ATF5 AS A REGULATOR OF A MAMMALIAN MITOCHONDRIAL UNFOLDED PROTEIN RESPONSE

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ATF5 AS A REGULATOR OF A MAMMALIAN MITOCHONDRIAL UNFOLDED PROTEIN RESPONSE

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Mitochondrial dysfunction is pervasive in human pathologies such as neurodegeneration, diabetes, cancer and pathogen infections as well as during normal aging. Cells sense and respond to mitochondrial stress or dysfunction by activating a protective transcriptional program known as the mitochondrial unfolded protein response (UPR^{mt}), which includes genes that promote mitochondrial protein homeostasis and the regeneration of metabolically defective organelles (Nargund, Pellegrino et al. 2012, Nargund, Fiorese et al. 2015). Work in *C. elegans* has shown that the UPR^{mt} is regulated by the transcription factor ATFS-1, which is regulated by organelle partitioning. Normally, ATFS-1 accumulates within mitochondria, but during respiratory chain dysfunction, high levels of ROS or mitochondrial protein folding stress, a percentage of ATFS-1 accumulates in the cytosol and traffics to the nucleus where it activates the UPR^{mt} (Nargund, Pellegrino et al. 2012). While similar transcriptional responses have been described in mammals (Zhao, Wang et al. 2002, Wu, Williams et al. 2014), how the UPR^{mt} is regulated remains unclear. Here, we describe a mammalian transcription factor, ATF5, which is regulated similarly to ATFS-1 and induces a similar transcriptional response. ATF5 expression can rescue UPR^{mt} signaling in *atfs-1*-deficient worms requiring the same UPR^{mt} promoter element identified in *C. elegans*. Furthermore,

mammalian cells require ATF5 to maintain mitochondrial activity during mitochondrial stress and to promote organelle recovery. Combined, these data suggest that regulation of the UPR^{mt} is conserved from worms to mammals.

BIOGRAPHICAL SKETCH

Christopher J. Fiorese attended Fordham University from 2003 to 2007, where he attained a B.A. in Natural Sciences. While at Fordham, Chris became involved in his first research project, using *Limulus Polyphemus* to study the effect of environmental pollution on the wild life of Jamaica Bay in New York City. He carried out this research under the supervision of Professors Dr. Mary G. Hamilton and Dr. Mark L. Botton. After graduation in 2007, Chris was hired as a research technician in Dr. Ralph Steinman's laboratory in Rockefeller University, which focused on Immunology. While at Rockefeller, Chris then continued his education in research and assisted with projects studying the biology of dendritic cells and how those cells control activation of adaptive immunity, specifically the role of dendritic cells in controlling tolerance to self-antigens through activation of regulatory T cells. In 2010, Chris applied to Weill Cornell Graduate School of Medical Sciences and joined the laboratory of Dr. Cole Haynes. This Thesis contains the substance of Chris' work in the Haynes laboratory.

ACKNOWLEDGEMENTS

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1.1 The Mitochondrion: The Seed of the tree Eucarya

Mitochondria are organelles descended from an endosymbioitic **α**-proteobacteria that was engulfed by pre-eukaryotic cells (Allen 2003, Lane and Martin 2010, Gray 2012). This engulfment event allowed energy production to be specialized to one area of the cell and contributed to the explosion of multicellular complexity found only in eukaryotic cells. Because of this contribution, the mitochondrion is primarily associated with energy production; however, it also serves important roles for production of essential metabolites and co-factors such as fatty acids, amino acids, and iron-sulfur clusters (Lill and Mühlenhoff 2008, Osman, Voelker et al. 2011, Wellen and Thompson 2012) and a signaling platform that is central in the activation of apoptosis and innate immunity (Zhao, Wang et al. 2002, Weinberg, Sena et al. 2015). Because of these diverse functions, the preservation of mitochondria homeostasis is centrally important to cellular and organismal health. Failure to maintain mitochondrial function results in a diverse array of diseases, from Parkinson's disease to various cancers (Nunnari and Suomalainen 2012, Vafai and Mootha 2012).

The central role mitochondria play in many cellular functions is in agreement with recent evidence that endosymbiosis is what drove the evolution of

eukaryotic cells (Martin and Muller 1998, Gray 2012). This model, known as symbiogenesis or the “hydrogen hypothesis” (Martin and Muller 1998) states that the engulfment of a eubacteria from the *Alphaproteobacteria* family by a host cell, likely from the *Archean* family came before all other features we associate with a eukaryotic cell. This theory has supplanted the classical theory of eukaryotic evolution, known as the “archezoan scenario” which held that eukaryotic cells were essentially developed before the endosymbiosis event (Margulis 1970), derived from the mistaken idea that some protists evolved without a discernable endosymbiont, the so-called “early-branching” eukaryotes (Embley and Hirt 1998). This revised timetable puts mitochondria in a leading role in the evolution of all eukaryotic cells, paving the way for the development of other features of the eukaryotic cell, such as a nucleus and further compartmentalization of the cell. The question now becomes: what makes mitochondria so special in contrast to the other features of the eukaryotic cell?

The key to the specialization of mitochondria is that they contain their own DNA, evidence of their endosymbiotic origins. Over time the genome of the mitochondria has had genes transferred to the nucleus or lost genes altogether (Gray 1999, Timmis, Ayliffe et al. 2004), which allowed mitochondria to become specialized for energy production, a phenomenon that is pronounced in multicellular eukaryotes. Animal mitochondria are gene-poor containing on average 40 genes, compared to protist mitochondria, some of which hold 100 genes (Burger, Gray et al. 2003). This shortening of the genome results in a

quicker replication time, making dynamic adjustments in the capacity of energy production easier. Mitochondria contain about 2-10 genomes per mitochondrion (Wiesner, Ruegg et al. 1992), and energy production of individual mitochondrion is tied to the transcriptional activity of local mitochondria DNA (mtDNA) (Williams 1986). Among the benefits of a specialized genome is more energy production per megabase of genetic information, which enables the cell to develop a genome with more complexity, such as non-coding regulatory regions, introns, and intergenic regions (Martin and Koonin 2006, Lane and Martin 2010). For example, note the greater density per Mb of genes found in prokaryotes, which have no specialized genome for energy production, versus those of mitochondria-containing animal cells (Lane and Martin 2010). Thus, the development of mitochondria enabled the eukaryotic genome to achieve the intricacy necessary to sustain complex organisms, and indeed is necessary to sustain the function of those organisms. In short, the presence of mitochondria in the eukaryotic cell allows the cell to increase the yield of energy per base pair and also dynamically adjust to energy requirements of the cell. It also raised a new problem for the cell: the coordination of nuclear and mitochondrial genomes for gene expression.

1.2 Energy production in mitochondria: a dance of two genomes

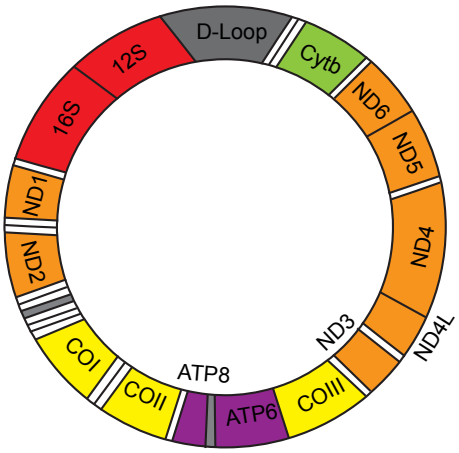
Although the mitochondrial genome is small in animals, about 16 kilobases in mammals, with a small number of genes encoded therein: 13 protein-coding, 22 tRNA, and 2 rRNA genes. In animal mtDNA, there is a core of gene products

always present that encodes components of the ETC and ATP synthase that produces the majority of energy for the cell (Figure 1.1). Transcription is polycistronic, producing two transcripts known as the heavy and light strands. This is due to the difference in density on a cesium chloride gradient caused by the high guanine content of the heavy strand while the light strand is relatively guanine poor (Falkenberg, Larsson et al. 2007).

The mitochondrial DNA encodes components for the ETC and ATP synthase, which are vitally important for the energy-producing capabilities of the mitochondrion. The ETC and ATP synthase combine to produce energy in a process known as oxidative phosphorylation (OXPHOS). This process takes fuel molecules and transfers their electrons to nicotinamide (NAD) or flavine adenine (FAD) through glycolysis, the tricaboxylic acid (TCA) cycle, and β-oxidation. NAD and FAD are then oxidized through the ETC. The ETC is comprised of four complexes, termed I-IV. Complexes I, III, and IV generate proton force pumping protons out of the matrix into the intermembrane space, aided by complex II and the electron transfer cofactors ubiquinone and cytochrome *c.* Proton translocation back into the matrix is then facilitated by ATP synthase which couples the proton translocation with ATP synthesis (Ghezzi and Zeviani 2012). Complex I is the largest of the ETC, a holoenzyme approximately 980 kDa in size. Complex I is comprised of 45 proteins total, with 39 being encoded by the nuclear genome and 7 being encoded by the mitochondrial DNA (Giachin, Bouverot et al. 2016). The complex takes the form

of an “L” shape, with the peripheral arm protruding into the matrix and catalyzing the electron transport function of the complex. The membrane integrated module handles the proton transport of the holoenzyme, and 40% f the proton motive force required for ATP synthesis is produced by complex I. The two functions are linked, as the oxidation of NADH to NAD^+ and transport of the electrons to ubqiuinone facilitates the transport of 4 protons from the matrix to the IMS. Interestingly, all seven of the mtDNA encoded complex I genes are components of this membrane domain (Vinothkumar, Zhu et al. 2014). The mitochondrial DNA components play a crucial role in complex I assembly. For example, it has been shown that the ND5 subunit encoded by mtDNA is rate-limiting step for assembly of complex I of the ETC (Bai, Shakeley et al. 2000). However, it remains unknown exactly why the cell does not simply have all the information for the building of multi-subunit complex all in one place, and rather the information be separate on two genomes, a seemingly unnecessary convoluted arrangement. While several theories have been proposed, from the idea that gene transfer between the mitochondrion and nucleus is ongoing, to the observation that the remaining genes in mitochondrion may be too hydrophobic to translate outside of the organelle, there remains little evidence for any one theory over the others (Allen 2003). Another possibility is the co-location of redox regulation (CORR) hypothesis.

A



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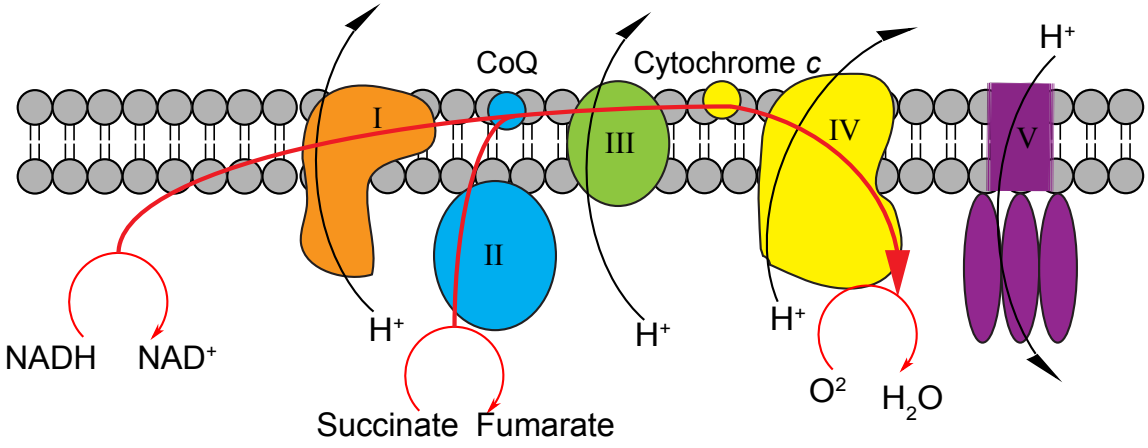

Figure 1.1 Human Mitochondrial DNA Map and Electron Transport Chain

(A) Map of human mitochondrial DNA showing non-coding regions (gray) and genes punctuated by tRNA genes (white) complex I (NADH dehydrogenase) genes (orange), complex III (cytochrome bc_1 complex) genes (green), complex IV (cytochrome c oxidase) genes (yellow), and complex V (ATP synthase) genes (purple). Corresponding electron transport chain complexes are matched in (B) with proton flow shown in black, and electron transport shown in red. Adapted from (Chial and Craig 2008)

The CORR hypothesis states that the nucleus is unable to respond to changes in O2 rapidly enough, and therefore genes whose gene products can respond to O2 changes through redox regulation have been retained in the mitochondria. While this hypothesis remains to be tested, this would explain the logic of splitting the information to build the respiratory chain into two genomes, as well as why the mtDNA always carry a core of genes that encode for the ETC and ATP synthase. This core of genes is involved with directly transporting electrons and would be able to sense changes in O2 concentration before any other system in the cell (Allen 2003). As we shall see later, this ability to detect oxygen concentration changes in the cell makes the mitochondria ideal for monitoring the overall health of the cell, a sort of canary in the coalmine for cellular homeostasis.

As attractive as the CORR hypothesis may be, there are many counter arguments to it that of varying plausibility. Amongst these hypotheses is the so-called “lock-in” theory, whereby certain genes must be expressed in the mitochondrion to direct the other components to the organelle to maintain proper localization of the respiratory complex to the mitochondria (Bogorad 1975). Another argument is that the gene transfer of mitochondrial genes to the nucleus is still in progress and has not yet been completed (Palmer 1997). Another hypothesis that does not necessarily contrast with the CORR hypothesis is the “hydrophobicity hypothesis”. This argument holds that a common feature of mtDNA encoded proteins is that they are hydrophobic. The

reason for this is that the proteins are simply too hydrophobic to be synthesized and imported in the cytosol and so must be made in the mitochondrial compartment(Beaudoin, Vachereau et al. 1986) In any case, having a cytoplasmic genome has necessitated the development of a cytoplasmic genetic system that is quite unlike the nuclear genetic system in many ways.

