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Long-lived *C. elegans* Mitochondrial mutants as a model for human mitochondrial-associated diseases

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Abstract

Mitochondria play a pivotal role in the life of cells, controlling diverse processes ranging from energy production to the regulation of cell death. In humans, numerous pathological conditions have been linked to mitochondrial dysfunction. Cancer, diabetes, obesity, neurodegeneration, cardiomyopathy and even aging are all associated with mitochondrial dysfunction. Over 400 mutations in mitochondrial DNA result directly in pathology and many more disorders associated with mitochondrial dysfunction arise from mutations in nuclear DNA. It is counter-intuitive then, that a class of mitochondrially defective mutants in the nematode *Caenorhabditis elegans*, the so called Mit (Mitochondrial) mutants, in fact live longer than wild-type animals. In this review, we will reconcile this paradox and provide support for the idea that the Mit mutants are in fact an excellent model for studying human mitochondrial associated diseases (HMADs). In the context of the 'Mitochondrial Threshold Effect Theory', we propose that the kinds of processes induced to counteract mitochondrial mutations in the Mit mutants (and which mediate their life extension), are very likely the same ones activated in many HMADs to delay disease appearance. The identification of such compensatory pathways opens a window of possibility for future preventative therapies for many HMADs. They may also provide a way of potentially extending human life span.
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1. Introduction

Mitochondrial dysfunction occurs in a wide variety of metabolic and degenerative diseases, cancer as well as aging (Wallace, 2005). Although this review will focus specifically on the unexpected relationship between human mitochondrial diseases and the long-lived *Caenorhabditis elegans* Mitochondrial (Mit) mutants, most of the consideration regarding human mitochondrial disorders will be potentially applicable to many other human pathological conditions.

Over the last few decades, mitochondrial dysfunction, mostly attributable to mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA), has been established as a cause for numerous human diseases, many of which are associated with neuronal degeneration. Most of these mutations directly or indirectly affect mitochondrial electron transport chain (ETC) function. The subsequent alterations in energy metabolism have been shown to reduce ATP production, decrease mitochondrial membrane potential (MMP), impair calcium buffering and lead to the generation of reactive oxygen species (ROS). The Mitochondrial Threshold Effect Theory (Rossignol et al., 2003) suggests that cells can cope with a certain degree of mitochondrial dysfunction, most likely through the activation of compensatory mechanisms which in turn act to sup-

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port cell viability; however, once beyond a threshold, pathology ensues.

Despite the many disorders induced by mitochondrial mutations in human, recent advances in the aging field have revealed, paradoxically, that mutations in mitochondrial genes (mainly affecting the ETC), can actually prolong *C. elegans* life span (Lee et al., 2003). The structure and function of the ETC as well as many pathways of intermediary metabolism, such as the Krebs cycle, and many other signal transduction pathways (for instance the insulin and the apoptotic pathways) are very well conserved between higher mammals such as humans and nematodes such as *C. elegans* (Wadsworth and Riddle, 1989). Given this, one can envision that those same early compensatory pathways, which, according to the Mitochondrial Threshold Effect Theory, allow cells to cope with mitochondrial mutations, are activated in mammals as well as in *C. elegans*. The induction of these compensatory pathways most likely accounts for increases in both stress resistance and life span in the *C. elegans* Mit mutants. The threshold effect theory also suggests that lowering mitochondrial function below a certain threshold inevitably leads to a condition whereby these compensatory mechanisms are no longer sufficient thus leading to deleterious consequences such as cell death and tissue degeneration. In the nematode, these effects presumably result in developmental arrest, sterility, decreased life span or lethality.

Friedreich's Ataxia is the most common heritable ataxia and it is caused by low level of frataxin, a nuclear encoded mitochondrial protein. In our attempt to generate a model organism for FRDA, we recently found that reduced expression of frataxin in *C. elegans* increases life span while frataxin knockout (KO) animals arrest as L2/L3 larvae (Ventura et al., 2005). This observation is consistent with the idea of the threshold.

In this review, we will explore the idea of taking advantage of the *C. elegans* Mitochondrial (Mit) mutants as a model for human mitochondrial disorders. We believe that our long-lived frataxin-deficient animals (and perhaps several other Mit mutants), may aid discovery of compensatory pathways which are induced in the early phase of human mitochondrial-associated diseases (HMADs). Most of these diseases have a chronic course with late onset and symptoms appearing only after mitochondrial dysfunction has become very severe. It is conceivable that compensatory pathways may be induced, well before phenotypic markers of an established pathology, during a phase when therapy could be more effective. The genetic nature of mitochondrial diseases allows the potential for pre-symptomatic diagnosis and preventive therapy. The long-lived *C. elegans* Mit mutants then represent a novel and powerful model for HMAD because they can be utilized to identify these initial compensatory pathways and test whether these innate supportive countermeasures can be exploited and manipulated as an early preventive therapy.

2. Mitochondria

2.1. Mitochondrial structure

About 2 billion years ago a partnership between a glycolytic proto-eukaryotic cell and an oxidative bacterium commenced (Margulis, 1996). The partnership promised huge benefits to both parties: it allowed the protoeukaryote to exploit the energy opportunities inherent in the emerging oxygen atmosphere and which was toxic to most other life forms, and it gave the endosymbiotic bacteria a ready supply of metabolites. The alliance was initially a shaky one and catastrophic conflicts in selection between the two genomes undoubtedly occurred. Once the new symbiotic organism moved into a fully aerobic world, life and death were controlled by the protomitochondria, which provided not only critical antioxidants but also a source of ROS as a by-product of oxidative phosphorylation (OXPHOS). Conditions that favored the protomitochondria over the host cell led to cell death and release of the free-living endosymbiont. Therefore, the symbiosis was perilously unstable until essential genes for mitochondrial metabolism and biogenesis were transferred to the nuclear genome, resulting in an obligate symbiosis. Thus, modern mitochondria retain a series of features that reflect their endosymbiotic origin - such as a double membrane structure and a circular mitochondrial genome with its own transcription, translation, and protein assembly systems (Schatz, 1996).

Over the past 20 years mitochondria have received increased attention because mitochondrial defects have been implicated in a wide variety of degenerative diseases, in aging and in cancer. This attention has improved our knowledge of the mitochondria's structure and functions. Mitochondria are highly dynamic organelles usually organized as a continuous reticulum that are able to fragment into smaller tubular structures depending on the cell state. The distribution and movements of the organelle are controlled by cytoskeleton components.

Two critical and highly specialized membranes surround each mitochondrion. Together the inner and outer mitochondrial membranes create two separate mitochondrial compartments: the internal matrix space and a much narrower intermembrane space (Frey et al., 2002).

The *outer mitochondrial membrane* contains many copies of a large protein-channel, called porin, permeable to all molecules of 5000 Da or less. Since most of these molecules can not pass the impermeable inner membrane, the intermembrane space is chemically equivalent to the cytosol. Other proteins in the outer membrane include enzymes involved in lipid metabolism. The *inner mitochondrial membrane* is folded into numerous cristae, which greatly increases its total surface area. Cristae number varies among different cell types, presumably depending on ATP demand (being higher in cells with higher demand such as cardiac cells). The inner membrane contains a high proportion of the phospholipid cardiolipin, which may help make it particularly impermeable to ions. The inner

membrane also contains proteins with three main types of functions: those that carry out the oxidation reactions of the respiratory chain, those involved in ATP synthesis and those that act to regulate the passage of metabolites into and out of the matrix. The *intermembrane space* contains several enzymes which use the ATP that is passing out of the matrix to phosphorylate other nucleotides. Finally the space bounded by the inner mitochondrial membrane, the *matrix,* contains a highly concentrated mixture of hundreds of enzymes, including those required for the oxidation of pyruvate and fatty acids and those necessary for the tricarboxylic acid (TCA) cycle. It also contains several identical copies of the mitochondrial DNA genome, mitochondrial ribosomes, tRNAs and various enzymes required for expression of the mitochondrial genes.

2.2. Mitochondrial function

Mitochondria have long been considered to play a straightforward but critical role in the life of the cell; namely to carry out energy-yielding oxidative reactions which create the vast majority of ATP necessary to support cellular functions. The fundamental mechanism of energy generation in mitochondria is chemiosmosis (Mitchell, 1979), in which the free energy of oxidation of carboxylic acids, generated by the TCA cycle and oxidative phosphorylation (OXPHOS), is used to pump protons out of the matrix and establish an electrochemical gradient across the membrane. An ATP synthase couples the transport of these protons back across the inner membrane with the subsequent phosphorylation of ADP to produce ATP.

