homologue of yeast Abf2, based on the conserved high-mobility-group (HMG) domains and its complementary effect in yeast cells devoid of Abf2.\(^{(55)}\) Both Twinkle and TFAM are essential for mtDNA maintenance.\(^{(56,57)}\) A fraction of POLG and mtSSB colocalize with Twinkle and mtDNA, as would be expected.\(^{(58)}\) Additional proteins co-purify with frog oocyte mtDNA,\(^{(59)}\) although the role of these, if any, in mtDNA metabolism is unknown.

Studies of yeast mitochondrial nucleoid dynamics have been aided by live-staining with 4’, 6-diamidino-2-phenylindole (DAPI). DAPI is a fluorescent dye that can pass through intact membranes to bind mtDNA and nuclear DNA in yeast. Unfortunately DAPI stains human mtDNA weakly. Recently PicoGreen, a fluorescent dye that binds specifically to double strand DNA in solution, has been used to stain mtDNA in living mammalian cells with good results.\(^{(60)}\)

6. Recent progress in mammalian mitochondrial nucleoids

A new procedure using the bacterial nucleoid protein HU to capture mitochondrial nucleoprotein from rat liver identified ATAD3, a member of the AAA+ family of ATPases.\(^{(61)}\) Repressing expression of the homologue of ATAD3 in C. elegans (F54B3.3) caused embryonic lethality, larval arrest, slow growth, and maternal and progeny sterility, indicating it is an important mitochondrial protein involved in cell viability (http://www.wormbase.org/db/gene?name=F54B3.3). Other members of the AAA+ family\(^{(62)}\) include the bacterial nucleoid protein FtsK,\(^{(63,64)}\) eukaryotic initiation protein Cdc6, and components of the origin recognition complex (Orcs 1, 4 and 5) required for the initiation of DNA replication.\(^{(66)}\)

ATAD3 was studied in further detail using RNA interference in human 143B cells.\(^{(61)}\) Dramatic reductions in the PicoGreen signal of mt-nucleoids were revealed in ATAD3 siRNA-treated cells, suggesting that it plays a role in mtDNA metabolism (Fig. 2). In ATAD3 gene-silenced cells, DNA analysis by neutral/neutral two-dimensional agarose gel electrophoresis indicated that ATAD3 binds within the NCR of human mtDNA and contributes to the maintenance or formation of mtDNA multimers.\(^{(61,67)}\) Hence, a coherent hypothesis is beginning to emerge. ATAD3 binds specifically to a region of mtDNA in the vicinity of the NCR, possibly the D-loop itself, and this brings together or stabilizes multiple
Fig. 2 The PicoGreen signal of mitochondrial nucleoids is greatly reduced after 2 rounds of ATAD3-siRNA. Panel A, human 143B cells mock transfected with nuclease free water (Ambion, Huntingdon, U.K.) were live-stained with PicoGreen (Invitrogen, Paisley, U.K.; green) and Mitotracker (Invitrogen, Paisley, U.K.; red in the panel). Panel B, human 143B cells with siRNA ATAD3 treatment were stained with PicoGreen (green) and Mitotracker (red). Magnifications were with a 60x objective lens with zoom x 4, 2, or 1, using a Radiance 2000 confocal microscopy system (BIORAD, Hemel Hempstead, U.K.).
mtDNA molecules.

The obvious next step was to investigate the DNA binding properties of ATAD3. Two fragments of recombinant ATAD3 for in vitro DNA binding assays showed that one (residues 44-247) preferentially binds to D-loop structures,(61) even in solutions where double-stranded DNA is 1,000 times more abundant. Coupled with the 2D-AGE data, it is strongly suggested that ATAD3 binds to clusters of mitochondrial D-loops through the N-terminal one-third of the protein. The three-stranded stretches of D-loop that frequently turn up in mtDNA might provide a scaffold for a protein that helps mitochondria organize their DNA.