1.3 Mitochondria: From Replication to Protein Synthesis

Mammalian mitochondrial DNA genetics are quite different from those of nuclear DNA genetics. As opposed to the two copies of nuclear DNA present in every cell, mitochondrial DNA is circular and can have thousands of copies per cell, given the cell type (Wallace 2010). In addition, mitochondrial DNA accumulates mutations at a greater rate than nuclear DNA (Schriner, Ogburn et al. 2000). Due to the many copies of mitochondrial DNA per cell, there is a threshold level of copy numbers whereby mutated mtDNA will have an effect (Figure 1.2). Generally, a mtDNA deletion will affect respiratory function when it is present at 50-60% of total mtDNA copies (i.e. 50-60% “heteroplasmy”) in a cell, while point mutations such as those found in mitochondrial tRNA genes typically must be found in 90% of the total copy number (Larsson and Clayton 1995). Futhermore, inheritance of mtDNA is maternal, with paternal mtDNA being eliminated sometime before or during fertilization (Sutovsky, Moreno et al. 1999). Transmissions from mother to offspring of point mutations in mtDNA are relatively common, while transmissions of deletions from maternal mtDNA are rare (Chinnery, DiMauro et al. 2004). The oocyte also contains approximately

one hundred thousand copies of mtDNA, generated from a small pool of mtDNA (Jenuth, Peterson et al. 1996). All this combines to give mitochondrial genetics a non-Mendelian mode of inheritance, as mitochondrial genes are passed down maternally and the state of heteroplasmy plays a role in whether a certain genetic phenotype is presented at all.

One would expect that mitochondrial genome replication and transcription would bear many similarities to bacteria, given its eubacterial origin. However, the system and proteins used to replicate mitochondrial DNA are an evolutionary patchwork. Many of the proteins involved in replication and transcription of mtDNA such as POLRMT, POLγA, and TWINKLE are actually of bacteriophage origin, and have conserved many of the mechanisms of that system. POLRMT is the RNA polymerase of the mitochondria, and contains a domain conserved from T7 bacteriophage to recognize promoter regions in the mtDNA and also can initiate DNA synthesis (Shadel and Clayton 1997, Shutt and Gray 2006). The DNA polymerase of mitochondria, POLγ, belongs to the same group of DNA polymerases as T7, the family A group (Fridlender, Fry et al. 1972). TWINKLE is the helicase of mtDNA, and is similar to the T7 primase/helicase but conserves only the helicase function of T7, with no evidence as of yet of primase function (Spelbrink, Li et al. 2001). Despite the conservation of these proteins from the bacteriophage line, mtDNA replication is a wholly unique process.

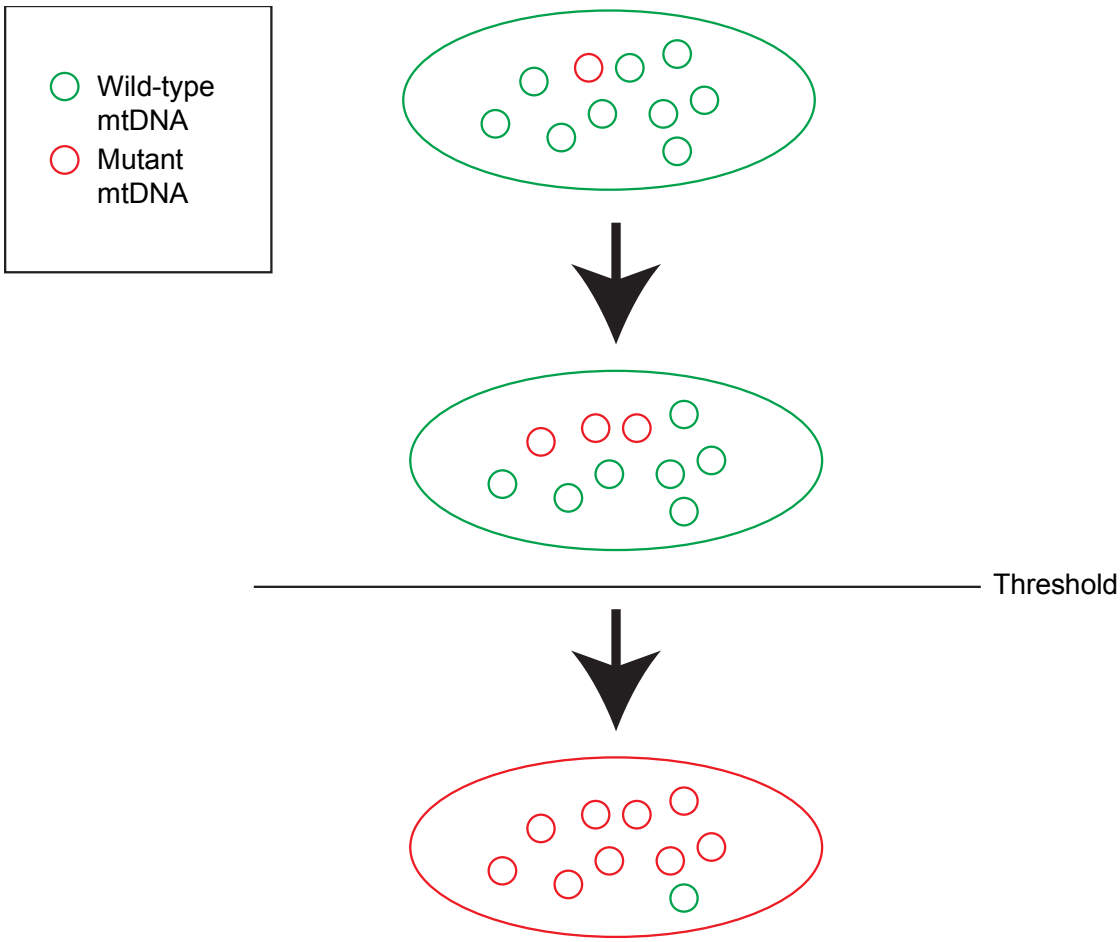

Figure 1.2 Heteroplasmy and the Threshold Effect

Mitochondrial heteroplasmy is the result of a mixed population of mutant (red circle) and wild-type (green circle) mitochondrial DNA within a mitochondrial population. In the case of a low level of heteroplasmy, mutant mtDNA will have no effect on mitochondrial function (green mitochondrion). However, due to the tendency of mutant mtDNA to proliferate more quickly than wild-type mtDNA, mutant mtDNA becomes more prevalent and subsequently affects mitochondrial function (red mitochondrion). This is known as the "threshold effect" (Stewart and Chinnery 2015).

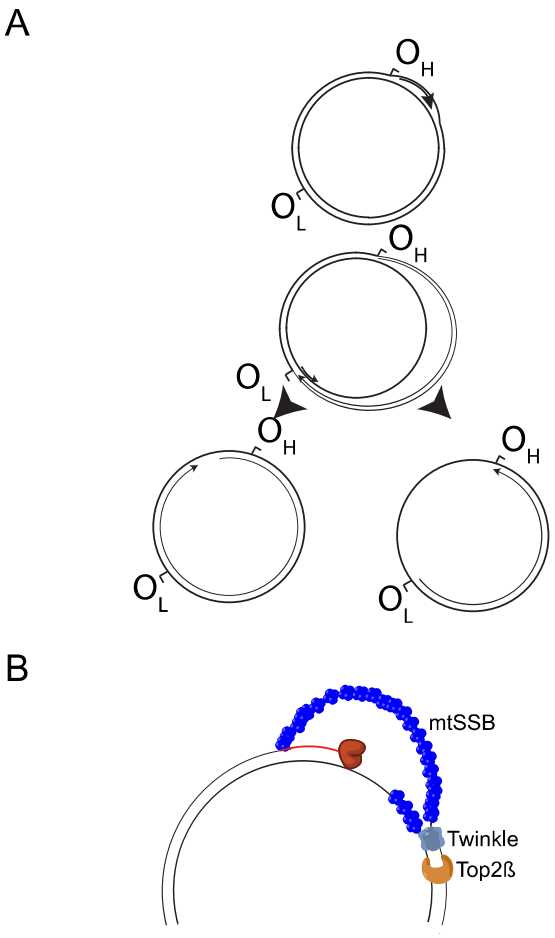

Figure 1.3 Strand Displacement Model

O_H (origin of heavy strand) where mtDNA replication begins in the strand displacement model. Replication proceeds until the O_L (origin of light strand) site, where replication temporarily stalls while the replication machinery assembles and begins processing from the O_L to begin lagging strand synthesis. (B) Minimal replisome required for mtDNA replication. Twinkle and Top2β unwind DNA and mtSSB binds to ssDNA to allow POLγ to begin replicating from POLRMT primers (Falkenberg, Larsson et al. 2007).

While there are competing models for mtDNA replication, one model that has gained prominence is a classical model known as the strand displacement model of replication (Hudson and Chinnery 2006). The strand displacement model proposes that mtDNA replication is continuous on each strand, with replication initiating from the “heavy”-strand of mtDNA (O_H) (Figure 1.3), displacing the parent heavy-strand as the new DNA is synthesized. The parental H-strand is protected by the mitochondrial single strand binding protein (mtSSB), which is bacterial in origin, in keeping with the theme of the crazy-quilt nature of mitochondrial DNA replication. Synthesis continues for two-thirds of the length of mtDNA until it is halted by the “light”-strand origin (O_L), forming a triple-stranded intermediate known as the D-loop (Clayton 1991). There, a stem-loop structure forms and recruits POLRMT, which primes the O_L for POLγ to begin lagging-strand synthesis. The mtSSB serves to protect the H-strand and also prevent aberrant synthesis from sites outside of the O_L, due to the low specificity of POLRMT (Miralles Fuste, Shi et al. 2014). Once the two daughter strands have been produced, the ends of the mtDNA are ligated into circular DNA and superhelical turns are introduced. Also present is a DNA gyrase known as Top2β, which prevents the parental and daughter strains of mtDNA from concatenating (Zhang, Zhang et al. 2014). After the mtDNA has been replicated, it must be packaged into a structure known as a nucleoid. The nucleoid acts as a unit of inheritance in the mitochondria, being divided and distributed across mitochondria as they fission and fuse. Recently, single mtDNA molecules have been shown to be packaged into nucleoids (Kukat,

Davies et al. 2015) and while they lack histones, mitochondrial proteins fill the same role. Namely, TFAM is abundantly bound to mtDNA at intervals of ~40 bp and TFAM levels have been shown to affect the copy numbers of mtDNA in a cell (Takamatsu, Umeda et al. 2002, Alam, Kanki et al. 2003, Kaufman, Durisic et al. 2007). Other proteins involved in mtDNA maintenance are also found in the nucleoid, such as mtSSB and TWINKLE. Surprisingly, mtDNA was also found to bind with mitochondrial membrane associated proteins ANT-1 and prohibitin (Bogenhagen, Wang et al. 2003, Wang and Bogenhagen 2006, Bogenhagen, Rousseau et al. 2008). This suggested that mtDNA was localized to the membrane when packaged in nucleoids. Recalling the CORR hypothesis, this localization of mtDNA fits as this positions the mtDNA and its gene products to quickly respond to any changes in oxygen consumption by the nearby ETC.

Transcription in the mitochondrial genetic system is also distinct from the nuclear system, due to the mosaic origins of the proteins involved in mitochondrial transcription, and a notable feature of mitochondrial transcription is the number of transcriptional proteins that also have another function within the mitochondria (Figure 1.4). The basal machinery for transcriptions consists of the RNA polymerase POLRMT, mitochondrial transcription factor B (TFB), and TFAM, which has a dual role in mtDNA packaging and transcription initiation (Kukat and Larsson 2013). POLRMT also has a dual role, acting as a primase for mitochondrial DNA replication and as an RNA polymerase for transcription.

POLRMT's origins as a bacteriophage protein has already been discussed but worth noting is that much of the protein's C-terminal region is conserved from the bacteriophage, and the method of promoter recognition is identical to that of the bacteriophage T7 RNA polymerase, as both use a specificity loop region that inserts into the DNA major groove to recognize promoter sequences (Cheetham and Steitz 2000, Schubot, Chen et al. 2001). Mitochondrial

transcription factor B belongs to a family of rRNA methyltransferases and originates from the mitochondrial endosymbiont, and also has a dual role in methylating 16S rRNA (Falkenberg, Gaspari et al. 2002). POLRMT requires DNA to be unwound to recognize promoter sequences, and the current model proposes that TFAM unwinds DNA, while TFB binds to and stabilizes single-stranded DNA, allowing POLRMT to function and initiate transcription (Falkenberg, Larsson et al. 2007). It has been proposed recently that replication and transcription is an either/or proposition in the mitochondria, and would go some way to explaining why a protein like POLRMT functions in both replication and transcription (Agaronyan, Morozov et al. 2015).

When transcription is initiated, transcription proceeds from the heavy strand promoter (HSP) and the light strand promoter (LSP). In animals, the polycistronic transcripts of each strand containing mRNA, rRNA, and tRNA

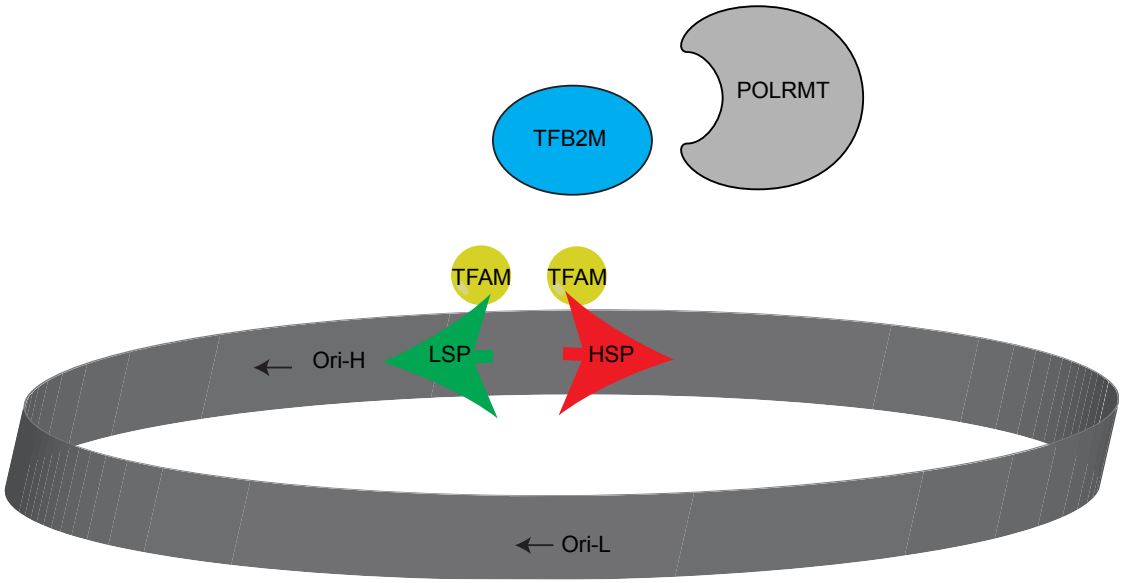

Figure 1.4 mtDNA transcription

Transcription begins when TFAM binds to mtDNA and unwinds DNA to allow for TFB2M to bind to Heavy strand promoter (HSP) or Light strand Promoter (LSP) and recruits POLRMT to HSP or LSP to begin transcription (Litonin, Sologub et al. 2010).

genes are punctuated at with the tRNA genes (Ojala, Montoya et al. 1981). These tRNA genes are believed to act as signals to be processed by the RNase

P complex at the 5' end of the tRNA gene and by the RNase Z complex at the 3' end (Hallberg and Larsson 2014). After this initial processing, mature mitochondrial transcripts look very different from transcripts processed in the nucleus. Mitochondrial transcripts often lack 5' UTR, as in the case of the ND6 gene, or otherwise have very short 5' UTRs. 3' UTRs are also absent in transcripts of many mitochondrial genes. Furthermore, mitochondrial mRNA's lack the 7' methylguanosine cap, a modification located on the 5' end of mRNA transcripts that is required for translation of all nuclear mRNA transcripts in protozoan and metazoan organisms (Grohmann, Amairic et al. 1978, Cowling 2009).