The proteins involved in OXPHOS include four respiratory enzyme complexes collectively referred to as the electron transport chain (ETC), an ATP synthase or complex V, and the adenine nucleotide translocator (ANT). Superficially, the ETC oxidizes hydrogen (derived from the oxidation of organic acids, such as pyruvate and fatty acid), with oxygen, to generate water. More specifically, electrons from NADH (nicotinamide adenine dinucleotide), are transferred to respiratory complex I (NADH:ubiquinone oxidoreductase) and then to ubiquinone (UQ), while electrons from succinate (as part of the TCA cycle) are transferred to complex II (succinate:ubiquinone-oxidoreductase) and then to UQ. From UQ, electrons are subsequently transferred to complex III (ubiquinone–cytocrome *c* oxidoreductase), then to cytocrome *c* (cyt-*c*), complex IV (cytocrome *c* oxidase, COX), and finally to molecular oxygen to give H_2O. Energy created by this electron transfer is used to pump protons out of the mitochondrial inner membrane creating an electrochemical gradient across the intermembrane space ($\Delta\psi_m$). This gradient represents the driving force for the transport of protons back into the matrix, leading to the catalytic condensation of ADP (adenosine diphosphate) and Pi (inorganic phosphate) via the ATP synthase to make ATP. Finally ATP is exported to the cytosol by ANT in exchange for spent ADP (Wallace, 1999).

In addition to being the site of respiration and oxidative phosphorylation, mitochondria also work as calcium buffers. Furthermore, they are essential for several other important functions such as the biosynthesis of heme, lipid, and amino acid, the Krebs cycle, the urea cycle, fatty acid oxidation, and iron homeostasis (Schatz, 1995).

2.3. The mitochondrial genome

The human mitochondrial genome is a double-stranded, circular DNA molecule of about 16,500 base pairs which is maternally inherited. This genome encodes for the two rRNAs and the 22 tRNAs required for mitochondrial protein synthesis, and also for 13 polypeptide subunits of the OXPHOS system. Specifically, it encodes for seven of the 42 subunits of complex I, one of the 11 subunits of complex III, three of the 13 subunits of complex IV and two subunits of the 14 of complex V. None of the four subunits of complex II are encoded by mitochondrial DNA (mtDNA). All remaining mitochondrial OXPHOS proteins, as well as all metabolic enzymes, both the mitochondrial DNA and RNA polymerases and all mitochondrial DNA regulatory factors are encoded by nuclear genes. mtDNA shows a higher mutation frequency than nuclear DNA (nDNA) mainly due to its close proximity to the ETC, the major source of intracellular ROS. This is further compounded by its absence of protective histones and introns, and because of a somewhat inefficient DNA repair system relative to that operating on nDNA (Mandavilli et al., 2002).

3. Human mitochondrial-associated disorders

3.1. Classification

A major advance in the last 20 years has been the recognition that many degenerative disorders associated with mitochondrial dysfunction and with severely compromised energy generation often arise because of genetic defects in the mitochondrial or nuclear genome (Wallace, 2005). A substantial number of pathogenic mitochondrial mutations are now known to be associated with the mitochondrial genome (over 400 within 16,500 bp (Servidei, 2004). Despite the high mutation frequency of mtDNA, the number of mutations occurring within nuclear genes that account for mitochondrial dysfunction is predicted to be even greater. Indeed, as mentioned above, most of the proteins from which mitochondria are built, as well as those synthesizing the machinery for building them, are nuclear-encoded and imported into mitochondria from the cytosol.

The number of mitochondria varies among cells depending in a large part on the metabolic requirements of each cell. Cells highly dependent on oxidative energy metabolism, such as neurons, cardiac and skeletal muscle, and pancreatic beta cells, contain more mitochondria and are consequently more sensitive to mitochondrial dysfunction.

Accordingly, most mitochondrial-related disorders present with signs and symptoms of neurodegeneration, myopathy and cardiac defects (encephalocardiomyopathies). The phenotype of mitochondrial diseases may be very complex since the same mutation can produce very different phenotypes while different mutations can produce quite similar phenotypes. Hence, mitochondrial disorders are usually classified by genetic defect rather then by clinical manifestations (DiMauro and Hirano, 2005). An extended classification of mitochondrial-associated diseases not only includes those directly associated with defects in mitochondrial genes and in nuclear genes encoding mitochondrial proteins, but also a variety of other disorders with indirect mitochondrial dysfunction as a cause or consequence (often indiscriminate) of their etiology. Hence, mitochondrial-related diseases can be divided into 3 main classes: (1) disorders ascribed primarily to mutations in mtDNA; (2) disorders ascribed to mutations in nuclear-encoded mitochondrial genes; (3) disorders not directly affecting mitochondrial proteins, but in which there is a secondary involvement of mitochondrial dysfunction responsible for the phenotypic outcome (Table 1).

3.1.1. Disorders ascribed to mutation in mtDNA

mtDNA-related diseases mainly originate from two classes of mutations: large DNA deletions and point mutations. Large mtDNA deletions, generally between 1 and 10 kb, give rise to pathologies such as Chronic Progressive Ophtalmoplegia (CPEO), Kearns-Sayre syndrome (KSS), and Pearson syndrome. Diseases caused by point mutations usually involve missense mutations in OXPHOS genes or in tRNA-encoding genes (which in turn lead to global disruption of mitochondrial protein synthesis). Most frequent point mutations in OXPHOS genes include several that affect complex I subunits, leading to Leber's Hereditary Optic Neuropathy (LHON), and others that affect subunit 6 of complex V (ATP6) which may result in either Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP) or to a lethal infantile form of Leigh syndrome. Common tRNAs mutations are those affecting tRNALys, leading to Myoclonic Epilepsy with Ragged Red Fibers (MERRF), and tRNALeu, which lead to Mitochondrial myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes (MELAS).

3.1.2. Disorders ascribed to mutations in nuclear-encoded mitochondrial genes

These disorders can be subdivided into three distinct classes: those that are caused by mutations affecting the sequence of a specific mitochondrial protein (most frequently OXPHOS components), those that affect the import and assembly of mitochondrial proteins, and finally

Table 1
Human Mitochondrial-Associated Diseases (HMADs)

Examples of human mitochondrial associated diseases	Mutated gene
1. *Primary mtDNA mutations*	
Deletions	
Chronic Progressive Ophtalmoplegia (CPEO)	
Kearns-Sayre syndrome (KSS)	
Pearson syndrome	
Point mutations (OXPHOS)	
Leber's Hereditary Optic Neuropathy (LHON)	MtDNA Complex Isubunits
Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP)/ Leigh syndrome	MtDNA ATPase 6
Point Mutations (tRNAs)	
Myoclonic Epilepsy with Ragged Red Fibers (MERRF)	tRNALys
Mitochondrial mvopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes (MELAS)	tRNALeu
2. *Nuclear gene mutations*	
Sequence of mitochondrial protein	
Leigh's syndrome	Complex I
Leigh's syndrome	Complex II
Import or assembly of mitochondrial protein	
Leigh's syndrome (with COX deficiency)	Surf-1 (complex IV)
Leigh's syndrome (with COX deficiency)	Sco-1 or 2 (complex IV)
Friedreich's Ataxia	Frataxin
Wilson's disease	ATP 7B
Mohr-Tranebjaerg syndrome	DPPI (TIMM8)
Hereditary spastic paraplegia	Paraplegin
mtDNA Maintenance	
3. *Mutations directly or indirectly affectina mitochondria:*	
Huntington's disease	Huntingtin
Parkinson's disease	Parkin, PINK1
Alzheimer's disease	APP, presenilins
Others: cancer, diabetes, obesity	

those that affect mtDNA stability, integrity and/or maintenance. Of the first class, mutations affecting OXPHOS components have been mainly found in complexes I and II, and are associated with Leigh syndrome. This syndrome can also be caused by mutations in Surf-1, Sco1 and Sco2, all genes belonging to the second class, and affecting the proper assembly of complex IV. Other diseases arising from defects in mitochondrial protein import and assembly include Friedreich's Ataxia (see next section); Wilson Disease, caused by a mutation in the ATP7B gene which normally encodes a copper transporting ATPase; Mohr-Tranebjaerg or Deafness-dystonia syndrome, which results from mutations in TIMM8 that normally encodes the Deafness-Dystonia Protein-1 (DDP1), a component of the mitochondrial import machinery; and finally Hereditary Spastic Paraplegia that occurs following mutations in SPG7 which normally encodes paraplegin (a mitochondrial protein similar to yeast metalloproteases). mtDNA stability disorders have been associated with mutations in genes required for mtDNA stability or integrity, such as the mtDNA helicase Twinkle, the mtDNA polymerase gamma (POLG) or the thymidine kinase 2 (TK2) and the deoxyguanosine kinase genes (these last two genes are both involved in the metabolism of the mitochondrial nucleotide pool). Mutations in these maintenance genes lead to depletions and/or deletions in mtDNA.