The N-terminal one-third of the protein (residues 44-247) had also been used to raise an antibody which was used to examine the distribution of ATAD3 relative to mtDNA. Confocal microscopy indicated that ATAD3 is localized exclusively within mitochondria (Fig. 3A). However, ATAD3 is not distributed uniformly throughout mitochondria but is restricted to discrete foci (Fig. 3A), a characteristic

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**Fig. 3** ATAD3 co-localized with mitochondria and mtDNA. Panel A, localization of mitochondria and ATAD3. Human 143B cells were live-stained with Mitotracker (red), then fixed, permeabilized, and immuno-labelled with a rabbit primary antibody against human ATAD3 (a kind gift from MRC Dunn Human Nutrition Unit, Cambridge, U.K.), and then with a goat anti-rabbit IgG secondary antibody pre-labelled with Oregon Green® 488 (Invitrogen, Paisley, U.K.; green). Panel B, localization of ATAD3 and DNA. Human 143B cells were fixed, permeabilized, and immuno-labelled for ATAD3 (as in panel A; green). In addition the cells were labelled with a mouse monoclonal anti-DNA IgM primary antibody (PROGEN Biotechnik, Heidelberg, Germany), and a goat anti-mouse secondary antibody Cy3 (Amersham Bioscience, Little Chalfont, U.K.; red). Panel C, enlargement of selected regions from the merged image of panel B. Cells were examined by confocal microscopy using an Olympus FluoView 1000 confocal microscopy system (Olympus, KeyMed House, U.K.), with magnifications from a 60x objective lens and zoom x 4 in panel A, and a 60x objective lens with zoom x 6 in panel B, in conjunction with the computer program Photoshop Elements (Adobe Systems Inc., Uxbridge, U.K.) in panel C.
of mt-nucleoid. (26,61,67) Confocal microscopy further revealed frequent co-localization (yellow in Fig. 3C-1, 3C-2, 3C-3) of ATAD3 (green in Fig. 3B, 3C) and mitochondrial nucleoids (red in Fig. 3B, 3C). Nevertheless a fraction of ATAD3 is in the form of ‘filamentous’ strings, flanked by mitochondrial nucleoids (Fig. 3C-4, 3C-5). Therefore, a model of mitochondrial nucleoid division has been proposed. (67) This model presumes that ATAD3 binding to mitochondrial D-loops enhances protein-protein interactions, thereby creating a filament, which drives apart the mitochondrial nucleoid. This also implies that mitochondrial nucleoids lacking ATAD3 are incapable of undergoing nucleoid division. Substantiating this model will require extensive investigation of cell biology and further in vitro analysis of ATAD3.

7. Human mitochondrial diseases

Mitochondrial diseases are a diverse group of human diseases characterised by defects in any aspect of mitochondrial function. Often for simplicity, defects relating to energy production, especially OXPHOS, are considered separately. Since the components of OXPHOS are encoded by mtDNA and nuclear DNA, and mtDNA is itself dependent on nuclear genes for its maintenance and expression, mitochondrial diseases of OXPHOS fall into three categories, mtDNA mutations as the primary cause, nuclear DNA defects affecting OXPHOS but unrelated to mtDNA, and nuclear DNA defects as a cause of mtDNA perturbation.

8. Mitochondrial DNA as the primary cause

Mitochondrial DNA mutations can themselves be divided into two broad categories, those that affect translation, and those affecting a specific protein-encoding gene. The 22 tRNA and 2 rRNA genes are all required for mitochondrial translation; hence, deleterious mutations in any one will affect the production of all 13 mitochondrially-encoded components of OXPHOS.

8.1. Large-scale mtDNA deletions

The occurrences of a variety of mtDNA deletions (∆mtDNA, ~1 to 8 kb in size) are mostly sporadic, always heteroplasmic, and always result in the loss of one or more tRNA genes, therefore affecting translation of all 13 mitochondrial genes. Such mtDNA deletions are invariably found in skeletal muscle but are often absent from blood. The clinical outcome is highly variable; the onset can range from birth to late adulthood (> 60 years). The reasons for this are not fully understood, but it relates in part to the mutant load in various tissues. The mildest clinical manifestation is progressive external ophthalmoplegia (PEO), which is characterised by droopy eyelids, and restricted eye movement, and may be accompanied by exercise intolerance. Much more severe is Kearns-Sayre syndrome (KSS), which involves cerebellar or cardiac dysfunction. Pearson’s syndrome (PS) is a fatal infantile disorder which is characterised by anemia and impaired synthesis of digestive enzymes in the pancreas. One third of PEO/KSS/PS patients have the so-called common deletion that removes 4977 bp of mtDNA (nucleotide position 8469 to 13447). (68,69)