Mitochondrial translation is also distinct from translation of nuclear proteins. In the nuclear genetic system, there are two forms of methionine tRNA ($tRNA^M$), one for initiation of translation, and another to be used in elongation of the protein. Mitochondrion, however, have only one form of $tRNA^M$ that is used in both, with a formylation modification serving to differentiate between the initiator and elongator $tRNA^M$ (Tucker, Hershman et al. 2011). The composition of mitochondrial ribosomes also differs from those of ribosomes in the cytosol,

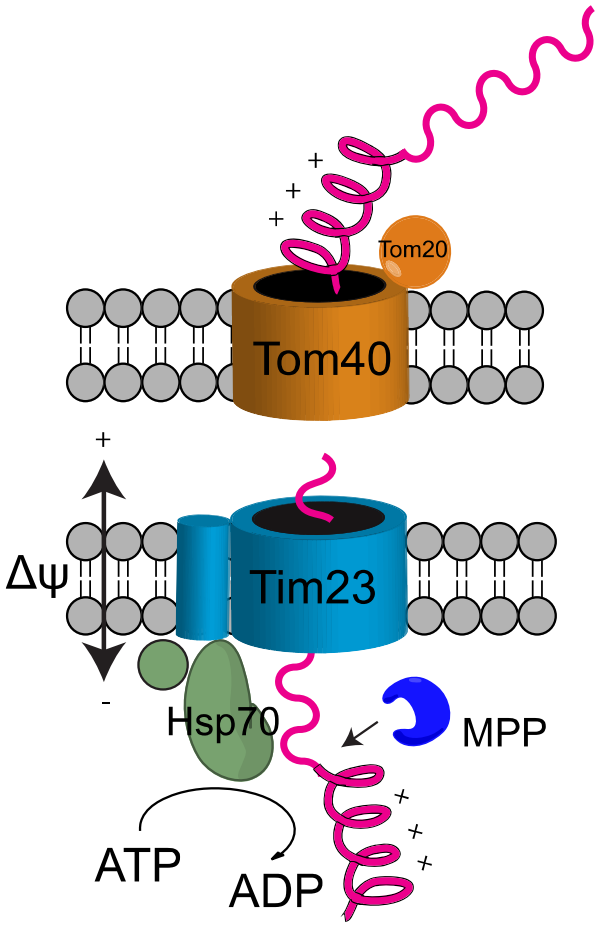

Figure 1.5 Protein Translocation into the mitochondrial matrix

99% of proteins are imported into mitochondria. The Translocase of the Outer Membrane (TOM) (orange) recognizes mitochondrial preproteins via the Tom20 component and passes the preprotein through the Tom40 channel. The preprotein then passes to the TIM channel (Tim23, blue) where it is pulled through channel by the PAM (protein assisted motor) by mitochondrial Hsp70 (green) in a membrane potential and ATP-dependent manner. The presequence is then cleaved by the mitochondrial processing peptidase (MPP) (Chacinska, Koehler et al. 2009).

with rRNA comprising 25-30% of the ribosome as opposed to nearly 60% in the cytosolic ribosome (Hallberg and Larsson 2014). This ribosome composition closely resembles those of bacteriophage ribosomes, rather than the bacterial ribosomes that one would expect mitochondrial ribosomes be similar to given mitochondria's eubacteria origins (Gray 2012). The actual synthesis of mitochondrial proteins, however, proceeds much like that of synthesis of proteins in the cytosol, with factors for initiation, elongation, and termination (Christian and Spremulli 2012).

While the mitochondrial genome has its own genetic system to produce its gene products, the proteins made by the mammalian mtDNA comprise only about 1% of the mitochondrial proteome. The other 99% of proteins located in the mitochondria are encoded in the nucleus and produced in the cytosol (Chacinska, Koehler et al. 2009). Since the mitochondrion is a double-membrane enclosed organelle known as the outer and inner mitochondrial membrane (OMM and IMM), proteins produced in the cytosol require a channel to be imported into the mitochondria. This channel, a complex of proteins known as the translocase of the outer/inner membrane (TOM/TIM channel) transport unfolded proteins through the outer and inner membranes of the organelle (Figure 1.5). This transport is dependent on the membrane potential ($\Delta\Psi$) generated by the ETC and ATP production (Chacinska, Koehler et al. 2009). Proteins targeted to the mitochondria can contain different signals directing their final localization in the cell, but mitochondrial proteins directed to

the mitochondrial matrix contain an N-terminal presequence for targeting to mitochondria. The presequence is in the form of an amphipathic helix, containing positive residues on one side of the helix, and hydrophobic residues on the other face of the helix (von Heijne 1986, von Heijne, Steppuhn et al. 1989). The positive residues target the protein to the overall negative charge of mitochondria that is a result of the pumping of protons out of the mitochondrial matrix, and the hydrophobic residues bind to a component of the TOM channel, Tom20 (Saitoh, Igura et al. 2007). Once bound to the TOM complex, the protein is passed through the main channel of the TOM, a β-barrel protein called Tom40 (Ahting, Thieffry et al. 2001). The protein is then passed to the TIM complex, and pulled through the Tim23 channel protein by the presequence translocase-associated motor (PAM) (Chacinska, Koehler et al. 2009). This complex is comprised of the chaperone mitochondrial heat shock protein 70 (mtHsp70), which binds to the unfolded
protein and imports it through a combined mechanism of ratcheting a pulling the protein in through cycles of ATP hydrolysis (Krayl, Lim et al. 2007). Once the protein is in the matrix of the mitochondria, the mitochondrial processing peptidase (MPP) cleaves off the presequence so the protein can fold properly (Luciano and Geli 1996)

1.4 Mitochondria: More than just energy production

While much of the mitochondria's genetic system is focused on producing and maintaining the electron transport chain's function, the mitochondrion is also a

platform for other vital cellular functions. Iron-sulfur cluster biogenesis is in fact the only mitochondrial process that is indispensible for life (Lill and Muhlenhoff 2008). Iron-sulfur clusters are small-inorganic molecules used as cofactors in a wide variety of cellular processes, from respiration, to DNA repair, and amino acid metabolism (Schagger and Pfeiffer 2000). Iron-sulfur (Fe-S) biogenesis begins in the mitochondria, facilitated by a complex known as the mitochondrial assembly machinery which consists of 17 proteins, and matured in the cytosol by the cytosolic iron-sulfur assembly complex (CIA) (Balk and Lill 2004) (Figure 1.6). Iron is imported in through the Mrs3-Mrs4 channel, which is dependent on the proton motive force generated by the electron transport chain. Iron and sulfur are combined in the mitochondria and reduced using electrons received from NAD(P)H, the cluster is then either inserted into mitochondrial proteins or shuttled to the cytosol for further processing (Lill and Muhlenhoff 2008). The iron-sulfur clusters are vital for heme synthesis, and are cofactors in proteins of the electron transport chain complexes I-III, as well as many other proteins with a diverse array of functions in the cell (Lill, Hoffmann et al. 2012).

Besides the synthesis of iron-sulfur clusters, the mitochondrion is also the source of many metabolic intermediates that are used by the cell during growth. The tricarboxcylic acid cycle (TCA) produces many of these intermediates. For example, citrate is produced by the TCA in the mitochondria and shuttled into the cytosol, where enzymes convert it into the small molecule acetyl-CoA which

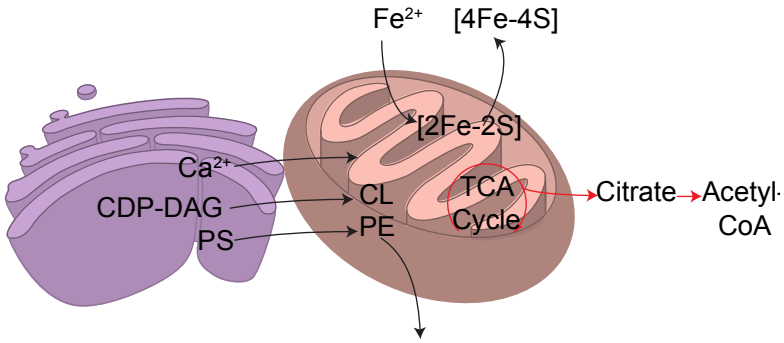

Figure 1.6 Essential Mitochondrial Products

Iron-Sulfur cluster production, Lipid biosynthesis, Calcium storage, and shuttling of TCA cycle products for use in other cellular processes such as acetylation. The proton motive force generated by oxidative phosphorylation is used to import molecules such as Iron (Fe^{2+}) and Calcium (Ca^{2+}). Iron is used to generate Iron-Sulfur clusters [2Fe-2S] which are then used in mitochondrial proteins or exported out of the mitochondria in the form of [4Fe-4S] to be used in enzymes involved in many different processes such as DNA repair(Lill and Muhlenhoff 2008). Mitochondria act as Ca^{2+} storage and regulate Ca^{2+} signaling by sequestering away excess Ca^{2+}(Rizzuto, De Stefani et al. 2012). Lipid synthesis also takes place on the mitochondrial membrane and is tightly linked to the endoplasmic reticulum. CDP-DAG is a precursor to cardiolipin, a lipid that plays many roles in the mitochondria. Phosphotidylserine (PS) is a precursor of phosphotidylethanolamine (PE) that is synthesized in mitochondria and exported to be used throughout the cell(Osman, Voelker et al. 2011). Intermediates of the TCA cycle are also used to synthesize various macromolecules in the cell, notably Acetyl-CoA is used in protein acetylation (Wellen and Thompson 2012).

is used for the acetylation of proteins in the nucleus and cytosol. Acetylation is a protein modification used in many applications by the cell, and often causes widespread changes in gene expression, through histone modification to use one example (Wellen and Thompson 2012). Acetyl-CoA is also an important precursor for fatty-acid synthesis (Morrish, Noonan et al. 2010).

Mitochondria have also long been recognized to play an important role in calcium handling in the cell. Although calcium is primarily stored in the endoplasmic reticulum (ER), mitochondria is also an important store for calcium and thus helps to regulate the concentration of calcium in the cytosol. Mitochondria also use Ca^{2+} for intramitochondrial signaling (Rizzuto, De Stefani et al. 2012). Calcium, a positively charged molecule, is drawn to the overall negative charge of the mitochondria, an example of the chemiosmotic gradient caused by the pumping of protons out of the mitochondria matrix by the ETC (Figure 1.6). Mitochondria are often located near calcium channels such as ryanodine-sensitive channels and facilitate proper function of these receptors by continuously removing calcium from the cytosol, providing a positive feedback for continued calcium transport (Szalai, Csordas et al. 2000). Once inside mitochondria, Ca^{2+} can influence oxidative phosphorylation rate as it interacts with several TCA cycle enzymes, notably pyruvate, α-ketoglutarate, and isocitrate–dehydrogenases (Forstner, Jabbal et al. 1979, Hansford 1994, Missale, Nash et al. 1998). This increases the amount of NADH produced by

the cycle and drives electron flow through the respiratory chain (Jouaville, Pinton et al. 1999).

In addition to the gradient that drives calcium into mitochondria, the proximity of the ER to mitochondria also facilitates the transport of calcium into the mitochondria. These domains of close association of the ER with mitochondria are known as the mitochondria-associated-membrane (MAM) of the ER. These contacts are <200 nm apart and seem to be mediated by proteins located on both the mitochondria and ER (Rizzuto, Pinton et al. 1998). In yeast, this protein complex is known as the Endoplasmic Reticulum Mitochondria Encounter Structure (ERMES) (Murley and Nunnari 2016). While a similar complex does not seem to exist in mammals, several proteins have been identified as important for tethering between the mitochondria and the ER, notably the protein mitofusin2 (Mfn2) (de Brito and Scorrano 2008). Calcium signaling is not the only role for the MAM; it is also the primary site of lipid biosynthesis in the mitochondria (Osman, Voelker et al. 2011). There, fatty acids such as phosphoethanolamine (PE) are synthesized by coordination of the ER and mitochondria and exported to be used by organelles throughout the cell (Voelker 1990). Another important lipid that is produced in mitochondria is the glycerophospholipid cardiolipin. Cardiolipin is a non-bilayer forming lipid with a conical shape, as opposed to the cylindrical shape of most phospholipids (Ortiz, Killian et al. 1999). Cardiolipin is crucial for many mitochondrial processes, from providing the curvature found in the cristae of mitochondria, to helping to form

microdomains where proteins can cluster in the inner membrane of mitochondria, in particular the proteins of the electron transport chain as cardiolipin has been shown to be required for the formation of the ETC (Pfeiffer, Gohil et al. 2003). Other pathways requiring cardiolipin include fusion of mitochondrial membranes, protein translocation across the mitochondrial membrane, and apoptosis (Osman, Voelker et al. 2011) (Figure 1.6).

Mitochondria also have crucial roles in signaling, most notably in programmed cell death. The mitochondrial-mediated caspase activation pathway consists of the interaction of anti- and pro-apoptotic Bcl-2 family proteins. Bcl-2 and other anti-apoptotic proteins bind the pro-apoptotic proteins and prevent pro-apoptotic downstream signaling. Pro-apoptotic signals increase the expression of the pro-apoptotic proteins Bax and other BH3-only proteins, which bind to the anti-apoptotic proteins and allow Bax and Bid to oligomerize and initiate permeabilization of the mitochondrial outer membrane and release of the electron transport chain cofactor cytochrome *c*, activating the caspase cascade that leads to apoptosis of the cell through assembly of the apoptosome (Xiong, Mu et al. 2014). The mitochondrial pathway also plays a role in other forms of apoptosis, as inhibition of mitochondrial pathway proteins such as Bmf-2 can inhibit death-receptor mediated apoptosis (Hitomi, Christofferson et al. 2008) and receptor-mediated necrosis (necroptosis) (Ben-Ari, Pappo et al. 2007). However, apoptosis is only one signaling cascade in which mitochondria plays a pivotal role.