3.1.3. Mutations indirectly affecting mitochondrial function and structure

These consist of a huge class of mutations which can lead to cancer, diabetes, obesity and neurodegenerative disorders. In general, any process that leads to elevated mitochondrial ROS production can potentially contribute to neoplastic transformation (Wallace, 2005). Mutations in the Bcl-2 family member genes, which can directly or indirectly affect mitochondrial function, have been found in many type of cancers (Armstrong, 2006). Mutations in other nuclear-encoded mitochondrial proteins, which should be properly classified in class II, are those in the subunits of the succinate dehydrogenase which lead to paraganglioma (Gottlieb and Tomlinson, 2005). While alteration in the mechanisms regulating the ratio of nuclear-encoded versus mitochondrially encoded subunits of the ETC complexes can also result in cancer (Herrmann et al., 2003). Mutations in genes regulating mitochondrial biogenesis have been associated with type 2 diabetes. Many mutations that directly affect mtDNA-encoded or nuclear encoded mitochondrial proteins (Class I and II mutations) are also known to result in diabetes (Wallace, 2005). Finally, important neurodegenerative disorders, such as Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD) and Amyotrophic lateral Sclerosis (ALS), are linked with mutations in non-mitochondrial proteins. Nevertheless, such diseases have been associated with features ascribable to mitochondrial dysfunction, including decreased mitochondrial membrane potential, increased ROS production, mitochondrial structure abnormalities and increased apoptosis (Schon and Manfredi, 2003). Whether mitochondrial dysfunction is a cause or a consequence of these neurodegenerative disorders remains unclear (Andersen, 2004).

3.2. Friedreich's Ataxia

Friedreich's Ataxia is a neurodegenerative disorder characterized by progressive gait and limb ataxia, areflexia, dysarthria, muscular weakness, skeletal abnormalities, increased incidence of diabetes and progressive hypertrophic cardiomyopathy, a frequent cause of premature death. FRDA is the most common inherited ataxia, with an estimated prevalence within the Caucasian population of ~1:30,000. FRDA is almost absent in other populations. It is inherited as an autosomal recessive trait, with a heterozygous carrier rate of ~1:90. Symptoms usually appear around puberty, but age at onset may range from 5 to 25 years. Loss of deambulation ensues 10–20 years from the onset (Puccio and Koenig, 2002).

FRDA is caused by the defective expression of the FXN (FRDA, X25) gene, located on chromosome 9q13. In most cases, this is due to a GAA triplet repeat expansion within the first intron of the FXN gene. GAA triplet repeats, up to ~40 in normal chromosomes, range from 70 to >1000 in FRDA chromosomes and may allow for the formation of triplex helical DNA. Long DNA triplexes associate with each other (so called "sticky DNA"), inhibiting downstream transcription (Sakamoto et al., 1999). Most patients are homozygous for intronic GAA expansions, while ~5% are heterozygous with intronic GAA expansions on one chromosome and relevant point mutations within FXN exons of the other chromosome (compound heterozygotes). A direct correlation exists between the size of the GAA expansion (and thus protein expression level), the age of onset, the severity of the phenotype and disease progression (Patel and Isaya, 2001), signs and symptoms only appearing when protein level expression is decreased below a critical threshold. The bigger the expansion, the lower the protein expression level, the worse the phenotype.

Impaired expression of the FXN gene causes the defective expression of the encoded protein frataxin, whose precise functions is not yet completely known. Frataxin is normally synthesized as a 210 aa precursor. An N-terminal mitochondrial localization sequence allows the import of the precursor into the mitochondria where it is proteolitically cleaved by a mitochondrial processing peptidase (MPP) to generate a 169 aa intermediate form and finally the 155 aa mature protein (Cavadini et al., 2000). Both the amino acid sequence and the overall structure of mature frataxin are remarkably conserved from plants to mammals. Frataxin is abundantly expressed in mitochondria-rich cells which have high oxygen consumption (Koutnikova et al., 1997). In the adult, frataxin is mostly expressed in the spinal cord, heart, liver, skeletal muscle, cerebellum and pancreas. Defective frataxin expression, however, affects specifically the large primary neurons in

the dorsal root ganglia, leading to degeneration of posterior columns, spinocerebellar tracts and corticospinal motor tracts of the spinal cord and atrophy of the large sensory fibers in the peripheral nerves. Also critically affected by frataxin deficiency are cardiomyocites, which eventually degenerate leading to dilated hypertrophic cardiomyopathy. Finally, the degeneration of pancreatic beta cells may account for the increased incidence of type I diabetes observed in FRDA patients.

Studies in yeast, as well as in mammalian cells, have shown that frataxin-deficient cells have impaired biosynthesis and function of iron–sulfur clusters (ISCs)-containing proteins (Rotig et al., 1997). Since several of the components of the mitochondrial respiratory chain are ISC-containing proteins, frataxin deficiency results in impaired mitochondrial respiration, low ATP production and higher production of ROS. Moreover, the reduced utilization of iron in ISCs may cause free iron toxicity and eventually iron accumulation in mitochondria. Accordingly, FRDA cells are generally more sensitive to oxidative stress (Wong et al., 1999; Condò et al., 2006), have increased protein glutathionylation (Pastore et al., 2003), and FRDA patients have increased blood levels of antioxidant enzymes (Tozzi et al., 2002). Yet, iron accumulation and oxidative stress may not be the sole pathogenic culprits in FRDA. Frataxin KO mice are embryonically lethal indicating that frataxin is essential for development (Cossee et al., 2000). Vital, conditional tissue-specific KO mice, however, have clarified that ISC protein deficiency precedes the onset of symptoms and that mitochondrial iron accumulation is a very late event (Puccio et al., 2001). Moreover, little pathogenicity attributable to oxidative stress could be found in the cardiac frataxin-deficient mouse (Seznec et al., 2005) suggesting that more explanations for the FRDA phatogenesis are necessary.

3.3. The threshold effect in HMADs

Most of the diseases associated with mitochondrial defects present with late onset and chronic degenerative course, with signs and symptoms only appearing when mitochondrial dysfunction is no longer tolerable by cells and tissues. Several important factors underlie this phenotypic threshold effect, the most important of which include mtDNA heteroplasmy, the existence of compensatory biochemical pathways (which can act to counter the disruptive effects of a mutation early in life) and finally age-related mitochondrial changes (Rossignol et al., 2003).

In mammalian cells the mitochondria exist as a mobile, interacting reticulum that contains thousands of copies of mtDNA which mutate ten times more frequently than nDNA (Johns, 1996). Because multiple copies of mtDNA reside inside each cell (poliplasmy), when a mutation arises and starts to propagate it necessarily exists as a mixture with wild-type mtDNA (heteroplasmy). Over many generations random segregation of mutant and wild-type mtDNA during cell division can give rise to cells with variable amounts of mutant mtDNA: in the extreme case it can give rise to cells with only mutated or only wild-type mtDNA (homoplasmy). For many mitochondrial diseases, it is only when a threshold amount of mutant mtDNA is present in a cell that energy metabolism becomes impaired severely enough to cause dysfunction; beyond this point disease symptoms appear. The proportion of mutant to wild-type DNA is the percent heteroplasmy and this can vary both between individuals and within an individual's own tissues and cells. Hence, patients with the same mtDNA mutation may exhibit very different clinical manifestations depending on their percentage heteroplasmy but also on which tissues or cells first reach the critical threshold (Wallace, 1999). One dramatic example of the effects of mtDNA heteroplasmy on phenotypic outcome is seen with the missense mutation MTND6*LDYT14459A, a G–A transition in the mitochondrial ND6 gene which encodes a subunit of the NADH dehydrogenase and which leads to a decrease in the activity of complex I. A low percentage of mutant mtDNA is associated with LHON which presents in mid-life with sudden onset blindness caused by optic nerve death. Patients with a high percentage of mutant mtDNA present instead with dystonia, a more severe syndrome, which appears earlier in life and is characterized by a generalized movement disorder, impaired speech and mental retardation. A second example of the threshold effect related to mtDNA heteroplasmy is the MTATP6*NARP8993G mutation, a T–G substitution in the ATP6 gene, which affects ATP synthase functionality. When mutant mtDNA is present only as a small percentage of total mtDNA the phenotypic outcome is NARP. When present in higher percentages it leads to the more severe Leigh's syndrome an early onset disease which is often lethal.