8.2. Point mutations affecting tRNA or rRNA

The most common pathological mtDNA mutations are located in tRNA genes; somewhat unexpectedly they produce distinct clinical syndromes. MELAS syndrome (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) usually presents before the age of 40, after normal early development. The majority of MELAS patients have an A to G transition at nucleotide position 3243 (A3243G) within the tRNALeu(UUR) gene, (70) which intriguingly is within the mTERF binding site (section 4.1). Another point mutation, A8344G, within the tRNALys gene, is associated with MERRF syndrome (myoclonus epilepsy and ragged-red fibres). (71,72) Ragged-red fibres (RRF) are a key indicator of mitochondrial dysfunction in skeletal muscle; high concentrations of mitochondria stain reddish-purple with the Gomori Trichrome stain, hence ragged red fibres mark areas of mitochondrial proliferation. In situ hybridisation studies indicate that the mitochondria in ragged red regions contain high levels of mutant mtDNA. (73) Hence, proliferation appears to be an attempt to boost mitochondrial output, although this is ultimately futile in the case of mutant mtDNA, as RRF are almost invariably cytochrome c oxidase negative. Both the MELAS and MERRF mutant tRNAs are non-functional, as they lack the usual post-transcriptional modification of uridine at the first position of the anticodon. (74)

The best-known rRNA mutation occurs at
nucleotide position 1555 in the 12S rRNA.\(^{(25)}\) This A1555G mutation is associated with aminoglycoside antibiotic-induced deafness, and maternally inherited non-syndromic deafness. It has been demonstrated that the new G-C pair, created by the mutation, facilitates the binding of aminoglycoside antibiotics to 12S rRNA,\(^{(26)}\) which explains the molecular pathophysiology of aminoglycoside antibiotic-induced deafness.

**8.3. Point mutations affecting protein-coding genes**

Point mutations T8993G/C and T9176G/C in human mtDNA convert a conserved leucine to an arginine or proline, near the C-terminus of subunit 6 of ATP synthase.\(^{(77)}\) Disease severity is directly related to the percentage of mutant mtDNA in the blood. When the percentage of T8993G mtDNA is less than 70\%, patients are asymptomatic or oligosymptomatic. When it exceeds the threshold of 70\%, NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa) develops. Point mutations of mitochondrial genes encoding subunits of complex I are associated with LHON (Leber’s hereditary optic neuropathy) syndrome, a form of adult onset blindness. The pathogenic mutations are G11778A in ND4, G3460A in ND1, and T14884C in ND6.

Primary mtDNA mutations can be sporadic, particularly in the case of large deletions. Where mutations are transmitted, they are invariably through the maternal line. Genetic counselling in the case of mtDNA mutations is a primitive art as penetrance and clinical phenotypes are so variable. However in the case of affected fathers, (almost) complete reassurance can be offered; they will not transmit the disease to their offspring.

**9. Nuclear DNA defects affecting OXPHOS rather than mtDNA**

Most of the 1000 or more mitochondrial proteins encoded in nuclear DNA do not directly affect mtDNA stability, but relate to mitochondrial metabolism and OXPHOS.\(^{(78,79)}\) However, since OXPHOS is formed through the complementation of nuclear and mitochondrial DNA, mutations in nuclear genes encoding either structural components of OXPHOS,\(^{(80,81)}\) or factors involved in the assembly of OXPHOS,\(^{(82)}\) can all lead to clinical phenotypes similar to mtDNA mutations.