Recently, the role of mitochondria in innate and adaptive immunity has been more greatly appreciated. The innate immune system can recognize extracellular mtDNA and N-formyl peptides as damage associated molecular patterns (DAMPs) through Toll-like Receptors (TLR) and activate production of cytokines (Weinberg, Sena et al. 2015). Mitochondria also act in anti-viral defense through the RIG-1/MAVS pathway (Kawai, Takahashi et al. 2005). RIG-1 can bind viral RNA, which exposes the proteins CARD domains, which then bind to ubiquitin chains associated with MAVS proteins. This allows MAVS to oligomerize and signal the NF-κβ transcription factor (Hou, Sun et al. 2011). The mitochondria also affect immunity through changes in metabolism. Innate immune cells such as macrophages use either glycolytic or oxidative phosphorylation after maturation to M1 or M2 phenotype respectively (Haschemi, Kosma et al. 2012). Dendritic cells also undergo changes in metabolism after activation, and a subset of dendritic cells known as tolerogenic DCs use fatty acid oxidation for energy primarily (Krawczyk, Holowka et al. 2010, Ibrahim, Nguyen et al. 2012). Adaptive immune cells also undergo metabolism changes during activation, as naïve T cells that differentiate into regulatory T cells (Tregs) require fatty acid oxidation while naïve T cells that differentiate into helper IL-17 T cells (Th17) increase glycolytic flux (Michalek, Gerriets et al. 2011). Memory T cells rely on fatty acid oxidation and show an increased spare respiratory capacity compared to activated or naive T cells (Pearce, Walsh et al. 2009, van der Windt, Everts et al. 2012). In B cells, mitochondrial ROS is required for antibody production in mature B cells

(Wheeler and Defranco 2012). In all, mitochondria function in immune cells is adjusted to suit the needs of that particular immune cell type. When the immune cells require mitochondria to synthesize metabolites for cellular growth, there is a shift to glycolytic metabolism. When the immune cell is requires maximum energy production, mitochondria are shifted primarily to oxidative phosphorylation or fatty acid oxidation.

1.5 Mitochondria: The Basis for Myriad Diseases

1.5.1 Introduction

Mitochondrial defects affect a wide range of cellular processes, resulting in a wide variety of diseases. The most common diseases which have mitochondrial dysfunction present include neurodegenerative disorders, cardiomyopathies, metabolic syndrome, cancer, and obesity. In addition, mitochondrial disorders can manifest in any organ, at any age, depending on whether the mutations are autosomal, inherited from the X chromosome, or from the maternal line (Nunnari and Suomalainen 2012). Further proof of mitochondria's importance in multiple processes is that many of the common mitochondrial mutations have a pleiotropic effect. This is due partially to heteroplasmy of mtDNA, the particular mix of wild-type and mutated mtDNA (Stewart and Chinnery 2015). As stated before, depending on the degree of heteroplasmy, high or low, mtDNA mutations can present as a more severe or less severe phenotype respectively (Wallace and Chalkia 2013).

1.5.2 Role of mtDNA Heteroplasmy in Mitochondrial Myopathies

A common type of mitochondrial mutation is mutations located in the mitochondrial tRNA coding genes. $tRNA^{Leu}$ in particular often contains a nt3243A>G mutation that can result in many different diseases depending on the level of heteroplasmy in the cell. High heteroplasmy is associated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). Low heteroplasmy of the mutation results in deafness and diabetes (Goto, Nonaka et al. 1990, van den Ouweland, Lemkes et al. 1992).

Similarly, mtDNA protein coding gene ATPase6 mutations with high heteroplasmy lead to pigment retinopathy, ataxia, and neuropathy in adults, while the same mutation with low heteroplasmy leads to inherited Leigh syndrome in infants (Holt, Harding et al. 1990, Tatuch, Christodoulou et al. 1992). Defects in complex I lead to atrophy of optic nerves when manifesting in adults and subacute necrotizing encephalopathy in infants (Wallace, Singh et al. 1988). As reactive oxygen species (ROS) is mainly produced by the respiratory complexes I and III, defects in these complexes often lead to aberrant ROS signaling. Leber's hereditary optic neuropathy (LHON) is caused by mutations in mtDNA encoded complex I subunits which affect electron transfer to ubiquinone (Patsi, Kervinen et al. 2008).

1.5.3 Mitochondrial Myopathies and Mutations of Nuclear Encoded Mitochondrial Genes

While many mitochondrially encoded genetic mutations result in various diseases, nuclear-encoded mitochondrial genes can underlie mitochondrial disease as well. For example, the majority of complex I defects found in patients derive from the nuclear-encoded genes of complex I (Ugalde, Janssen et al. 2004). Most pathogenic mutations of complex I are located in the highly conserved core of the complex (Yano 2002). While the exact effect of many of these mutations is unknown, studies of lower organisms have shown that complex I mutants can affect everything from electron transfer (Kerscher, Kashani-Poor et al. 2001) to complex I's association with complex III (Ugalde, Janssen et al. 2004), which is necessary for complex I stability(Calvaruso, Willems et al. 2012).

As one would imagine, mutations in any of the core mtDNA replisome proteins cause major mitochondrial dysfunction, and many of the mutations in nuclear-encoded genes such as *POLG* and *TWINKLE* have similar phenotypes. Both *POLG* and *TWINKLE* mutations can result in progressive external opthalmoplegia (PEO) (Spelbrink, Li et al. 2001, Longley, Clark et al. 2006). Characteristics of PEO include peripheral eye muscle weakening (ophthalmoparesis), muscle wasting, neurological and neuromuscular defects. A study found that a mouse model containing a *Twinkle* mutation found in patients with PEO reconstituted many of the symptoms of the disease. These "Deletor" mice accumulated mtDNA deletions and showed respiratory

deficiencies in skeletal muscle, cerebellar and hippocampal neurons (Tyynismaa, Sembongi et al. 2004).

1.5.4 Mitochondrial Dysfunction and Cancer Cells

Cancer cells and mitochondria dysfunction have long been linked, first in the description of the Warburg effect in cancer cells (Koppenol, Bounds et al. 2011). The Warburg effect describes a phenomenon in cancer cells where glucose uptaken by cancer cells is fermented into lactic acid despite the ready availability of oxygen. While Warburg initially proposed that this phenomenon was due to mitochondrial dysfunction, the reality of how mitochondrial dysfunction interacts with cancer biology has been shown to be more complicated than that hypothesis. Mitochondrial function is required for cancer cell viability, and depletion of functional mitochondria has been shown to impair tumor cell growth (Weinberg, Hamanaka et al. 2010). However, rather than oxidative phosphorylation, mitochondria are shifted to produce macromolecules for the cancer cell to use for cell proliferation (Vander Heiden, Cantley et al. 2009). Mutations in TCA cycle genes have been shown to result in many different cancers, in particular the isocitrate dehydrogenase-1 (*IDH1*) has been shown to be a tumor suppressor, as *IDH1* mutations found in gliomas (Yan, Parsons et al. 2009).

Mitochondrial DNA mutations are also associated with tumor growth, as many different types of cancers have been shown to accumulate mtDNA mutations as

they grow and some tumors contain homoplasmic mtDNA mutations (Polyak, Li et al. 1998, Petros, Baumann et al. 2005). These mutations can affect electron transport chain efficiency, and increase the production of ROS by the mitochondria (Ishikawa, Takenaga et al. 2008, Ralph, Rodriguez-Enriquez et al. 2010). Further evidence that mtDNA mutations drive cancer formation have shown that efficient mitochondrial function is tumor suppressive in some cases (Santidrian, Matsuno-Yagi et al. 2013)

1.6 Cellular Responses To Mitochondrial Dysfunction

As mitochondria are responsible for many essential cellular functions and as mitochondrial dysfunction results in pleiotropic diseases, maintenance of mitochondrial homeostasis is critical. Given the importance of this function, eukaryotes have several ways of monitoring mitochondrial function. Referring back to the CORR hypothesis, one of the tenets of CORR is that in addition to mitochondria sensing changes in oxygen levels through regulation of its own genome, a secondary signal must come from the mitochondria that reports to the nucleus on the current state of the organelle population (Allen 2003). This mitochondria to nuclear communication can happen through several pathways. Some signals that convey mitochondrial status to the nucleus include calcium, ROS, import efficiency, or energy production levels (Topf, Wrobel et al. 2016) (**Error! Reference source not found.**)

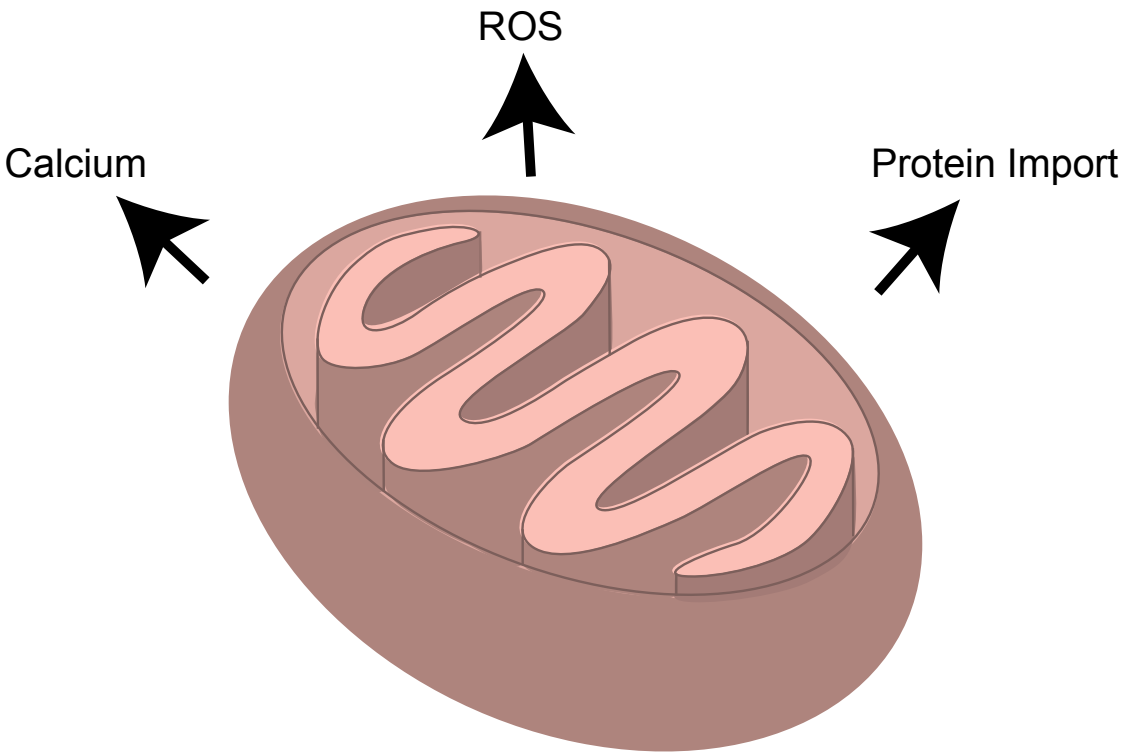

Figure 1.7 Mitochondrial Retrogade Signals

Shown are retrograde signals that facilitate communication between the mitochondria and nucleus. Mitochondrial stress can cause altered levels of Calcium and ROS, and act as signals to the nucleus to activate stress responsive genes that restore mitochondrial function. Protein import can also be affected by mitochondrial stress, as mitochondrial dysfunction can affect oxidative phosphorylation, which drives the proton motive force and ATP production that fuels mitochondrial protein import. A slowdown in import can then signal to the nucleus mitochondrial stress, in *C. elegans* through the transcription factor ATFS-1.

In yeast, mitochondria use a system of signaling known as the retrograde response (RTG). Mitochondrial stress caused by mtDNA depletion activates the transcription factors Rtg1 and Rtg3 by the sensor of mitochondrial stress Rtg2 via an unknown mechanism. Rtg1 and Rtg3 are phosphorylated and sequestered from the nucleus in the absence of mitochondrial stress. During stress, Rtg2 is activated and Rtg1 and Rtg3 is dephosphorylated and translocates to the nucleus to activate the RTG response. Once the RTG is activated, the gene *CIT2* is upregulated, a key enzyme of the glyoxylate cycle that is a modified tricarboxylic acid cycle that allows for alternate carbon sources to be used for energy. The RTG also controls TCA cycle gene expression once activated (Butow and Avadhani 2004).

However, a direct homolog of the RTG pathway has not been found in mammals. Rather, the RTG response seems to be broken up and its role taken by multiple pathways. The transcription factors NRF1 and NRF2 control expression of the respiratory genes, VDAC, mtTFA and mtTFB (Scarpulla 2002). Peroxisome proliferator activated receptor (PPAR) α and γ control gene expression of genes involved in fatty acid metabolism and β-oxidation (Puigserver and Spiegelman 2003). PPARγ coactivator-1 (PGC-1) controls mitochondrial biogenesis as well as rate of respiration in brown fat and skeletal muscle cells (Wu, Puigserver et al. 1999).

As calcium regulation is an important function of mitochondria, Ca^{2+} can also act as a retrograde signal from mitochondria to the nucleus. Ca^{2+} is imported into

mitochondria due to $\Delta\Psi_m$, so any perturbation of $\Delta\Psi_m$ will increase the cytosolic Ca^{2+}. Sustained increase of Ca^{2+} cytosolic levels results in activation of the Ca^{2+} responsive phosphatase calcineurin, which results in an upregulation of genes involved in calcium storage and transport (Biswas, Adebanjo et al. 1999). Calcineurin was also shown to activate NF-κB signaling downstream of mitochondrial stress by dephosphorylating IkBβ, a factor that sequesters NF-κB in the cytosol. This dephosphorylation event disassociates IkBβ from NF-κB and allows NF-κB to translocate to the nucleus and activate a transcriptional response that includes hexokinase, ryanodine receptors, and cathepsin L (Butow and Avadhani 2004).

ROS generated by the respiratory complexes are also able to signal mitochondrial dysfunction to the nucleus. ROS have been shown to lower the mitochondrial membrane potential and interfere with proper protein import (Wright, Terada et al. 2001). It has also been proposed that accumulation of oxidized proteins due to ROS exposure serves as a possible mechanism for NF-κB activation and upregulation of ROS-protective genes seen in mitochondria-stressed cells (Kriete and Mayo 2009). Additionally, ROS activates the Nrf2-antioxidant response element pathway (ARE), which also upregulates ROS protective genes (Johnson, Johnson et al. 2008). Much like with other pathways ROS affects, the mechanism of how elevated ROS levels activate these various pathways is unknown.