Heteroplasmy is not sufficient to explain all instances of the phenotypic threshold effect. It cannot explain the tissue specific appearance of pathogenesis when an individual is homoplasmic for a mtDNA mutation. Similarly, it cannot explain cases where there is no correlation between the percentage of a mutant mtDNA and phenotype, nor can it explain the phenotypic variability associated with certain nuclear-encoded mitochondrial mutations or with other more generic disorders associated with secondary mitochondrial dysfunctions (diabetes, cancer, obesity). Several reasons can, however, be envisioned to explain the threshold effect in these instances. These can be summarized as follows: the concomitant presence of environmental factors or of other mitochondrial and/or nuclear mutations; association with a specific haplotype or with tissue-specific nuclear gene expression patterns; compensation by activation of reserve amounts of the same or of other mitochondrial proteins by means of increasing their transcription, translation, enzyme assembly and/or activity; a shift in energy producing pathways away from oxidative phosphorylation to glycolysis; and finally activation of cellular signaling pathways that can act to delay loss of cell viability (some of these will be discussed further later). Finally,

several studies have described an age-related decline in OXPHOS enzyme activity in multiple tissues including skeletal muscle, liver and brain (Lu et al., 2004; Trounce et al., 1989). In these same tissues an age-associated accumulation of somatic mtDNA rearrangements has been observed, most likely attributable to continuous exposure to free radical production by the ETC (Loeb et al., 2005). It is possible that this age-related decline in OXPHOS functionality, and consequent decrease in the bioenergetic capacity of cells, could ultimately impinge on an inherited mitochondrial defect aggravating it into phenotypic existence.

4. Long-lived *C. elegans* Mit mutants

One of the most powerful animal models currently in use for studying the aging process is the nematode *C. elegans.* Several classes of mutations that lead to life span extension have been identified in this organism. One of the most fascinating classes encompasses alterations which paradoxically lead to mitochondrial dysfunction (Hamilton et al., 2005). Indeed, most of the affected genes and interventions that characterize this class directly or indirectly affect ETC functionality and mitochondrial energy metabolism. However, not all mitochondrial mutations, increase lifespan; some actually decrease it (Hartman et al., 2001). To date there is no satisfactory explanation for why knocking down mitochondrial components can increase life span. Some interesting hypotheses have been proposed (review in Rea, 2005), most of which, according to the free radical theory of aging (Harman, 1956), could potentially increase life span by reducing free radical production.

4.1. Mit mutant classification

The long-lived *C. elegans* Mit mutants of can be classified into 3 major categories based on how life extension is achieved: the first category is the largest, and derives from gene inactivation by RNA interference (RNAi), the second category is somewhat smaller and can be ascribed to classical genetic mutations, the third category involves external interventions which increase life span by impinging directly on mitochondrial functionality (Table 2).

4.1.1. Gene inactivation by RNAi

Large-scale screening of whole genome by RNAi bacterial-feeding libraries led to the identification of most of the genes in category one (Hamilton et al., 2005; Lee et al., 2003). Several genes fall into this class, including frataxin which we recently found while establishing our *C. elegans* model for Friedreich's Ataxia (Ventura et al., 2005). Many of these Mit mutants display a reduction in body and brood size, have lowered oxygen consumption and/or ATP production rates, exhibit reduced behavioral rates, have altered mitochondrial morphology and affected sensitivity to oxidative stress. Most of them also increase life span in a DAF-16 independent manner (DAF-16 is a key gene involved in regulating lifespan via an insulin-like pathway).

Table 2
C. elegans mitochondrial (Mit) mutants

Examples of *C.elegans* mitochondrial mutants	Gene affected
1. *Inactivation by RNAi*	
nuo-1	Subunits of NADH–ubiquinone-oxidoreductase
nuo-2	(complex I)
cyc-1	Subunit of cytochrome *c* reductase (complex III)
cco-1	Subunit of cytochrome *c* oxidase (complex IV)
atp-2	Subunits of ATP synthase (complex V)
atp-3	
frh-1	Iron–sulfur proteins (complex I–II–III)
Many others	ETC and energy metabolism
2. *Mutations (deletion or point mutation)*	
nuo-1 (ual)	
atp-2(ua2)	Subunit of complex I
clk-l(qm30-qm51-e2519)	Subunit of complex V
isp-l(qm50)	Ubiquinone biosynthesis (complex II)
frh-l(ok610)	Rieske iron–sulfur protein of complex III
Irs-2(mg312)	Iron–sulfur proteins (complex I–II–III)
gro-l(e2400)	Mitochondrial Leucine t-RNA synthase
	Isopentenylphosphate:tRNA transferase
3. *Intereventions*	
EtBr	
Chloramphenicol or doxycycline	
Antimycin A	
Antioxidants (Conzime Q, Vitamin E, superoxide dismutase and catalase mimetics)	
Caloric restriction	

4.1.2. Gene mutations (deletion or point mutations)

Relatively few classical genetic mutants that fall into the second category of Mit mutants have been identified. This probably highlights both the importance of the ETC in nematode survival and the difficulty in obtaining hypomorphic mutations. clk-1 is the best characterized genetic Mit mutant. Clk-1 encodes a demethoxyubuiquinone (DMQ) monoxygenase necessary for the synthesis of ubiquinone (Wong et al., 1995). Three different clk-1 alleles have been described – e2519, qm30 and qm51; each displays a different degree of phenotypic severity but all accumulate the same amount of DMQ_9 – hence ruling out a role for this quinone intermediate in regulating life span (Miyadera et al., 2001). It is now thought that, like other Mit mutants, the life span increase in clk-1 mutants is due to decreased ETC functionality with consequent decrease in free radical production (Kayser et al., 2004). gro-1(e2400) is a another genetically defined Mit mutant and it has a phenotype similar to clk-1. Gro-1 normally encodes isopentenylphosphate:tRNA transferase, an enzyme that modifies a subset of mitochondrial tRNAs and is necessary for the efficient translation of mtDNA genes. Both clk-1 and gro-1 display increased life span, reduced brood size, delayed development and slowed behavioral rates (Lemieux et al., 2001). isp-1(qm150) is a third genetically defined Mit mutant and it was identified in a screen for mutants that displayed a Clk-like phenotype (Feng et al., 2001). Isp-1 encodes the Rieske iron-sulphur protein subunit of complex III and the qm150 allele contains a missense point mutation that most likely affects its redox potential. This long-lived mutant is characterized by low oxygen consumption, decreased sensitivity to ROS, a very low egg-laying rate and a dramatic reduction in both embryonic and post-embryonic development. Like both clk-1 and gro-1, isp-1 acts independently of daf-16. Finally, lrs-2(mg312) is a fourth genetically-defined Mit mutant that was identified in a screen for genetic alterations increasing C. elegans life span in a daf-16 independent manner (Lee et al., 2003). Lrs-2 encodes mitochondrial leucine tRNA synthetase and the mg312 allele is predicted to form a truncated and non-functional version of the protein. The mitochondrial C. elegans genome encodes 12 polypeptides all of which are components of the ETC and the ATP synthase. Since mitochondrial tRNA syntetase is necessary for mitochondrial gene expression, the mg312 mutant very likely has reduced or no ETC activity. lrs-2 mutants slowly develop into L4-sized, sterile adults with arrested gonad development. nuo-1(ua1), atp-2(ua2) (Tsang et al., 2001b) and frh-1 (ok610) (Ventura et al., 2005) are three loss-of-function deletions mutants which arrest at the L3 larval stage but nonetheless are long-lived, (thus technically placing them in this second category of Mit mutants). The fact that development can be uncoupled from long-life implies that different deleterious effects can appear before others when mitochondrial function is increasingly lowered (see below for threshold effects).

4.1.3. External interventions

The third and final category of factors that affect mitochondrial ETC functionality and lead to long life mainly involves exposure to various chemicals. Compounds falling into this category include ethidium bromide (EtBr), a DNA cross-linker used extensively for modeling mitochondrial dysfunction in mammalian cells (Tsang and Lemire, 2002), and antimycin A, an inhibitor of complex III (Dillin et al., 2002). Like EtBr and antimycin A, animals treated with the mitochondrial translational inhibitors chloramphenicol and doxycycline cause L3 larval arrest but life span measurements have not been undertaken on these animals (Tsang et al., 2001b). Other interventions which may directly or indirectly affect mitochondrial functionality involve mostly antioxidants such as ubiquinone, vitamin E (Ishii et al., 2004), and superoxide dismutase and catalase mimetics (Melov et al., 2000). This class of treatments opens the door to possible therapeutic interventions aimed at controlling mitochondrial functionality in both mitochondrial related diseases and aging.