**10. Nuclear DNA defects as a cause of mtDNA perturbations**

Nuclear genes encode all the mtDNA binding proteins. Some are essential for replication and others are presumed to play roles in maintenance and segregation. As mitochondria are the major site of free radical production, protection from oxidative stress is another key function of the mtDNA binding proteins. Mutations in these nuclear genes can therefore produce mutations in mtDNA, but unlike primary mtDNA mutations, these are heterogeneous (multiple deletions and/or point mutations). Predictably, the clinical phenotypes associated with nuclear gene defects of proteins involved in mtDNA metabolism are often similar to those linked to specific mtDNA mutations, but the transmission follows Mendelian rules rather than a maternal pattern of inheritance.

**10.1. POLG1, Twinkle, and Adenine Nucleotide Translocator 1 (ANT1)**

Autosomal dominant progressive external ophthalmoplegia (adPEO) is characterised by multiple large deletions of mtDNA in not only the ocular and skeletal muscles, but also the brain and heart.\(^{(83-85)}\) adPEO is genetically heterogeneous with disease loci mapped to chromosome 15q25, 10q24, and 4q34-35.

Chromosome 15-linked adPEO is caused by mutations in the POLG1 gene.\(^{(86)}\) In fact, mutations of POLG1 are the most frequent cause of adPEO, accounting for approximately half of family cohorts.\(^{(87)}\) Amino acid position 955 of POLG1, which is located within the catalytic polymerase domain, or C-domain, is the most frequent mutation site.\(^{(87)}\) Mutations within the 3’→5’ exonuclease domain, or N-domain, are associated with a recessively inherited form of PEO. Mutations of POLG1 are also associated with Parkinsonism.\(^{(88)}\)

Chromosome 10-linked adPEO is caused by mutations in Twinkle, a DNA helicase, which co-localizes with mtDNA\(^{(26)}\) and is a component of mitochondrial nucleoids.\(^{(57)}\) Mutations in Twinkle include various missense mutations and an in-frame duplication of 13 amino acids (dup352-364).\(^{(26,89)}\) A recent study showed that transgenic mice expressing mutant Twinkle with in-frame dup352-364 develop multiple mtDNA deletions and a late-onset mitochondrial disease.\(^{(90)}\)

Chromosome 4-linked adPEO is caused by
mutations in adenine nucleotide translocator 1 (ANT1).\textsuperscript{(91)} The familial mutation substitutes a proline for a highly conserved alanine at position 114 in the ANT1 protein. ANT1 is a heart- and muscle-specific form of the ADP/ATP transporter, which is located in the mitochondrial inner membrane and is responsible for transporting ADP in and ATP out of mitochondria. It is likely that the mutation disturbs mitochondrial nucleotide pools, although it is not clear why this should cause multiple mtDNA deletions rather than depletion (as in the next section).

10.2. Thymidine Kinase 2 (TK2) and Deoxyguanosine Kinase (dGK)

Mutations in the genes encoding thymidine kinase 2 (TK2)\textsuperscript{(92)} and deoxyguanosine kinase (dGK)\textsuperscript{(93)} are associated with mtDNA depletion syndrome (MDS), a severe infantile disease inherited in an autosomal recessive pattern. Patients develop tissue-specific depletion of mtDNA, with its level in the liver dropping to less than 10% of normal.\textsuperscript{(94,95)} In mitochondria, both TK2 and dGK are required for the synthesis of deoxyribonucleoside triphosphates (dNTPs), the building blocks of DNA. In differentiated neurons and muscle cells, nuclear DNA replication is suspended and the cytosolic dNTP pool is lowered accordingly. There is no known de novo synthesis of dNTP in mitochondria, and so deoxyribonucleosides have to be recycled to replenish the mitochondrial dNTP pool. The salvage pathway for pyrimidines initially generates dTMP and dCMP by the action of TK2, whereas dGK generates dAMP and dGMP for purine biosynthesis. There is evidence showing that dNTPs need to be in balance for optimal DNA synthesis,\textsuperscript{(96)} an excess or deficiency in any one dNTP can result in error-prone or complete cessation of DNA synthesis.\textsuperscript{(97,98)}