1.7 The UPR^{mt} : A brief history

An additional pathway in mitochondria to nuclear communication is known as the mitochondrial unfolded protein response (UPR^{mt}) (Figure 1.8). This pathway was initially described as a way for mitochondria to maintain protein homeostasis in the organelle, as mitochondria are constantly importing and processing proteins in their unfolded state. Hoogenraad et al. initially described the pathway in rat hepatoma cells that had their mitochondrial DNA depleted through ethidium bromide (EtBr) treatment (Martinus, Garth et al. 1996). This group also used a mutated form of ornithine transcarbamylase (OTC), a mitochondrial protein that has an in-frame deletion (ΔOTC) that is imported into mitochondria, does not properly fold into a functional enzyme, and accumulates in the organelle as an unfolded protein (Zhao, Wang et al. 2002). These stresses were shown to upregulate mitochondrial chaperones and proteases such as Cpn60 (HSP60) and ClpP, which was reminiscent of the response to unfolded protein accumulation in the endoplasmic reticiulum, the UPR^{ER} (Ron and Walter 2007). The transcription factor CHOP was proposed to activate the UPR^{mt} after mitochondrial stress, although the evidence for this is indirect and the mechanism by which CHOP senses mitochondrial stress is unknown. Another aspect of the mammalian UPR^{mt} that has been proposed is the involvement of the double-stranded RNA-activated protein kinase (PKR) as a molecule that detects mitochondrial stress and signals downstream to the c-Jun

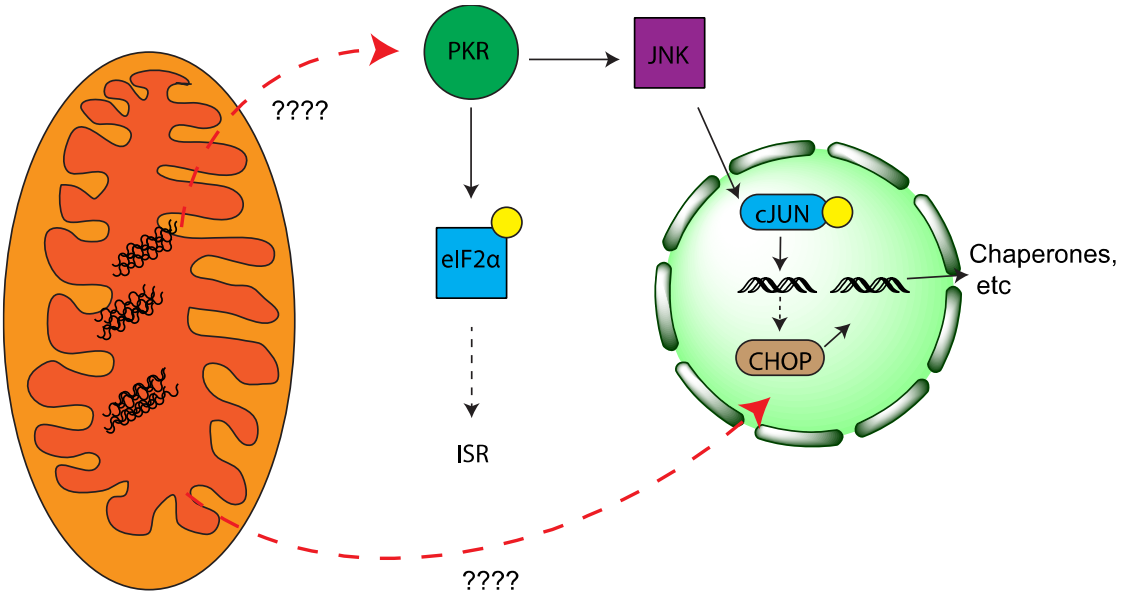

Figure 1.8 Mammalian Mitochondrial UPR

While much remains unknown about the UPR^{mt} in mammals, some studies have identified possible components of the UPR^{mt}. Although no mechanism is known, current proposals are that accumulation of unfolded proteins or other stresses activates transcription of chaperones and proteases either by direct activation of the CHOP transcription factor, or activation of the kinase PKR. PKR signaling goes through the cJUN/AP1 pathway to possibly activate CHOP (Arnould, Michel et al. 2015). PKR is also known to phosphorylate eIF2α although how this might affect the UPR^{mt} is unknown.

through eIF2α phosphorylation, although how the PKR kinase is activated by mitochondrial stress remains unknown. Exactly what the c-Jun/AP1 pathway can activate in response to mitochondrial stress is not known (Rath, Berger et al. 2012).

Another aspect of retrograde signaling is the involvement of sirtuin proteins, a family of deacetylases. NAD^+ is required for the function of sirtuins, and in particular, increased NAD^+ levels activate sirtuin-1 and lead to upregulation of mitochondrial mRNA protein translation (Houtkooper, Mouchiroud et al. 2013). Another sirtuin with a role in mitochondrial stress is sirtuin-3, which responds to mitochondrial proteotoxic stress by inducing antioxidant genes through the transcription factor FOXOA3 and increasing mitochondrial turnover (Papa and Germain 2014).

While there are a few proposals about the downstream effectors of mitochondrial stress, not much is known about the mechanism or the signal that communicates from the mitochondria to the nucleus to indicate the presence of mitochondrial stress. The aim of this thesis is to elucidate the mechanism of mitochondrial stress sensing in both the worm *Caenorhabditis elegans* and the mammalian system.

Chapter 2. Activation of UPR^{mt} is Controlled by ATFS-1 via Mitochondrial Import Efficiency and Activates a Broad Transcriptional Response

2.1 The UPR^{mt} communicates mitochondrial stress to the nucleus by monitoring import efficiency

While the initial studies of the UPR^{mt} were carried out in mammalian cell culture, most of the mechanism of how the UPR^{mt} is activated has been carried out in *Caenorhabditis elegans*. The UPR^{mt} in *C. elegans* was activated by conditions that cause mitochondrial stress, such as depletion of mtDNA, (Yoneda, Benedetti et al. 2004) ETC genes (Durieux, Wolff et al. 2011), depletion of mitochondrial proteases (Aldridge, Horibe et al. 2007, Nargund, Pellegrino et al. 2012), and reagents that generated a high concentration of ROS in the cell (Yoneda, Benedetti et al. 2004). Furthermore, worms lacking UPR^{mt} show impaired growth and survival during mitochondrial stress (Haynes, Yang et al. 2010, Nargund, Pellegrino et al. 2012). To determine how the UPR^{mt} was being regulated, an RNAi screen using worms expressing a GFP reporter under the mitochondrial chaperone *hsp-60* promoter that were exposed to mitochondrial stress revealed a bZIP transcription factor known as ATFS-1 that was required for *hsp-60$_{pr}$::gfp* activation and thus UPR^{mt} signaling (Haynes, Petrova et al. 2007). What was unknown was how the transcription factor detected mitochondrial stress.

To determine how ATFS-1 regulated the UPR^{mt}, worms were fed RNAi against the mitochondrial protease *lon* to determine if ATFS-1 was a substrate of the protease. Indeed, ATFS-1 accumulated in the mitochondria after depletion of LON protease, suggesting that ATFS-1 is imported into the mitochondrial matrix where it is degraded by LON protease under normal conditions. To determine if ATFS-1 was targeted to the mitochondria, the first 100 amino acids was N-terminally tagged to GFP and expressed in HeLa cells. ATFS-1$^{1-100}$::GFP indeed localized to mitochondria in HeLa cells, confirming the mitochondrial localization of the protein (Figure 2.1). Immunoblot against fractionated HeLa cells confirmed ATFS-1$^{1-100}$::GFP was enriched in the mitochondrial fraction in a mature form, while the precursor form of the protein was enriched in the cytosolic fraction (Nargund, Pellegrino et al. 2012). This data shows that ATFS-1 is imported into mitochondria, and its MTS is cleaved upon import. Other experiments in our lab found that ATFS-1 is expressed constitutively, and is constantly degraded by LON protease during normal conditions. This method of regulation echoes other stress-responsive transcription factors that are regulated by constant synthesis followed by turnover, such as p53 and HIF-1α (Haupt, Maya et al. 1997, Maxwell, Wiesener et al. 1999).

Next, experiments were performed to determine wanted to confirm whether mitochondrial localization of ATFS-1 negatively regulated activation of the UPR^{mt}. To test this, a construct of ATFS-1 was created that replaced the N-terminal 23 amino acids of ATFS-1 with the protein-tag myc. This construct was

then expressed transgenically in worms that lacked atfs-1 expression. Indeed, expression of this construct in the absence of ATFS-1 was enough to activate *hsp-60*$_{pr}$*::gfp* expression in the worm (Figure 2.2), consistent with the hypothesis that ATFS-1 is imported into mitochondria to prevent activation of the UPR^{mt}, and blockage of ATFS-1 import is sufficient to activate the UPR^{mt}.

Microarrays performed by our lab found that besides the usual chaperones and proteases, ATFS-1 also upregulates 381 genes involved in many other processes, from ROS detoxification to glycolysis (Nargund, Pellegrino et al. 2012). To further validate the direct involvement of ATFS-1 in upregulating these processes during mitochondrial stress, our lab performed ChIP-Seq. ChIP-Seq data confirmed that ATFS-1 bound to genes important for UPR^{mt} activation such as *hsp-60* and other genes found to be upregulated in an *atfs-1* dependent manner by microarray. There were also genes that *atfs-1* appeared to activate indirectly, as ATFS-1 was found to not bind directly to the promoter regions of these genes, but according to the microarray are upregulated in an *atfs-1* dependent manner (Nargund, Fiorese et al. 2015).

Because ATFS-1 is a transcription factor, it must recognize a specific sequence in the promoter regions of genes in order to activate gene expression. Promoter search of genes that are activated by mitochondrial stress in an ATFS-1 dependent manner revealed an 14-base pair element that was enriched in the promoter regions of UPR^{mt} target genes (Nargund, Fiorese et al. 2015). To determine if that element is required for UPR^{mt} activation, a transgene

containing the *hsp-60$_{pr}$::gfp* reporter with the UPR^{mt} element in the *hsp-60* promoter mutated was expressed in worms (hsp-60$_{pr*}$::gfp). These transgenic worms were then subjected to mitochondrial stress and showed impaired UPR^{mt} activation.

An UPR^{mt} element was revealed to also be located in the D-loop region of mtDNA as well (Nargund, Fiorese et al. 2015). This implied that ATFS-1 was able to bind both nuclear and mtDNA. To test this hypothesis, an EMSA was performed on an oligonucleotide generated containing the UPR^{mt} element found in mtDNA, and incubated with recombinant ATFS-1 protein. Indeed, ATFS-1 bound to the UPR^{mt} Element containing oligonucleotide but not an oligonucleotide that contained a scrambled sequence (Figure 2.3). This data is consistent with ATFS-1 binding a UPR^{mt} element in both nuclear and mitochondrial DNA. To confirm that ATFS-1 was binding to mtDNA during stress, qPCR was performed on worms with and without mitochondrial stress in the presence or absence of *atfs-1*. This data showed that during mitochondrial stress, *atfs-1* effected a small upregulation of mtDNA transcripts, however in the absence of *atfs-1*, mtDNA transcripts were increased to 10-fold above wild-type, indicating that ATFS-1 was acting as a repressor of gene expression during stress.

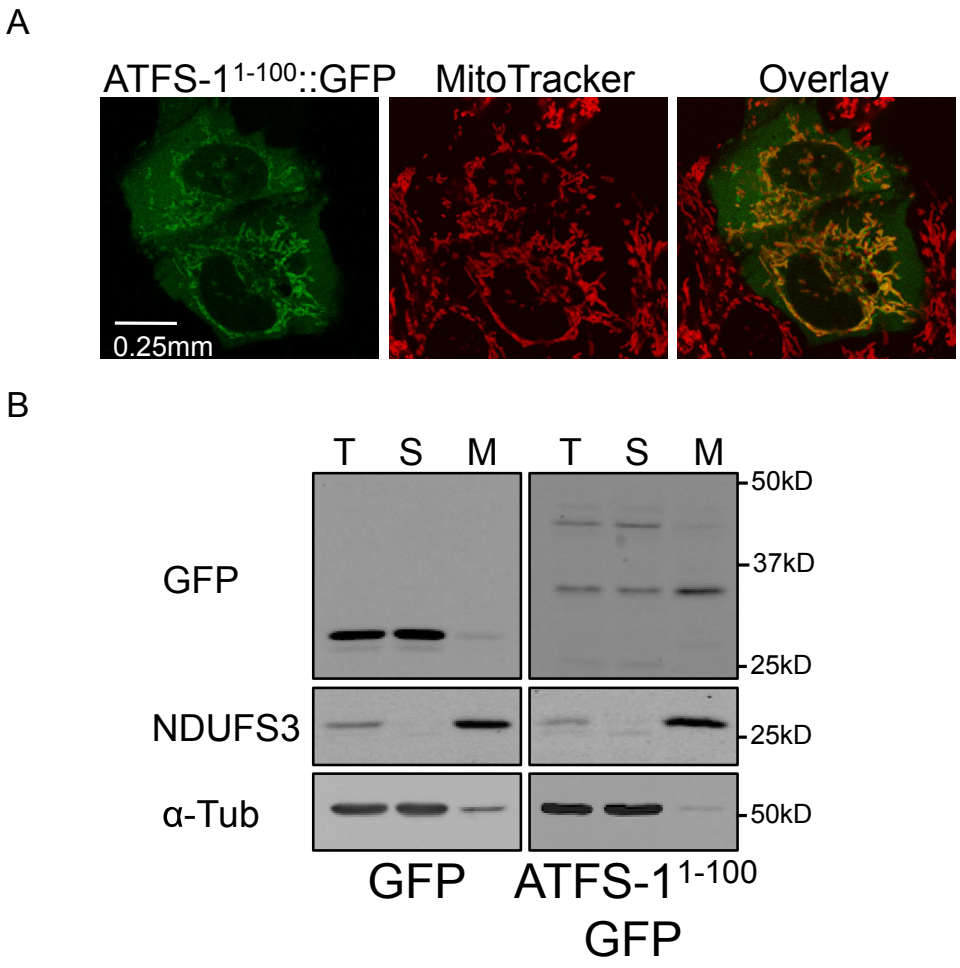

Figure 2.1 In the absence of stress, ATFS-1 is imported into mitochondria

A. Photomicrographs of HeLa cells expressing ATFS-11-100::GFP or GFP stained with MitoTracker. Scale bar, 0.25 mm (n = 3).

B. Immunoblots of HeLa cells expressing GFP or ATFS-11-100::GFP following fractionation into total lysate (T), postmitochondrial supernatant (S) and mitochondrial pellet (M). Longer exposure of the ATFS-11-100::GFP panel was required due to toxicity and weak expression.