4.2. The threshold effect in Mit mutants

As described in Section 3.3 many human mitochondrial disorders often present with a delayed onset, their symptoms only appearing when a threshold of mitochondrial dysfunction is reached. It has been shown that for some complexes of the ETC, as much as an 80% reduction in activity can be tolerated before mitochondrial function becomes disrupted (Rossignol et al., 2003). As we will see shortly, many cells likely have a collection of pathways at their disposal to offset reduction in their ETC activity. It is in this light that we might begin to understand how numerous genes in the *C. elegans* Mit mutants can be disrupted and yet still result in long life. The simplest explanation is that all Mit mutants retain a degree of ETC functionality and that they operate at a level just above the threshold for overt dysfunction. Compensatory pathways that are activated to counter reduced ETC functionality presumably act to also increase life span as a byproduct. Total disruption of ETC activity (by complete KO or a more robust RNAi effect) would therefore be predicted to lead to a much more severe phenotype such as developmental arrest, sterility, short life span or perhaps even lethality. Is there any evidence to support this proposition?

There is indeed a wealth of indirect evidence to support this notion. First, bacterial feeding RNAi is a knock-down rather than a knock-out technology (Ventura et al., 2005). Some degree of ETC functionality is therefore almost guaranteed to exist in RNAi-induced Mit mutants. Consistent with this concept, in the large-scale RNAi library screens undertaken by Lee and colleagues (Lee et al., 2003), some of the RNAi's directed against mitochondrial components were found to induce sterility or larval lethality, suggestive of a more severe loss of mitochondrial ETC functionality by these RNAi clones.

A similar threshold type effect can be seen when comparing different mutations in the same gene. The two known alleles of Isp-1 provide an excellent example. The *gk267* allele of Isp-1 is a knock-out mutation that results in early larval arrest. This phenotype is in stark contrast to the incredibly long-lived phenotype that is induced by the *isp-1(qm150)* missense allele encoding a protein with only reduced functionality.

Another example of a threshold effect clearly acting to control longevity in the Mit mutants can be observed when *clk-1* mutants are cultured on bacteria unable to produce ubiquinone (Q_8). Clk-1 mutants lack the ability to produce endogenous quinone (Q_9) and in order to survive they relay on bacterial Q_8 acquired from their diet (Jonassen et al., 2001). Exogenous Q_8, when present at levels corresponding to only 5% of the normal endogenous Q_9 levels, was found to be sufficient to rescue for both the development and fertility of *clk-1* mutants. Yet, when *clk-1* is cultured on bacteria unable to produce Q_8, they arrest as L2 larvae revealing a threshold effect which was otherwise masked by the presence of bacterial Q_8.

Another explanation for the threshold effect can be ascribed to a so called "maternal effect". *C. elegans* is unusual in that a significant contribution of maternal mRNA and/or protein can be transferred to progeny and still remain active well into adulthood. In some instances this maternal effect is likely to act in the Mit mutants to counter the appearance of an overt threshold effect for mitochondrial dysfunction. Specifically this phenomenon may explain why homozygous *nuo-1(ua1)* and *atp-2(ua2)* nullomorphs derived from a heterozygous mother arrest as L3 larvae (Tsang et al., 2001b), yet wild type animals fed RNAi against these same genes produce progeny that die as early embryos. Maternal effect has clearly been shown to be the responsible factor leading to the absence of *clk-1* (Wong et al., 1995) and *gro-1* (Lemieux et al., 2001) phenotypes in the first generation of progeny derived from their respective heterozygous mothers. Similarly maternal effect might also be part of the reason why *frh-1* RNAi takes two to three generations to induce a phenotype in wild type animals (Ventura et al., 2005). Finally, long-lived *lrs-2* animals are only derivable from a parental strain containing wild-type Lrs-2, since homozygous lrs-2 animals are infertile (Lee et al., 2003). It is therefore very likely that residual mRNA or protein activity contributes to the somatic development of these animals and likewise their life span extension.

Two mitochondrial mutations that shorten life span in *C.elegans* have been described so far: *mev-1(kn1)*, a missense allele in the gene encoding the cytochrome b subunit of complex II (Senoo-Matsuda et al., 2003) and *gas-1(fc21)* which affects the 49-kDa subunit of complex I (Hartman et al., 2001). These two mutations most likely represent instances in which a threshold for healthy mitochondrial function has been surpassed, leading to overt mitochondrial damage and the inability to invoke compensatory counter-measures. At least in the case of *mev-1*, it has been shown that the number of apoptotic bodies in embryos relative to normal animals is increased. Furthermore these supernumerary apoptotic deaths are, in part, responsible for the shortened life span of these animals. Thus, once beyond the threshold, apoptosis could be triggered by severe mitochondrial dysfunction (see below).

In summary, many mitochondrial mutations in *C. elegans* result in long life. Nonetheless, it appears that in *C. elegans* just like in many HMADs, severe loss of mitochondrial function results in dramatic consequences. Hence, the ability of the long-lived Mit mutants to avoid reaching some critical threshold for mitochondrial dysfunction, like in mammalian cells, can likely be ascribed to the presence of residual gene expression, protein activity, or mitochondrial functionality. Moreover, as we shall see shortly, it is very probable that several kinds of compensatory pathways are actively invoked in these long-lived animals to counter their mitochondrial dysfunction and energy deficit. These same kinds of mechanisms may also be responsible for maintaining cell viability in HMAD, at least before overt appearance of an established phenotype.

4.3. Long-lived frataxin-defective nematodes

We recently generated a new genetic tool to gain insight into the molecular pathogenesis of Friedreich's Ataxia and to characterize the function of frataxin at the organismal level. We microinjected RNAi against the *C. elegans* frataxin homolog *frh-1* into the gonad of wild type animals, and assessed their affected progeny. Consistent with other Mit Mutants we found that interfering with the expression of this mitochondrial protein increases mean life span by about 25%. We also observed that even though larval development was largely unaffected, RNAi-treated animals were smaller and paler compared to control RNAi-treated animals. These animals also layed eggs at a much slower rate and overall they also had a reduced total brood size, but their period of fertility was extended relative to control animals. Similar to other Mit mutants, we found *frh-1* RNAi-microinjected animals to be resistant to hydrogen peroxide but hypersensitive to juglone, a superoxide generator (Ventura et al., 2005).

Using a related approach, we also generated a large population of *frh-1* animals utilizing a bacterial feeding RNAi against frataxin. This allowed us to monitor both the phenotype and quantitative effects of continuous *frh-1* depletion over several generations. In the initial generation of worms exposed from eggs to *frh-1* RNAi, there was little phenotypic effect. After two generations, however, worms displayed a phenotype almost identical to that observed in progeny of *frh-1* RNAi microinjected animals: pale with small body size, reduced egg laying rate and a lifespan extension of ~25%. In these animals, quantitative RT-PCR analysis indicated that *frh-1* expression was reduced by 30% (F1) to 70% (F3).

Finally, we also obtained a frataxin knock out (KO) strain. Homozygous *frh-1(ok610)* animals were found to

arrest at the L2/L3 larval stage, yet were still long-lived. This result is reminiscent of the effect seen in the *nuo-1(ua1)* and *atp-2(ua2)* mitochondrial mutations (Tsang et al., 2001a). In an effort to improve the efficiency of our *frh-1* RNAi feeding in the nervous system (in order to obtain a better model for the human pathology), we took advantage of the *rrf-3* mutant strain, which shows an enhanced sensitivity to RNAi in all cells. Interestingly, while these animals did not exhibit larval arrest, we found that they were in fact short-lived (entura, S.L.Rea, N. Ventura and T.E. Johnson, manuscript in preparation). It is possible that in the *rrf-3* mutants *frh-1* RNAi did not reduce frataxin to the same extent in all tissues and so the life shortening effect is a result of tissue imbalances in their degree of compensatory pathway activation. A similar explanation could account for the lack of larval arrest in these animals. This would not be the case for the *frh-1(ok610)* KO animals. Differences in RNAi efficacy presumably also account for why Palau and co-workers saw shortened life span after they microinjected wild type-worms with *frh-1* RNAi, in contradiction to our earlier studies (Vazquez-Manrique et al., 2006).

5. Significance of the “Threshold”

Mitochondrial dysfunction is a normal consequence of aging, the obvious trigger of the human disorders described above, and it is likely the cause of many other disorders associated with defects in energy metabolism. As we have seen in the previous chapters, in both mammals and nematodes, mitochondrial dysfunction seems to be tolerated until a certain critical threshold is reached, beyond which a phenotype appears. Cells can thus cope with some degree of alteration in mitochondrial functionality, most likely by the activation of compensatory mechanisms that are possibly similar to the well recognized retrograde response in yeast (Butow and Avadhani, 2004). Yeast cells with defective mitochondria cannot respire normally and compensate for this metabolic insult through activation of the retrograde response that increases transcription of genes coding for glycolytic enzymes, peroxisomal and mitochondrial biogenesis, antioxidants, the glyoxylate pathway, and proteins involved in damage repair mechanisms. One consequence of this concerted response is increased replicative life span (Jazwinski, 2005).