10.3. Thymidine Phosphorylase (TP)

Mutations in the gene encoding thymidine phosphorylase (TP)\textsuperscript{(99)} are associated with depletion and/or multiple deletions of mtDNA in the muscles of patients with MNGIE (Mitochondrial neurogastrointestinal encephalomyopathy), an autosomal recessive disease. Intriguingly TP is not located in mitochondria, but in the cytosol where it is involved in the salvage of thymidine by catalysing the conversion of thymidine to phosphoribose and thymine. Mutations of TP lead to a decrease in enzyme activity to 5% of that in controls,\textsuperscript{(99)} which results in accumulation of thymidine and an imbalance of dNTP pools.\textsuperscript{(100)}

10.4. TFAM

TFAM is a component of mitochondrial nucleoids.\textsuperscript{(54,55)} Although TFAM has no known genetic relationship with any mitochondrial disease, in mice TFAM knockout causes severe mtDNA depletion and death in the fetus.\textsuperscript{(56)} Tissue-specific disruption of TFAM causes dilated cardiomyopathy, anaesthesia-related conduction block in the heart,\textsuperscript{(100)} and impaired insulin secretion in pancreatic-β-cells.\textsuperscript{(102)}

11. The importance of dissecting the mitochondrial nucleoid in the pathogenesis of mitochondrial DNA instability

Since there are more than 1000 copies of mtDNA in each cell, the percentage of mutant mtDNA needs to exceed a threshold to cause cell dysfunction. The threshold varies according to energy requirements, with the brain, heart, and skeletal muscle being the most susceptible to mitochondrial dysfunction. Crossing the threshold may result from random genetic drift\textsuperscript{(103)} or biased segregation.\textsuperscript{(104,105)} From studies on cultured cells it is known that different nuclear backgrounds confer selective advantages to particular mitochondrial genotypes.\textsuperscript{(105,106)} Identifying and characterizing all the nucleus-encoded proteins that are functionally associated with mitochondrial nucleoids will help elucidate the production or biased segregation of mutant mtDNA. These studies are relevant to patients as the same mechanisms are likely to be key determinants of mutant load in particular individuals and tissues.

12. Summary

This review reports the basic facts about mitochondria and mtDNA, and introduces nucleus-encoded proteins that are involved in the metabolism of mtDNA, with special emphasis on POLG, Twinkle, and TFAM. Integrated knowledge about the protein components of the genetic units of mtDNA, namely mitochondrial nucleoids, is also included. Among them Twinkle, TFAM, and the recently identified ATAD3 are detailed. Based on the molecular causes of mtDNA instability, mitochondria DNA diseases are grouped into two categories, mtDNA as the primary cause, and nuclear DNA defects as a cause of
mtDNA perturbations. However, key questions remain to be answered, in particular the production and biased segregation of mtDNA instability in specific tissues. These fields may be greatly advanced by further identification and investigation of the individual components of mitochondrial nucleoids.

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Mitochondrial DNA instability
粒線體 DNA 不穩定所致疾病之臨床及分子面向

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人類細胞的粒線體內擁有數眾多的小型環狀雙股 DNA 分子，其正常功能有賴於核 DNA 製造並輸入到粒線體的特殊蛋白質。因而粒線體 DNA 的不穩定，可源於自身也可次發於核 DNA 異常。本文首先介紹粒線體 DNA 的特徵、複製、轉錄、轉錄等機轉及參與其中的蛋白質，特別是 DNA polymerase γ (POLG) 、DNA helicase Twinkle (Twinkle)、mitochondrial transcription factor A (TFAM)。接者闡述粒線體之類核體結構對保護並促進粒線體 DNA 代謝的重要性和少數已知的類核體組成蛋白，特別著重於 Twinkle、TFAM，以及新近發現的 ATAD3。本文作者針對粒線體 DNA 的原發性異常及源於核 DNA 的次發性異常來分類粒線體 DNA 疾病；前者包含粒線體 DNA 的原發大片段缺損及點狀突變，後者包含異常的核基因 POLG1、Twinkle、ANT1、TK2、dGK、TP。藉著將粒線體 DNA 疾病作統整的分子及臨床分類，我們希望幫助醫療人員了解複雜的粒線體 DNA 疾病並激發研究的興趣。(長庚醫誌 2009;32:354-69)

關鍵詞：粒線體 DNA，粒線體之類核體，粒線體疾病