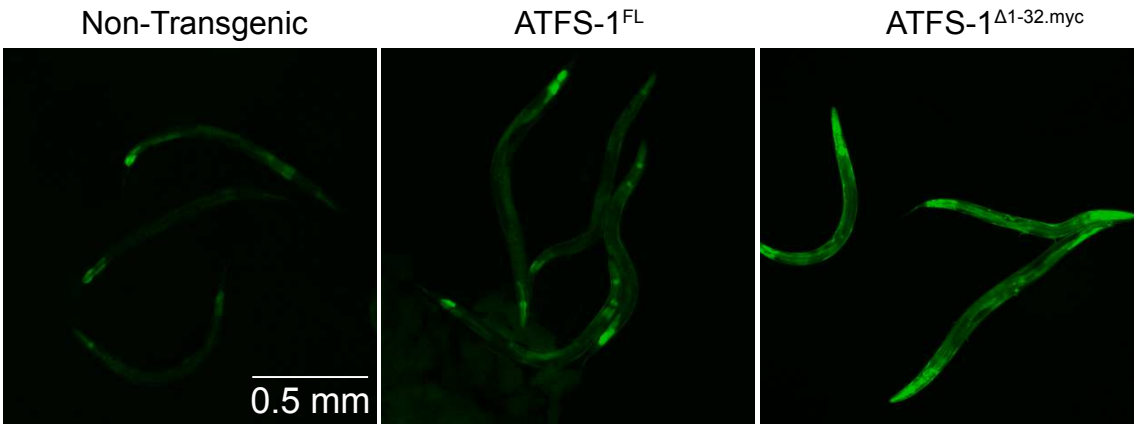

Figure 2.2 ATFS-1 is negatively regulated by mitochondrial import

Photomicrographs of *atfs-1(tm4525); hsp-60pr::gfp* worms expressing wild-type full-length (FL) ATFS-1, ATFS-1$^{\Delta 1\text{-}32.myc}$ or ATFS-1$^{\Delta 1\text{-}32.myc.\Delta NLS}$ (n = 3).

2.2 ATFS-1 Regulates a Broad Transcriptional Response Including Innate Immunity

As stated before, the role of mitochondrial biology in immunity has recently begun to be appreciated (Weinberg, Sena et al. 2015). While the effect of mitochondria on specialized immune cells has been discussed already, mitochondria are also a target for attack by pathogens as a way for those pathogens to gain a foothold in the organism for proliferation. Examples of such include a species of the Gram-positive bacteria *Streptomyces* that produces the mitochondrial poisons antimycin and oligomycin, which inhibit complex III and V respectively (Pellegrino and Haynes 2015). The pathogen *Pseudomonas aeruginosa* produces the mitochondrial complex IV inhibitor cyanide, a complex IV inhibitor (Pellegrino and Haynes 2015). Clearly, the mitochondria is an important target for pathogens as well as an organelle that is integral to immune signaling and function.

Activation of the UPR^{mt} results in a wide range of genes being activated, from mitochondrial chaperones and proteases typical of an unfolded protein response, to ROS scavengers, and interestingly, those involved in innate immunity (Nargund, Pellegrino et al. 2012). Other studies have shown that perturbation of vital cellular functions is able to activate an immune response, as many pathogens interfere with these vital functions (Melo and Ruvkun 2012). Among those that activate the immune response are any that interfere with mitochondrial function. To test whether the UPR^{mt} was involved in this

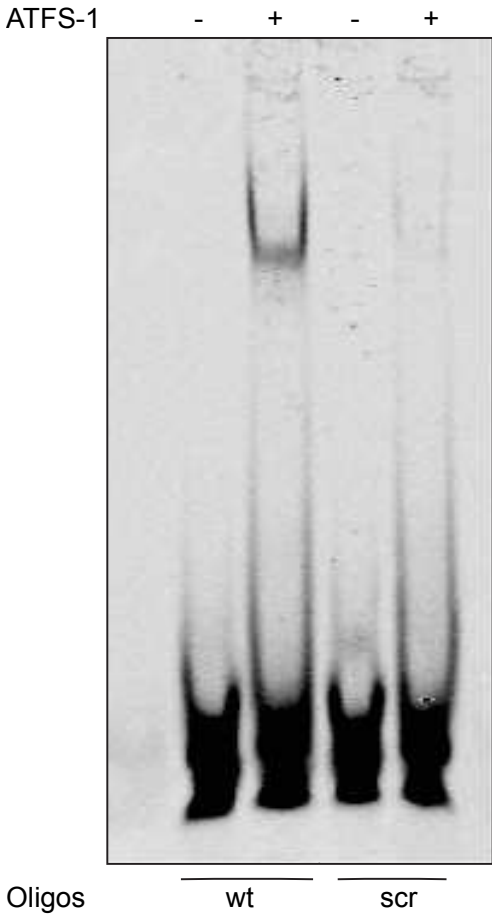

Figure 2.3 ATFS-1 binds to UPR(mt) Element located in mtDNA

EMSA using recombinant ATFS-1 and wild-type D-loop oligonucleotides or D-loop oligonucleotides with the 9 base pair UPRmtE scrambled. Unbound oligos (black square), ATFS-1-DNA complex (black arrow) and the loading well (gray arrow) are indicated. (n = 3)

response, worms expressing the UPR^{mt} reporter *hsp-60$_{pr}$::gfp* were fed *P. aeurginosa*. After exposure to *P. aeurginosa*, worms showed activation of UPR^{mt} in an ATFS-1 dependent manner (Pellegrino, Nargund et al. 2014). In addition, worms that are primed with mitochondrial stress prior to exposure to *P. aeurginosa* were more resistant to the pathogen than worms that had not been primed with mitochondrial stress beforehand. ATFS-1 was also required for this increased resistance to *P. aeurginosa* in these worms that had the UPR^{mt} pre-activated, indicating that UPR^{mt} activation is necessary for *P. aeurginosa* resistance in worms.

It has also been shown that mitochondria has a role in resistance to bacterial infection in mammals. In particular, ROS generated by mitochondria is crucial in activation of the immune response to bacterial infection (Pellegrino and Haynes 2015). Mitochondrial poisons rotenone and antimycin were able to activate the NRLP3 inflammosome (Traba, Kwarteng-Siaw et al. 2015). As the UPR^{mt} activates innate immune genes in worms, the next question is whether mitochondrial stress can activate innate immune genes in the mammalian system. Indeed, transgenic expression of misfolded protein ΔOTC or a dominant negative version of mitochondrial protease AFG3L2 caused upregulation of innate immune genes, particularly anti-microbial peptides (Figure 2.4). This data is consistent with mitochondrial stresses known to activate the UPR^{mt} lead to an activation of innate immune genes.

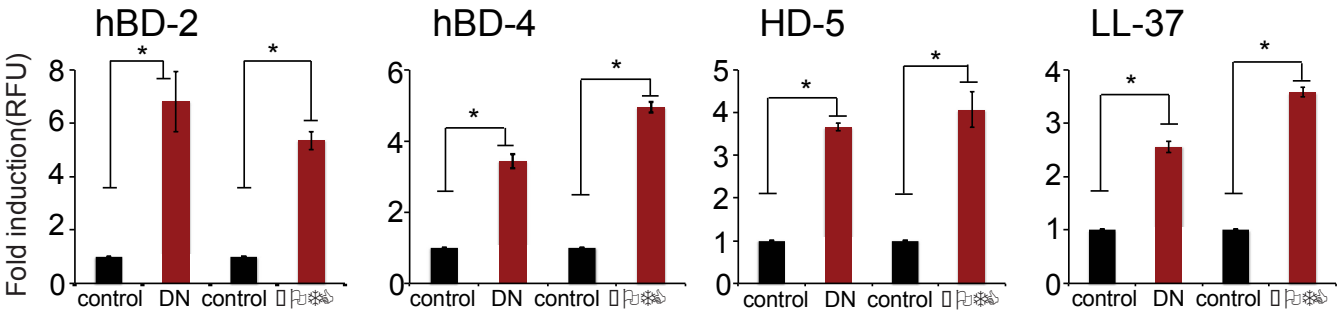

Figure 2.4 Mitochondrial stress results in upregulation of innate immune genes in human cells

Expression levels of *hBD-2*, *hBD-4*, *HD-5*, and *LL-37* mRNA in HEK 293T cells expressing dominant negative AFG3L2 (DN) or HeLa cells expressing transgenic ΔOTC (ΔOTC) or the respective control parent plasmids. DN cells were induced with tetracycline for 48h or ΔOTC cells were transfected and collected 72h later (N=3, ± SD), * $p<0.05$ (Student's *t* test).

Studies in our lab have shown that ATFS-1 is a dually localized bZIP transcription factor, with an MTS targeting it to the mitochondria in the absence of stress and an NLS that allows the protein to translocate to the nucleus if it accumulates in the cytosol due to mitochondrial import slowdown during mitochondrial stress (Figure 2.5). Once the transcription factor is localized to the nucleus, it activates a broad transcriptional response that upregulates genes from a wide range of cellular processes to repair and restore mitochondrial function, including genes involved in the immune response. ATFS-1 does this by recognition of an element known as the UPR^{mt} element, which is a sequence located in the promoter region of genes that are either activated or repressed by ATFS-1. ATFS-1 is also still able to be imported into mitochondria during stress and bind to an UPR^{mt} element in mtDNA to affect mtDNA expression.

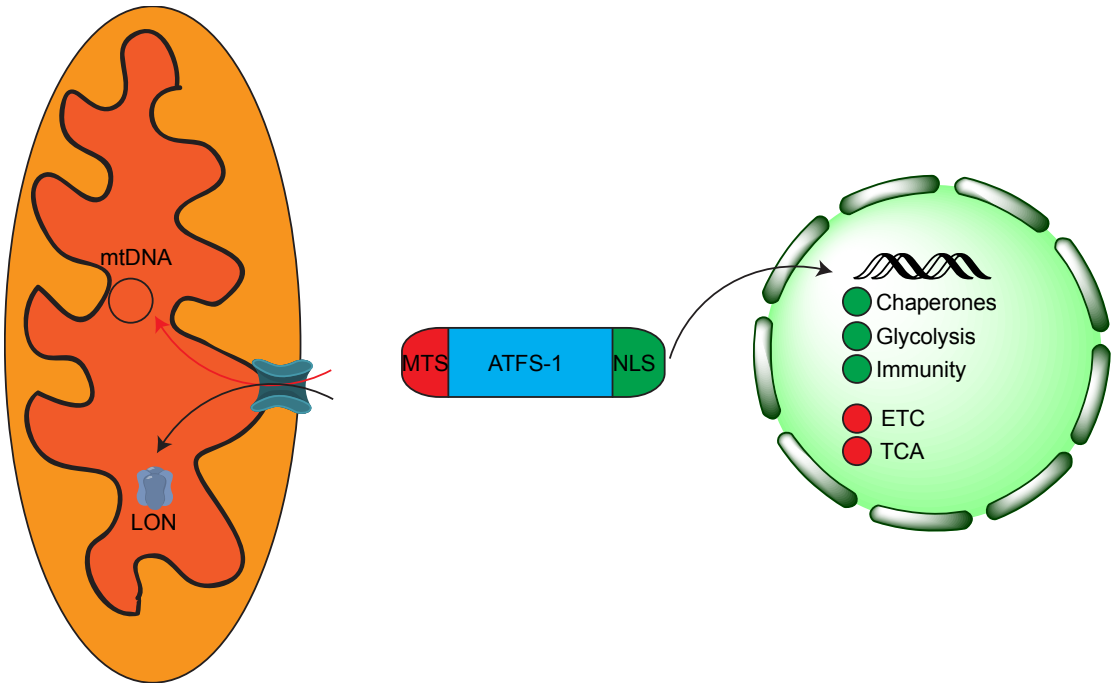

Figure 2.5 Model of UPR(mt) Regulation in *C. elegans*

ATFS-1 is a bZIP transcription factor. In the absence of stress, ATFS-1 is imported into mitochondria via its MTS and degraded by the protease LON. During stress, ATFS-1 translocates to the nucleus due to a slowdown in import and activates or represses genes to rehabilitate mitochondria. ATFS-1 also has a role in mitochondria repressing transcription.

Chapter 3. ATF5 as a Regulator of Mammalian Mitochondrial UPR

3.1 Introduction

Considerable evidence suggests the UPR^{mt} is conserved in mammals where it was originally discovered. Expression of the mitochondrial protein ornithine transcarbamylase (OTC) lacking 84 amino acids rendering it unable to fold (ΔOTC), or exposure to ethidium bromide (EtBr) which depletes mitochondrial DNA (mtDNA), induces transcription of mitochondrial chaperone and protease genes in cultured cells (Martinus, Garth et al. 1996, Zhao, Wang et al. 2002). Furthermore, perturbation of mitochondrial ribosomes activates a similar transcription response in cultured cells and mice (Wu, Williams et al. 2014, Moullan, Mouchiroud et al. 2015), strongly suggesting the existence of a homologous regulatory mechanism to that described in *C.elegans*. Perhaps most intriguing, an element in the promoters of those genes induced in a mouse model of mitochondrial myopathy is nearly identical to the UPR^{mt}E to which ATFS-1 binds to induce chaperone and protease transcription in *C. elegans* (Tyynismaa, Carroll et al. 2010, Nargund, Fiorese et al. 2015).

To identify potential regulators of a mammalian UPR^{mt}, we searched for bZip proteins homologous to ATFS-1 with potential MTS. ATF4 and ATF5 had considerable homology within the bZip domain to ATFS-1, however ATF5 also had a putative, but relatively weak, MTS as determined by Mitoprot (Claros and Vincens 1996) (Figures 3.1 and 3.2A). Interestingly, several studies suggest a

role for ATF5 during mitochondrial dysfunction. A recent transcription profiling study from patients with autosomal dominant ataxia caused by a mutation in a gene encoding a mitochondrial protease had increased *ATF5* transcripts (Mancini, Roncaglia et al. 2013), consistent with *atfs-1* transcripts being induced in *C. elegans* when the orthologous mitochondrial AAA protease (*spg-7*) is impaired (Nargund, Pellegrino et al. 2012). Furthermore, mouse models of respiratory chain dysfunction caused by impaired mtDNA replication or a defective mitochondrial aspartyl-tRNA synthetase also caused induction of *ATF5* transcripts (Tyynismaa, Carroll et al. 2010, Dogan, Pujol et al. 2014).