Pathways similar to those activated by the retrograde response in yeast are known to be induced in mammalian cells following increased energy demand, from exercise, cold exposure and starvation (Biswas et al., 2005; Mitchell et al., 2002). These same pathways may well be activated to meet the energy request in mitochondrial-associated diseases (Heddi et al., 1999). The existence of a threshold effect has important implications in the study, diagnosis and treatment of human mitochondrial-associated disorders. The threshold theory implies that it may not be necessary to completely correct a defect at the genetic level in order to rescue the pathology. Instead, it might be sufficient to just compensate for it with supporting therapies in order to keep mitochondrial activity just above the critical threshold. In the following section we will provide a non-comprehensive description of signal transduction pathways which, we hypothesize, might help to compensate for mitochondrial dysfunction in both HMADs and in the *C. elegans* Mit mutants (Fig. 1). Activation of these pathways may account for increased stress resistance and increased life span in the *C. elegans* Mit mutants while being critical for rescuing cell viability in mammals, thus offering targets for potential therapeutic intervention.

6. Mitochondrial compensatory stress Responses: Mit mutants as a model for HMAD (Fig. 1)

6.1. Mitochondrial biogenesis and control of energy metabolism

When a metabolic stress places chronic demand upon ATP, cells respond by altering their metabolism and increasing mitochondrial biogenesis (Lopez-Lluch et al., 2006). Disruption of ETC function, as in many HMADs, is expected to place severe restrictions on ATP production. The transcriptional coactivator protein PGC-1 (peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1), represents a master regulator that controls mitochondrial biogenesis in mammalian cells (Wu et al., 1999). Several factors are known to regulate PGC-1, both transcriptionally or post-translationally, including the forkead transcription factor FoxO, AMP kinase, p38/JNK stress/mitogen-activated kinase, mammalian target of rapamicin (mTOR), the deacetylase sirtuin 1 (SIRT1) and calcium/calmodulin-dependent protein kinase IV (CaMKIV) (Corton and Brown-Borg, 2005). Each of these activators directly sense metabolites that are intimately associated with mitochondrial function, such as ROS, Ca^{2+}, NADH, AMP. PGC-1 in turn regulates a suite of genes necessary for the proper assembly and integration of new mitochondria into the existing mitochondrial reticulum. These include mitochondrial transcription factor, mtDNA maintenance genes (such as aconitase), mitochondrial energy generation genes (ETC complexes) and many other genes necessary for mitochondrial-related metabolic pathways (such as those regulating glucose and fatty acid metabolism).

All the pathways regulating PGC-1 expression and function are very well conserved between species and many act to regulate aging in *C.elegans.* Therefore they are optimal candidates to be investigated for a potential role in the mitochondrial stress response activated in HMADs and in the long-lived Mit mutants.

Of special interest, PGC-1 family members have emerged as central regulators of the adaptive response to caloric deprivation (Corton and Brown-Borg, 2005). Many genes which are under control of PGC-1 in mammals and belong to mitochondrial and energy metabolism pathways are repressed with aging. This repression can be partially

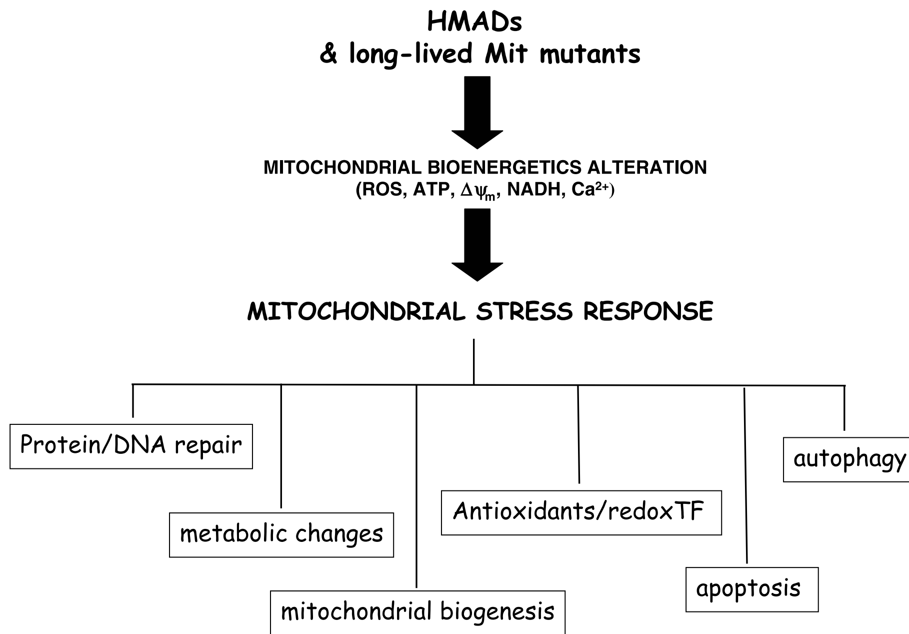

Fig. 1. Mitochondrial disruption – cell effects and responses. Mitochondrial dysfunction leads to alterations in several intracellular parameters including free radical production (ROS), mitochondrial membrane potential ($\Delta\psi_m$), ATP and NADH production, and also Ca^{2+} homeostasis. Cells actively counter these changes by inducing several compensatory pathways. Those focused on in the text include mitochondrial biogenesis and changes in energy metabolism, induction of redox-regulated transcription factors (TF) and antioxidant processes, DNA and protein repair, autophagy and apoptosis. In HMADs activation of such pathways rescues cell viability, while in *C. elegans* Mit mutants they increase cell resistance and lead to life span increase.

reversed by caloric restriction (CR) (Mootha et al., 2003; Patti et al., 2003; Paul et al., 2005). A fascinating hypothesis is that the long-lived frataxin animals, as well as other Mit mutants, are under a sort of CR and hence they mimic all the effects normally activated by CR intervention.

6.2. Antioxidants

Studies on purified mitochondria show that when the ETC is affected, ROS generation can increase (Kushnareva et al., 2002). Given the nature of the defects in many HMADs and Mit mutants, it is likely that elevated ROS production is also occurring in many of these patients as well as in the nematode mutants. Chronic, elevated ROS production negatively impacts cell survival and this is underscored by studies showing that aging and age-related damage to macromolecules, both correlate with increased production of free radical species and/or decreased antioxidant defenses (Finkel and Holbrook, 2000; Harman, 1956). Additional evidence for the impact upon aging of ROS-producing mitochondria comes from studies of p66shc KO mice. Mice lacking this mitochondrial adaptor protein, which regulates intracellular level of ROS, have been found to display increased resistance to oxidative stress and to be long-lived (Migliaccio et al., 1999).

Cells normally counter oxidative stress by upregulating the expression of several antioxidant genes such as SOD, catalase, glutathione peroxidase and peroxiredoxines. Many of these genes, are upregulated in the long-lived *C. elegans* mutants (Hsu et al., 2003) and the overexpression of antioxidant genes and anitoxidant mimetics increase *C. elegans* life span (Sampayo et al., 2003). Consistently, *C. elegans* that lack thioredoxin-1 are short lived (Miranda-Vizuete et al., 2006). Many of the long-lived Mit mutants have also been shown to display enhanced resistance to peroxide (Lee et al., 2003). Conversely, the same long-lived Mit mutants were shown to be more sensitive to the superoxide generators paraquat and juglone (Lee et al., 2003). This capability of Mit mutants to deal with only some kinds of oxidative stress but not others, might be ascribable to the induction of very specific antioxidant responses and/or to the activation of specific repair mechanisms. Whether this selectivity occurs in HMADs, and what role, if any, such processes might play in countering the threshold effect, remains to be determined.

6.3. Redox transcription factors

AP-1 (Jun/Fos), NF-kB, Nrf-2 and p53 are among the most extensively characterized transcription factors (TFs) which regulate cellular response to oxidative stress (Hansen et al., 2005). Their *trans*-activation activity relies upon two necessary redox-sensitive steps: an initial one in the cytoplasm, consisting of either phosphorylation and/or dissociation from inhibitory complexes and a second one occurring after translocation into the nucleus, where cysteine residues within the DNA binding domain of each transcription factor are reduced by thioredoxin. One important implication of this two-steps system is that even though oxidative stress in the cytoplasm may be sufficient to cause nuclear translocation of a TF, the presence of too much oxidative stress inside the nucleus, will block

TF activation. One of the most important redox-sensitive TFs is p53 (Hainaut and Milner, 1993). p53 operates as a sentinel against cellular damage by invoking multiple layers of protection. Indeed, beyond its transcription factor activity, p53 is also necessary for base excision repair (Seo and Jung, 2004) and it directly binds to oxidatively damaged DNA lesions. Recently, p53 was also shown to participate in the antioxidant defense of the cell (Sablina et al., 2005). Interestingly, two papers have now linked p53 activation to mitochondrial dysfunction and energy deprivation via AMP kinase-dependent phosphorylation (Jones et al., 2005; Mandal et al., 2005). p53 activation in these conditions inhibits the cell cycle until ATP levels are restored. Metabolic stress and/or increased free radical production, can also activate the stress-activated kinases JNK and p38, (Pelletier et al., 2005); (Veal et al., 2004)), both of which have been shown to directly regulate p53 activity (Wu, 2004). Finally p53 is interconnected in a complex network with the insulin growth factor (IGF) and the Target Of Rapamicin (TOR) pathways (see below). Hence, it is evident that p53 sits at the nexus of a complex scenario of signal transduction pathways that together integrate information necessary for cell survival under conditions of stress. In the case of HMADs it is conceivable to imagine that p53, and other redox-regulated TFs, might play key roles in preventing cells from reaching a critical threshold of disruption by inducing cell cycle arrest to repair cell damage and co-incidentally uphold energy requests. If damage is too severe, p53 can induce apoptosis (Sablina et al., 2005).

In *C. elegans*, CEP-1 is orthologous to p53. CEP-1 has been shown to promote caspase-independent germ line apoptosis in response to DNA damage. It is also required in the soma for normal resistance to environmental stressors such as hypoxia and starvation (Derry et al., 2001). Many of the pathways described above responsible for regulating p53 activity in mammalian cells could very possibly also act on CEP-1 to directly impinge on Mit mutant aging. One imaginable order of events in the etiology of Mit mutant longevity might proceed as follows: Mitochondrial dysfunction initially causes mild metabolic and/or oxidative stress. A variety of signaling cascades, involving ROS or AMP, induce transient or sub-lethal activation of p53. p53 in turn provides immediate defense against ROS damage and ATP depletion. For mitochondrial mutations that surpass a critical threshold of mitochondrial dysfunction, de-regulation of the expression and or activity of p53 could lead to all of the detrimental phenotypes observed in the Mit mutants – such as arrest, sterility, short life span or lethality.

6.4. DNA and protein repair

Oxidative damage to cellular macromolecules has been postulated to be a major contributor to the aging of diverse organisms. This damage can be limited not only by maintaining high levels of antioxidant defenses but also by the efficient clearing and repairing of damaged molecules (Tavernarakis and Driscoll, 2002). We already mentioned the role of p53 in nuclear DNA damage and protection. Recent findings have also shown a role for p53 in maintaining the integrity of mtDNA against oxidative stress. This function is mediated through the ability of p53 to bind mtDNA specific DNA-polymerase gamma (POLG) and enhance its proofreading capability (Achanta et al., 2005). The importance of maintaining POLG proofreading fidelity is evident from a mouse model containing a proofreading-deficient version of POLG which was shown to accumulate mtDNA mutations at a greater rate and display features of accelerated aging (Kujoth et al., 2005). Interestingly, mtDNA mutations are also know to accumulate in yeast with chronic frataxin deficiency (Karthikeyan et al., 2003) and with age in *C. elegans* (Melov et al., 1995).

In yeast, it has been recently shown that the iron–sulfur cluster (ISC) containing protein aconitase (Aco1p), not only functions as an enzyme of the Krebs-cycle but also as an essential component of the machinery needed for maintaining mtDNA stability (Chen et al., 2005). Aco1p is associated with protein–mtDNA complexes called nucleoids and hence it could potentially influence mitochondrial gene expression in response to changes in both cellular metabolism and oxidative stress. Consistent with this idea, aconitase, by virtue of its redox active iron–sulfur center (Bulteau et al., 2003), displays sensitivity to oxidative conditions, which becomes increasingly apparent with aging (Yan et al., 1997). Interestingly the activity of aconitase is known to be regulated by frataxin (Bulteau et al., 2004). Shadel has hypothesized that loss or oxidation of the ISC in aconitase may result in the translocation of this protein from the TCA cycle to mtDNA nucleoids (Shadel, 2005). He suggests that this would provide a double protective checkpoint in conditions of oxidative stress since protection of mtDNA by nucleoid-associated Aco1p would also be associated with reduced production of ROS because of the attenuation of the TCA cycle. Therefore, protecting mtDNA from damage in HMADs and Mit mutants is likely an important process for the avoidance of irreversible establishment of mitochondrial dysfunction.

The repair and turnover of damaged proteins are additional routes that could contribute to the maintenance of cell viability in HMADs and the Mit mutants. In *C. elegans* many long-lived mutants display increased expression of heat shock proteins and consistently both overexpression of HSPs (Walker et al., 2001) or mild heat shock treatment (Cypser and Johnson, 2002) are sufficient to increase the life span of this organism. Of note, it has been shown that mitochondrial respiratory deficiency (Kuzmin et al., 2004) or the presence of unfolded proteins in mitochondria (Hoogenraad et al., 2002) are both able to induce an up-regulation of heat shock proteins. With regard to protein turnover, it has been shown that decrease in protein degradative capacity correlate with aging, while elevated protein turnover has been shown to promote longevity. Interestingly, in long-lived calorically restricted animals, protein turn-

over is maintained at high levels (Tavernarakis and Driscoll, 2002).

6.5. Autophagy

Nutrient limitation in yeast cells, and/or hormonal stimulation in mammalian cells, triggers a conserved catabolic "self-eating" process called autophagy. This process is responsible for the degradation and recycling of nonessential cellular components (ranging from macromolecules to whole organelles), during periods of energy deprivation (Lum et al., 2005). Recently it was show that the autophagic process can also be induced by mitochondrial damage (Lemasters, 2005). In this case autophagy can be envisioned either as a process of mitochondrial quality control, or as an ultimate cellular response triggered when cells are overwhelmed with damaged mitochondria. Central to the autophagic program is Target Of Rapamycin (TOR), a phosphatidylinositol kinase-related protein kinase that is conserved from yeast to mammals (Sarbassov dos et al., 2005). In response to nutrient limitation TOR is inactivated leading to autophagy and inhibition of cell growth. TOR inactivation upon nutrient deprivation is driven by inactivation of the insulin/IGF pathway and by activation of the AMP kinases (Feng et al., 2005; Levine et al., 2006). DNA damage and energy deprivation can also lead to inactivation of TOR by rapid phosphorylation of p53.

Mouse models for FRDA reveal autophagic neurodegeneration (Simon et al., 2004). Moreover, FRDA patients often are glucose intolerant or develop type II diabetes due to alteration in the activity of the Krebs's cycle (Ristow et al., 1998). Thus, cells from such patients may perceive these alterations in glucose metabolism as a sort of nutrient deprivation signal and, along with the need to get rid of dysfunctional mitochondria, might activate the autophagic pathway as a compensatory survival mechanism.

In *C. elegans*, autophagic genes are essential for embryonic development, dauer formation and life-span extension (Melendez et al., 2003). A fascinating hypothesis then, is that the autophagic pathway is activated in the *C. elegnas* Mit mutants in order to survive and live longer. Deleterious phenotypes might appear when autophagy is unable to meet cellular energy requirements, ultimately leading to apoptosis.

6.6. Apoptosis

Apoptosis, or programmed cell death, occurs by a genetically regulated program that is well conserved from nematodes to humans (Putcha and Johnson, 2004). Apoptosis can be invoked for either developmental reasons or for purposes of removing damaged cells. Apoptotic features have been found in degenerating neurons of FRDA patients and in FRDA conditional KO mice (Puccio et al., 2001). We hypothesize that the many pathways described in earlier sections, which are normally activated in response to mitochondrial disruption, presumably reach a point where their combined actions are no longer effective in the face of massive mitochondrial damage and this inevitably leads to the activation of the apoptotic pathway.

In *C. elegans*, the core apoptotic machinery is comprised of four proteins: EGL-1, CED-9, CED-4 and CED-3. EGL-1 is induced in cells that are destined to die. It interacts with the cell death inhibitor protein CED-9 thereby displacing the adaptor protein CED-4, which in turn promotes the activation of the executioner protease CED-3. Parallel studies in vertebrates have identified the counterparts of these nematode proteins as pro-apoptotic "BH3-only" BCL-2 proteins (e.g. BIM and BID), anti-apoptotic BCL-2 proteins (e.g., BCL-2 and BCL-XL), Apaf-1 and the caspase family of cysteine proteases, respectively (Horvitz, 2001).