3.2 ATF5 Complements UPR^{mt} mutations in worms

To determine if either ATF4 or ATF5 can regulate a UPR^{mt}, the mammalian transcription factors were expressed in worms lacking ATFS-1. Identification of components that regulate the UPR^{mt} has been facilitated by the use of mitochondrial chaperone transcriptional reporter *C. elegans* strains (Yoneda, Benedetti et al. 2004) (Figure 3.2B). Induction of *hsp-60$_{pr}$::gfp* during mitochondrial stress requires both *atfs-1* and the UPR^{mt}E (Nargund, Fiorese et al. 2015). Interestingly, *atfs-1*-deletion worms expressing transgenic ATF5, but not worms expressing transgenic ATF4, were able to induce the *hsp-60$_{pr}$::gfp* reporter during mitochondrial stress caused by depletion of a mitochondrial protein import component, *timm-23,* or protease, *spg-7* (Figure 3.2C). In

Figure 3.1 – Mitochondrial targeting sequence heat map of mammalian bZIP proteins and BLAST homology to ATFS-1. ATFS-1 protein sequence was used in a protein to protein BLAST search of mammalian proteins. Alignment scores were taken from the BLAST search, any protein marked NS had no significant alignment with ATFS-1. Each bZIP N-terminal sequence was then entered into Mitoprot, the heat map represents the percentage chance the protein is imported into mitochondria.

Family	Protein	BLAST score	Mitoprot Score
CEBPG	CEBPG	16.2	17
CEBP	CEBPA	27.7	12
	CEBPB	28.9	6
	CEBPD	27.7	2
	CEBPE	15.8	1
DDIT3	DDIT3	17.7	10
CREB	ATF1	18.9	11
	CREB-1	15.0	2
	CREM	25.4	4
OASISA	CREB3	30.4	0
	CREB3L3	38.1	0
	CREB3L4	17.3	0.4
OASISB	CREB3L1	22.7	0.7
ATF6	ATF6	25.0	0.7
	ATF6B	28.9	6
CREBZF	CREBZF	21.9	35
XBP1	XBP1	31.2	0.3
NFIL3	NFIL3	19.2	0.3
ATF2	ATF2	27.7	19
	ATF7	31.6	4.5
	CREB5	31.2	6
JUN	JUN	33.1	18
	JUNB	16.5	15
	JUND	31.6	4
FOS	FOS	20.8	3
	FOSB	15.4	11
	FOSL1	18.5	9
	FOSL2	18.5	7
ATF3	ATF-3	21.9	1
	JDP2	20.8	3
ATF4	ATF-4	42.0	12
	ATF-5	40.0	30
B-ATF	B-ATF	25.4	8.6
	B-ATF2	21.2	0
	B-ATF3	28.1	18
PAR	DBP	18.9	56
	HLF	21.9	10
	TEF	30.0	1
SMAF	MAFF	23.9	6
	MAFG	13.1	3
LMAF	MAFB	23.1	5
	MAF	23.9	3
	MAFA	NS	1
	NRL	NS	2
NFE2	NFE2-p45	25.0	20
	NFE2L1	25.0	10
	NFE2L2	26.9	0
	NFE2L3	26.9	59
BACH	BACH1	27.3	4
	BACH	26.6	3

Mitoprot Score
≥25
≥10
≥5
≥0.1

Figure 3.1 Mitochondrial targeting sequence heat map of mammalian bZIP proteins and BLAST homology to ATFS-1

addition, UPR^{mt} activation by ATF5 required the UPR^{mt}E in the *hsp-60* promoter (Figures 3.2D). ATF5 activation was mitochondrial stress-specific as perturbing endoplasmic reticulum (ER) protein folding with *ero-1*(RNAi) failed to activate *hsp-60$_{pr}$::gfp* in transgenic ATFS-1 or ATF5 worms (Figure 3.3B), but did activate the ER chaperone reporter *hsp-4$_{pr}$::gfp* (Figure 3.3C). Furthermore, transgenic ATF5 was unable to rescue induction of the ER UPR in worms lacking the ER stress-specific transcription factor XBP-1, further suggesting that ATF5 functions specifically during mitochondrial stress (Figure 3.3A).

3.3 Response to Mitochondrial Stress Requires ATF5 in Mammalian Cells

Analysis of all mammalian gene promoters (Bucher and Trifonov 1986) revealed putative UPR^{mt}Es (Figure 3.2B) in the promoters of *HSP60* (*HSPD1*), *HSP10* (*HSPE1*), *mtHSP70* (*HSPA9*), the mitochondrial protease *LONP1*, as well as *ATF5* (data not shown) suggesting they may be regulated by ATF5. Exposure of HEK 293T cells to paraquat, which perturbs the respiratory chain causing toxic reactive oxygen (ROS) generation (Castello, Drechsel et al. 2007), resulted in increased transcription of *HSP60*, *mtHSP70*, *LONP1* and *HD-5*, a secreted anti-microbial peptide (Figure 3.4B), consistent with activation of a UPR^{mt}. Impressively, transcription of all four genes was significantly reduced when ATF5 was impaired by two different shRNAs (Figures 3.4B and 3.4C). Similarly, expression of ΔOTC, but not OTC (Figures 3.5B-D) caused modest induction of *mtHSP70, HSP10, and LONP1*, but not *HSP60* (Figure 3.4B) consistent with ΔOTC being less toxic than paraquat.

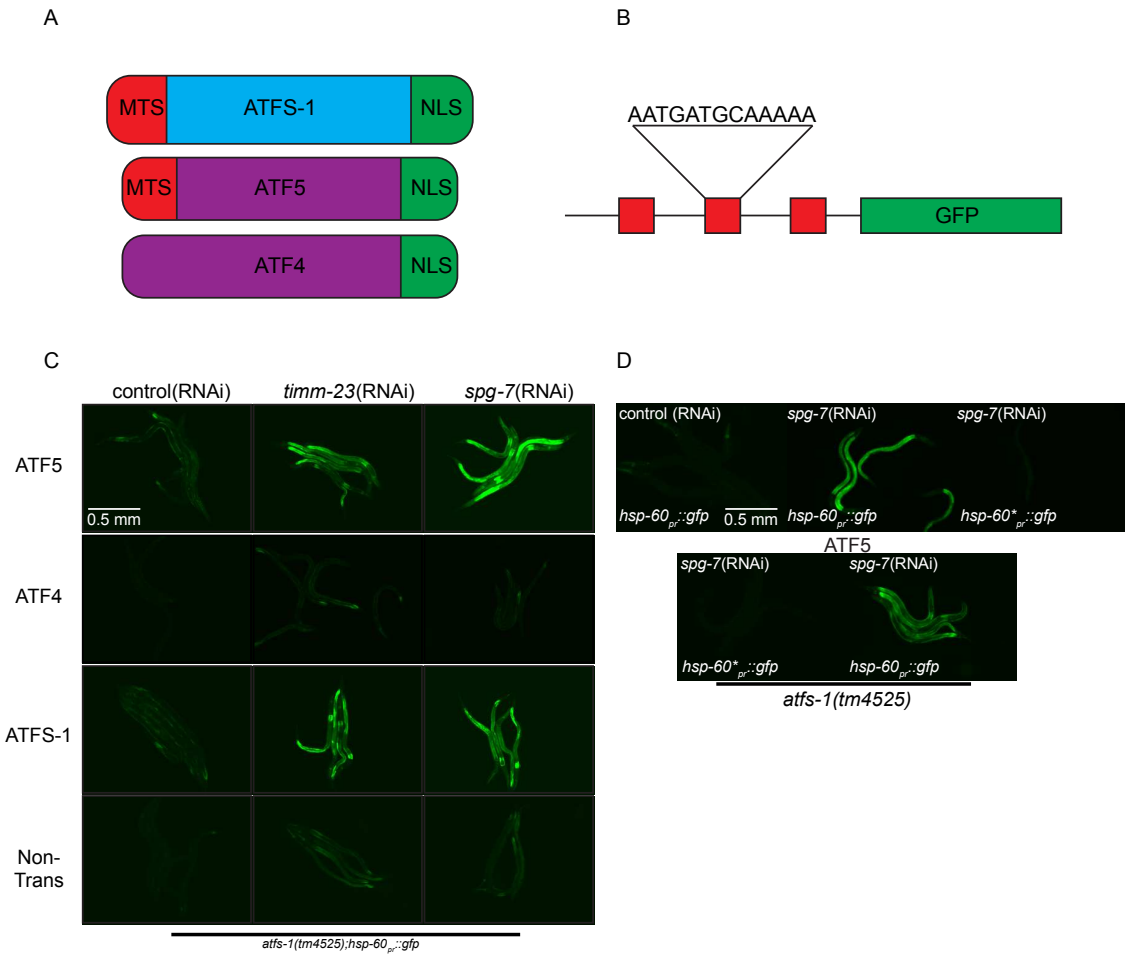

Figure 3.2 ATF5 is able to rescue UPR(mt)

(A) Schematic comparing the bZip transcription factors ATFS-1 and ATF5 including the mitochondrial targeting sequence (MTS), nuclear export sequence (NES) and the nuclear localization sequence (NLS).

(B) Schematic of the *hsp-60$_{pr}$::gfp* reporter highlighting the three UPR^{mt} elements in the promoter. The mutated element used in Figure 1D is marked with an asterisk (*).

(C) Photomicrographs of *atfs-1(tm4525);hsp60$_{pr}$::gfp* worms expressing transgenic ATF5, ATF4 or ATFS-1 and raised on control, *timm-23*, or *spg-7.* Scale bar, 0.5 mm.

(D) Photomicrographs of wildtype and *atfs-1(tm4525)* worms expressing either *hsp-60$_{pr}$::gfp* or *hsp-60$_{pr}$::gfp* lacking a UPR^{mt}E (*) (Figure 3.2B) raised on control or *spg-7*(RNAi). Worms in the right two panels express transgenic ATF5. Scale bar, 0.5 mm.

However, all four transcripts were reduced in ΔOTC expressing cells when treated with either *ATF5* shRNA (Figures 3.5A-C), similar to *C. elegans* (Figure 3.5A). Lastly, *ATF5* transcripts were also increased during mitochondrial stress (Figure 3.4B and 3.5B) consistent with what has been observed for *atfs-1* in worms (Nargund, Fiorese et al. 2015), for *Atf5* in mouse models of mitochondrial disease (Tyynismaa, Carroll et al. 2010, Dogan, Pujol et al. 2014) and *ATF5* in patient samples (Mancini, Roncaglia et al. 2013). Of note, mRNAs encoding ER resident chaperones were not induced during mitochondrial stress in an ATF5-dependent manner (Figures 3.3D-E) suggesting ATF5 specifically promotes mitochondrial protein homeostasis.

We next sought to determine the impact of OXPHOS activity and mitochondrial membrane potential (Δψ) on ATF5-dependent UPR^{mt} activation. Interestingly, simultaneous inhibition of respiratory chain complexes I, III and V, which dissipates Δψ (Pfanner and Neupert 1986) induced *LONP1* expression in an ATF5-dependent manner (Figure 3.4E) indicating that the UPR^{mt} can be activated upon loss of membrane potential. However, because separate treatment with either piericidin (complex I), antimycin (complex III), or oligomycin (complex V), which does not deplete Δψ, also increased *LONP1* expression (Figure 3.4E), depletion of Δψ is not required for UPR^{mt} activation.

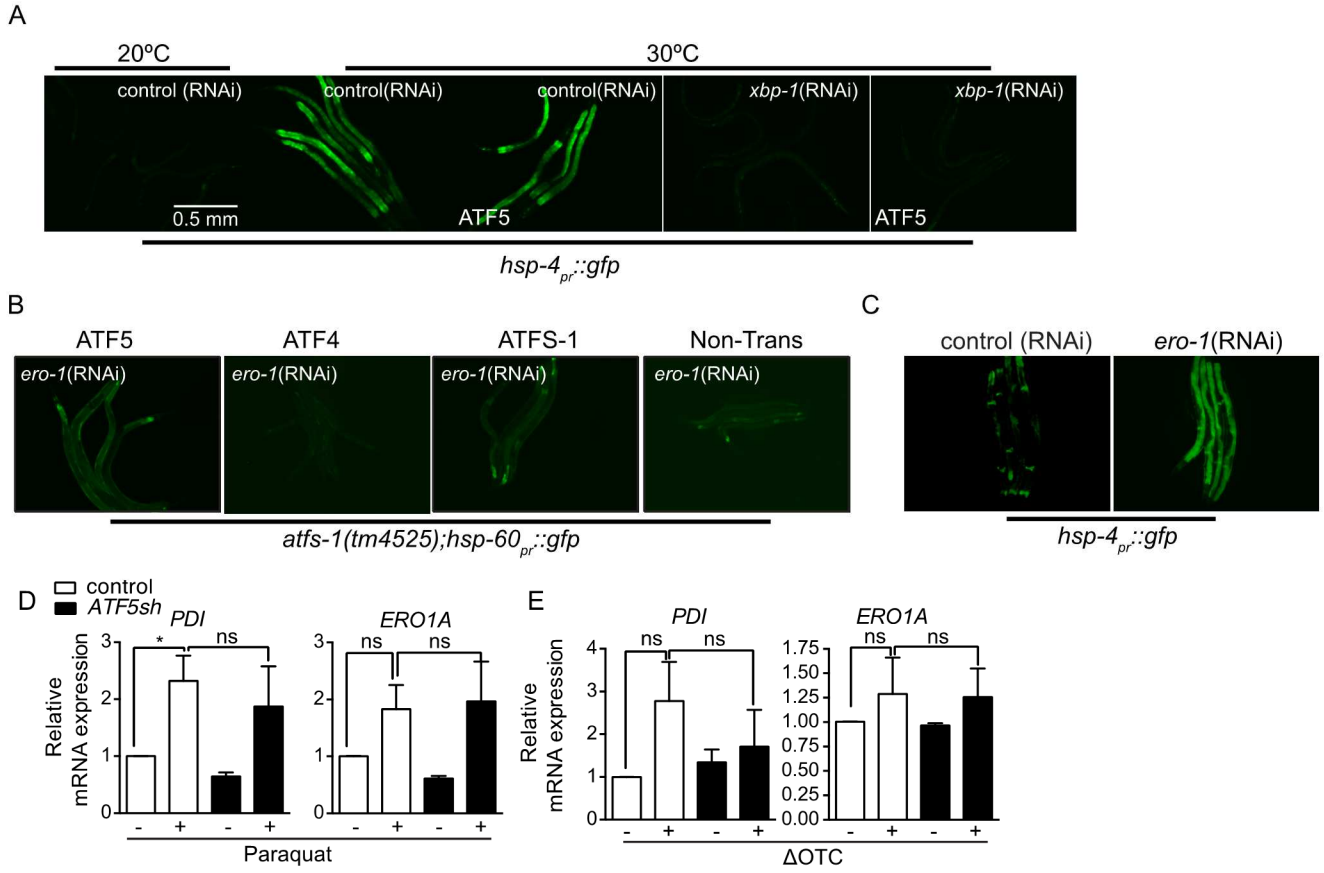

Figure 3.3 Activation of UPR(mt) by ATF5 is specific to mitochondrial stress

(A) Photomicrographs of control or transgenic ATF5 expressing *hsp-4*$_{pr}$*::gfp* worms, raised on control or *xbp-1*(RNAi) incubated at 20°C or 30°C. Scale bar, 0.5 mm.