Three overlapping but distinct types of apoptosis have been described in *C. elegans.* One type occurs in the germ line in response to genotoxic stress (Gartner et al., 2000) or infection (Aballay and Ausubel, 2001). The second, and most extensively explored, does not occur in response to any stress, but instead, is developmentally regulated. Of the 1090 somatic cells generated during development of the *C. elegans* hermaphrodite, only 959 remain in the adult – the rest being removed by apoptosis. (Horvitz, 2001). The third type of apoptosis that has been described in worms, and germane to our current discussion, is specific to the mitochondrial mutant *mev-1*. Several supernumerary apoptotic events have been observed to occur in this mutant during somatic development and the number is enhanced under hyperoxia. Similar to other forms of apoptosis this type is characterized by mitochondrial ultrastructural abnormalities, loss of mitochondrial membrane potential and *ced-3* and *ced-4* dependency. Indeed a *ced-3* mutant is able to partially revert the decrease in life span of the *mev-1* mutant (Senoo-Matsuda et al., 2003). Increased apoptosis due to oxidative stress is therefore very likely to play an important role in many *C. elegans* mitochondrial mutants once they surpass an intolerable threshold of mitochondrial dysfunction.

In *C. elegans*, cell loss due to apoptosis might therefore account for the arrest phenotype of frataxin knockout animals and for the deleterious consequences observed in other mitochondrial mutants such as sterility and short life span. On the other hand, apoptosis of specific subsets of cells may be responsible for the increased life span of the Mit mutants. In *C. elegans*, several pathways are known to regulate metabolism and longevity (such as the IGF, TGF, and the TOR pathways) through signaling perceived by sensory neurons (Walker et al., 2005). Mutations have been identified which affect the function and development of the sensory neurons and which lead to increases in *C. elegans* life span. (Alcedo and Kenyon, 2004). In the Mit mutants, it is possible that intrinsic mitochondrial damage induces apoptosis of a subset of neurons causing worms to believe they are under nutrient deprivation and consequently leading to lifespan extension. Similarly, ablation

of the germline precursor cells in *C. elegans*, or inhibition of germ cell expansion by genetic means, have both been shown to increase adult life span (Hsin and Kenyon, 1999). Accelerated germ cell apoptosis during the life of the Mit mutants, may therefore also act to prolong life span. Possible increases in the rate of germline apoptosis could explain why *frh-1* RNAi-treated animals, as well as other Mit mutants, have a smaller brood size and lower rate of egg laying compared to control animals (Ventura et al., 2005).

In mammalian cells the connection between apoptosis and aging is less clear. Several data show that oxidative stress over the life of an organism increases the amount of mitochondria-dependent apoptosis. CR and a deletion mutation in p66shc both increase resistance to oxidative stress and apoptosis and both lead to life span extension (Migliaccio et al., 1999). Such data suggest a critical role for apoptosis in connecting oxidative stress with aging. On the other hand, deregulation of apoptosis can have a negative impact on life span and normal aging by contributing to the development of cancer and degenerative diseases (Zhang and Herman, 2002). At present it therefore remains unclear whether the alterations in apoptosis observed during aging are consequences of this process or in fact an active part of the normal aging process. Whether an increase in life span can be achieved through intervention into the apoptotic pathway, and what role apoptotic regulation will have on the outcome of HMADs, are an area in need of further investigation.

7. A window of Hope: conclusions and perspectives (Fig. 2)

The availability of the complete *C. elegans* DNA sequence has facilitated the rapid investigation of gene function using forward and reverse genetic approaches. Since a number of human disease genes have homologues in *C. elegans*, the nematode system has yielded important insights into the function of many of these (Aboobaker and Blaxter, 2000; Culetto and Sattelle, 2000).

In humans, chronic frataxin deficiency, and other mitochondrial-associated disorders in general, induce a vicious cycle that ultimately results in the dismantling of cell physiology. Currently, the progressive nature of many of HMAD diseases makes it difficult to distinguish pathological causes from effects. Thus, once a pathology presents with symptoms, cellular damage is already so severe that clinicians can only intervene with symptomatic treatment. The existence of a mitochondrial threshold effect indicates that affected cells actively try to compensate for their mitochondrial dysfunction in an effort to prevent irreversible cellular damage. These processes most likely begin well before the appearance of an established pathology. Indeed it was recently shown in mice with moderate levels of frataxin inactivation, that there was a significant alteration in the pattern of expression of mutliple genes, despite the fact that these animals manifest with no classical features of neurodegeneration, neither at the phenotypic or biochemical level (Coppola et al., 2006). When coupled with the fact that FRDA and other HMAD patients can be diagnosed by genetic analysis (either pre-symptomatically or early in the course of their disease), it become clear that targeted control of such pathways offers the potential for new therapeutic and/or preventive interventions.

Aging is one of the major risk factors for the appearance of mitochondrial-associated diseases, indicating there is likely overlap in the pathogenic alterations of these two processes. Moderate damage to mitochondria might induce a mild stress response within cells. The same stress response activated to compensate for mitochondrial dysfunction in HMADs might be responsible for ameliorating age-associated decline in mitochondrial and cell functionality. This idea is similar to the mechanism by which dietary restriction is thought to benefits cells: a mild metabolic stress causes cells to respond by modulating their energy metabolism, redox status, protein biosynthesis, mitochondrial function and genomic integrity (Lee et al., 2000; Yu et al., 1999).

It is in this light that we have explored the possible connection between HMADs and the long lived *C. elegans* Mit mutants. These animals, with their paradoxical consequences from defects in the ETC leading to life span extension, tell us there can be an advantage in lowering mitochondrial functionality, but only when it is kept within defined limits. Changes in the activity of ETC complexes, ATP production, free radical production and mitochondrial membrane potential have all been described as biochemical parameters affected in FRDA and other HMADs cells. Many of these parameters are likely also affected in the Mit mutants – but just to a different degree. This leads us to hypothesize that mitochondrial dysfunction is probably more appropriately characterized by two thresholds. In worms the first threshold coincides with the point at which mild mitochondrial dysfunction activates compensatory mechanisms giving rise to specific phenotypes such as slowed development and increased life span. The second threshold is delineated by more severe mitochondrial dysfunction, the initial compensatory mechanisms no longer benefit the cell and deleterious phenotypes such as sterility, lethality and short life span begin to appear. In the case of frataxin, we have observed that protein levels must be kept between 25 and 50% of normal amounts to see life span extension. Below 25% animals arrest and become inviable. Thus, it is more accurate to talk about a "window" in which there exists the opportunity to increase longevity if you are a worm (and perhaps a human). By corollary it is in this window that resides the opportunity to apply preventive measures to delay or prevent established HMAD (Fig. 2).

In summary, throughout this review we have introduced many pathways which may be involved in compensating for mitochondrial dysfunction in HMADs and the Mit mutants. Testing the role of such pathways in maintaining cell viability and for their potential therapeutic value is a promising area open for further investigation.

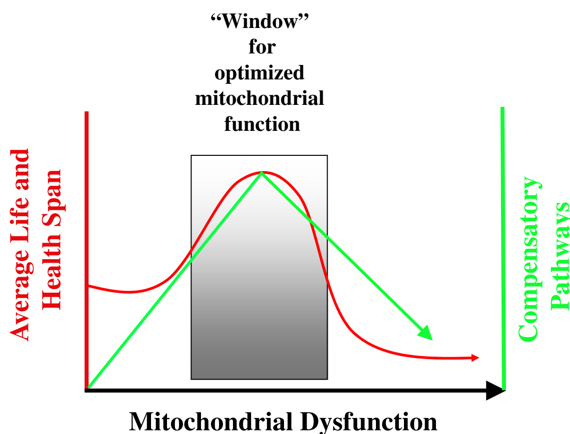

Fig. 2. "Window of Hope" for HMADs preventive therapy and for increasing life span. Graphic depiction of the effects of increasing mitochondrial dysfunction on mean life, health and compensatory pathway activation. Organisms respond to mild mitochondrial dysfunction by activating compensatory mechanisms (*green line*) that act to sustain cellular and animal viability. When mitochondrial dysfunction becomes too extreme these compensatory mechanisms cannot counter the resulting crisis and so both health and life span are compromised (red line). A discrete "window" (*box*) exists in which compensatory mechanisms not only counter mitochondrial dysfunction but incidentally lead to an increase in life span in the *C. elegans* Mit mutants. The same window is most likely responsible for the late onset of many HMADs. This provides the potential to treat mitochondrial diseases and increase human life span by external intervention with the intent of optimizing mitochondrial function and compensatory pathway activation.

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