(B) Photomicrographs of *atfs-1(tm4525);hsp60*$_{pr}$*::gfp* worms expressing transgenic ATF5, ATF4 or ATFS-1 and raised on *ero-1*(RNAi). Scale bar, 0.5 mm.

(C) Photomicrographs of *hsp-4*$_{pr}$*::gfp* treatd with control or *ero-1*(RNAi). Scale bar, 0.5 mm.

(D-E) Expression levels of *PDI*, and *ERO1A* mRNA in control or *ATF5* shRNA HEK293T cells treated with either (D) PQ, or (E) expressing ΔOTC (n=3 combined experiments, mean ± SEM, *p<0.05).

3.4 ATF5 is Dually Localized to Mitochondria and Nucleus

Because ATF5 regulates mitochondrial chaperone and protease transcription during mitochondrial stress similarly to ATFS-1, we hypothesized ATF5 may also be regulated via organelle partitioning. ATF5-dependent transcription has been shown to coincide with its nuclear accumulation (Monaco, Angelastro et al. 2007, Dalton, Lyons et al. 2013), consistent with the presence of a nuclear localization sequence (Figure 3.2A). Therefore, we sought to determine if ATF5 localizes to mitochondria in the absence of mitochondrial stress. Subcellular fractionation in *C. elegans* indicated that in the absence of stress, ATF5 co-fractionated with a mitochondrial protein (Figure 3.6A), consistent with ATF5 being inactive in the absence of stress (Figure 3.2C). Endogenous ATF5 is difficult to detect in cultured mammalian cells as translation of the *ATF5* transcript is impaired by the presence of upstream open reading frames (uORFs) (Zhou, Palam et al. 2008), and its relatively short half-life (Uekusa, Namimatsu et al. 2009). To increase ATF5 expression, HeLa cells were cultured in the presence of a proteasome inhibitor that leads to phosphorylation of the translation initiation factor eIF2α and increases synthesis of proteins encoded by uORF containing mRNAs (Teske, Fusakio et al. 2013). Bortezomib treatment resulted in increased expression of ATF5, which was impaired by

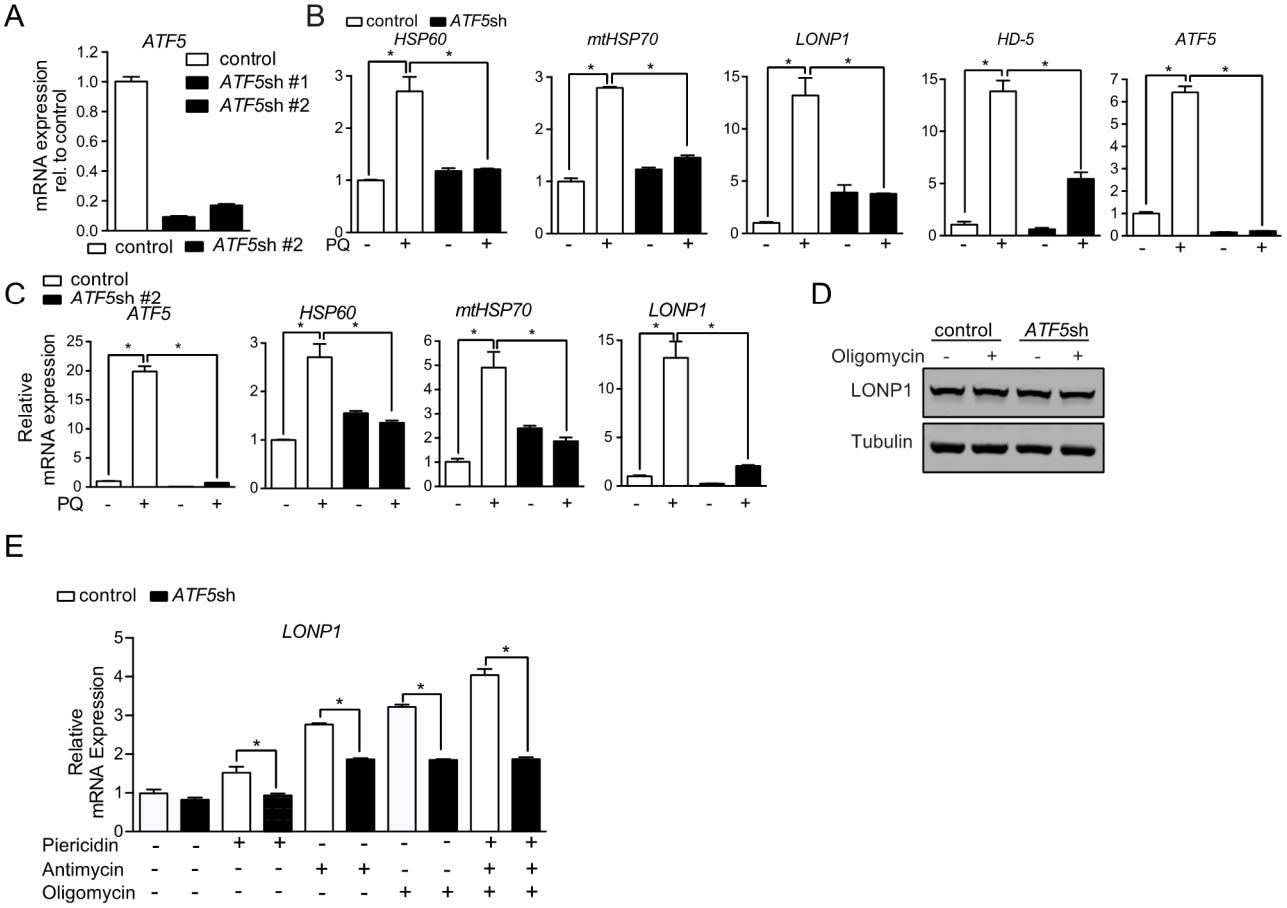

Figure 3.4 UPR(mt) is activated in ATF5-dependent manner in HEK 293T cells

(A) Expression levels of *ATF5* mRNA in HEK 293T cells treated with vector shRNA, *ATF5* shRNA #1, or *ATF5* shRNA #2 (see Experimental Procedures) (n=3, mean ± SEM, *p<0.05).

(B) Expression levels of *HSP60*, *mtHSP70*, *LONP1*, *HD-5*, and *ATF5* mRNA in control or *ATF5* shRNA HEK 293T cells with or without paraquat (PQ) (n=3, mean ± SEM, *p<0.05).

(C) Expression levels of *ATF5*, *HSP60*, *mtHSP70* and *LONP1* mRNA in control or *ATF5* shRNA #2 HEK 293T cells with or without paraquat (PQ) (n=3, mean ± SEM, p<0.05,).

(D) Immunoblots from control or *ATF5* shRNA HEK293T cells with or without oligomycin treatment.

(E) Expression levels of *LONP1* mRNA in control or *ATF5* shRNA HEK 293T cells treated with oligomycin, antimycin, piericidin, or all three inhibitors (n=3, mean ± SEM, *p<0.05).

ATF5 shRNA (Figure 3.6B). Interestingly, ATF5 was enriched in the mitochondrial fraction further supporting localization of ATF5 inmitochondria in the absence of mitochondrial stress. Of note, we were unable to detect cleavage of the MTS upon import into mitochondria (Figure 3.6B), suggesting the MTS remains intact not unlike several other mitochondrial proteins (Amaya, Arakawa et al. 1988).

Because we were only able to detect endogenous ATF5 in cultured cells upon proteasome inhibition (Figure 3.6B), we expressed ATF5::GFP via the CMV promoter in HeLa cells. Given the strong over-expression, considerable ATF5::GFP localized to the nucleus as expected, but ATF5::GFP also co-localized with mitochondria (Figures 3.7A and B), unlike GFP fusion proteins directed to the cytosol or nucleus (Figures 3.7A and B). Unlike in cultured cells, ATF5 is highly expressed in mouse and human liver cells Interestingly, sucrose fractionation of mouse liver homogenates demonstrated co-fractionation of ATF5 with a mitochondrial, but not an ER-resident protein (Figure 3.6C). Given the difficulties in detecting ATF5 perhaps due to its low expression and short half-life (Zhou, Palam et al. 2008, Li, Xu et al. 2011), it has been challenging to observe stress dependent shifts in ATF5 localization, but because ATF5-dependent transcription is induced during mitochondrial stress and ATF5 localizes to mitochondria in the absence of stress, our data are consistent with ATF5 being regulated post-translationally similarly to (Pascual, Gomez-Lechon et al. 2008), ATFS-1.

3.5 ATF5 is required during and in recovery from Mitochondrial Stress

Lastly, we sought to determine the role of ATF5 in protecting or maintaining mitochondrial function. Interestingly, in HEK 293T cells, knockdown of ATF5 reduced basal respiration, overall respiratory capacity and maximal respiration (Figures 3.8A-D) suggesting a basal role for ATF5 in mitochondrial maintenance in these cells. *ATF5* knockdown also impaired cell proliferation specifically in cells expressing ΔOTC (Figures 3.9A-B), further suggesting ATF5 promotes mitochondrial function during stress. We next examined the role of ATF5 during the recovery from mitochondrial stress caused by depletion of mitochondrial genomes (mtDNAs) via EtBr exposure. mtDNA was depleted to ~10% of normal levels in both control and *ATF5* shRNA cells (Figures 3.9C).

Upon EtBr removal, both cell types recovered mtDNA levels at similar rates (Figure 3.9D), suggesting ATF5 does not affect mtDNA replication. However, the *ATF5* shRNA cells proliferated much more slowly (Figures 3.9D and 3.9E) suggesting ATF5 and the regulation of mitochondrial protein homeostasis machinery promotes the recovery from mitochondrial stress. Furthermore, *ATF5* shRNA reduced the steady state expression of *HSP60*, *mtHSP70*, *LONP1* and impaired cell growth (Figures 3.10A-B) in an oncocytic cell line harboring multiple mtDNA lesions that impair respiratory chain activity further supporting

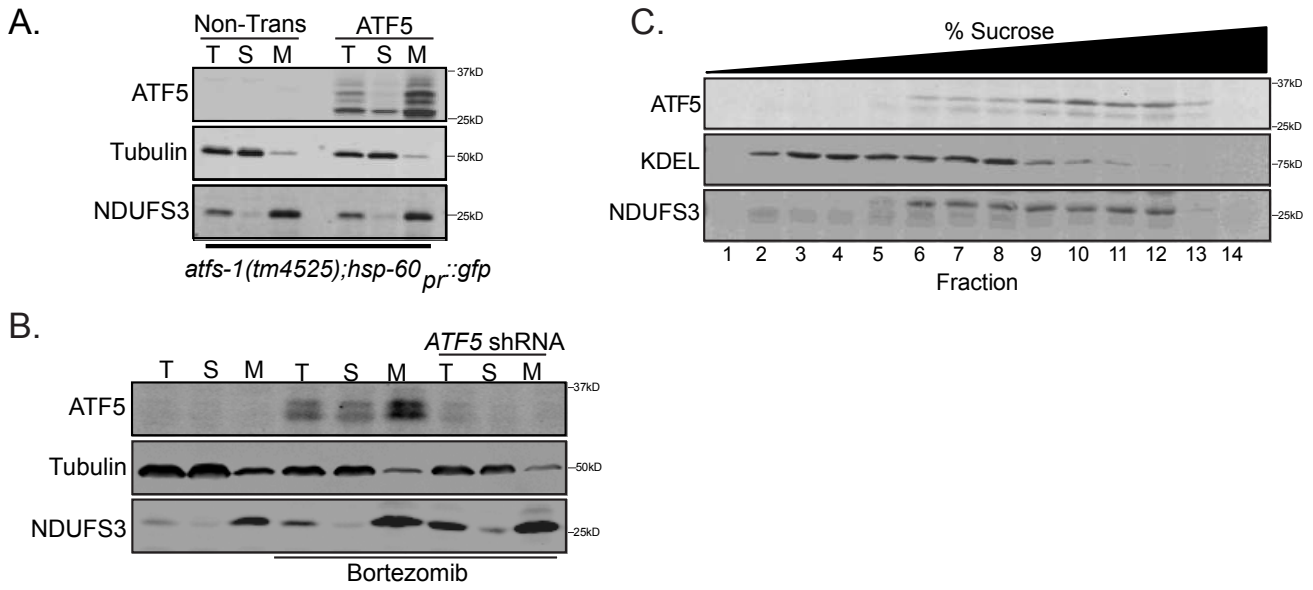

Figure 3.5 ATF5 localizes to mitochondria in worms, humans, and mice

(A) Immunoblots of lysates from control or ATF5 expressing *atfs-1(tm4525);hsp60$_{pr}$::gfp* worms following fractionation into total lysate (T), postmitochondrial supernatant (S), and mitochondrial pellet (M). NDUFS3 serves as a mitochondrial marker and tubulin as a cytosolic marker.

(B) Immunoblots of control or *ATF5* shRNA HeLa cells treated with DMSO or Bortezomib and fractionated into total lysate (T), postmitochondrial supernatant (S), and mitochondrial pellet (M).

(C) Immunoblot of mouse liver fractions following centrifugation on a sucrose gradient. Endogenous KDEL serves as an ER marker and NDUFS3 as a mitochondria marker.

the role for ATF5-dependent transcription during mitochondrial dysfunction (Bonora, Porcelli et al. 2006).

Combined, our data suggest that ATF5 regulates a UPR^{mt} in mammalian cells that is similar to the response regulated by ATFS-1 in *C. elegans*. Our data support a model that when expressed, ATF5 localizes to mitochondria in the absence of stress. However, during mitochondrial stress, there is induction of mitochondrial protective transcripts, or a UPR^{mt}, in an ATF5-dependent manner. This role is consistent with a previously established function of ATF5 as being anti-apoptotic by inducing *BCL-2* transcription (Persengiev, Devireddy et al. 2002) and promoting survival of a number of cancer cells including glioblastomas (Angelastro, Canoll et al. 2006, Sheng, Li et al. 2010), which are known to have mitochondrial dysfunction (Griguer and Oliva 2011). Of note, our work also suggests an interaction between the UPR^{mt} and the integrated stress response (ISR) because increased eIF2α-phosphorylation results in preferential ATF5 synthesis (Zhou, Palam et al. 2008). This is consistent with several studies demonstrating increased eIF2α phosphorylation during mitochondrial dysfunction mediated by the kinases GCN2 and PERK (Hori, Ichinoda et al. 2002, Baker, Nargund et al. 2012) and suggests an additional layer of regulation in addition to organelle partitioning. Our findings indicate a protective role for ATF5 during mitochondrial dysfunction by regulating a UPR^{mt}

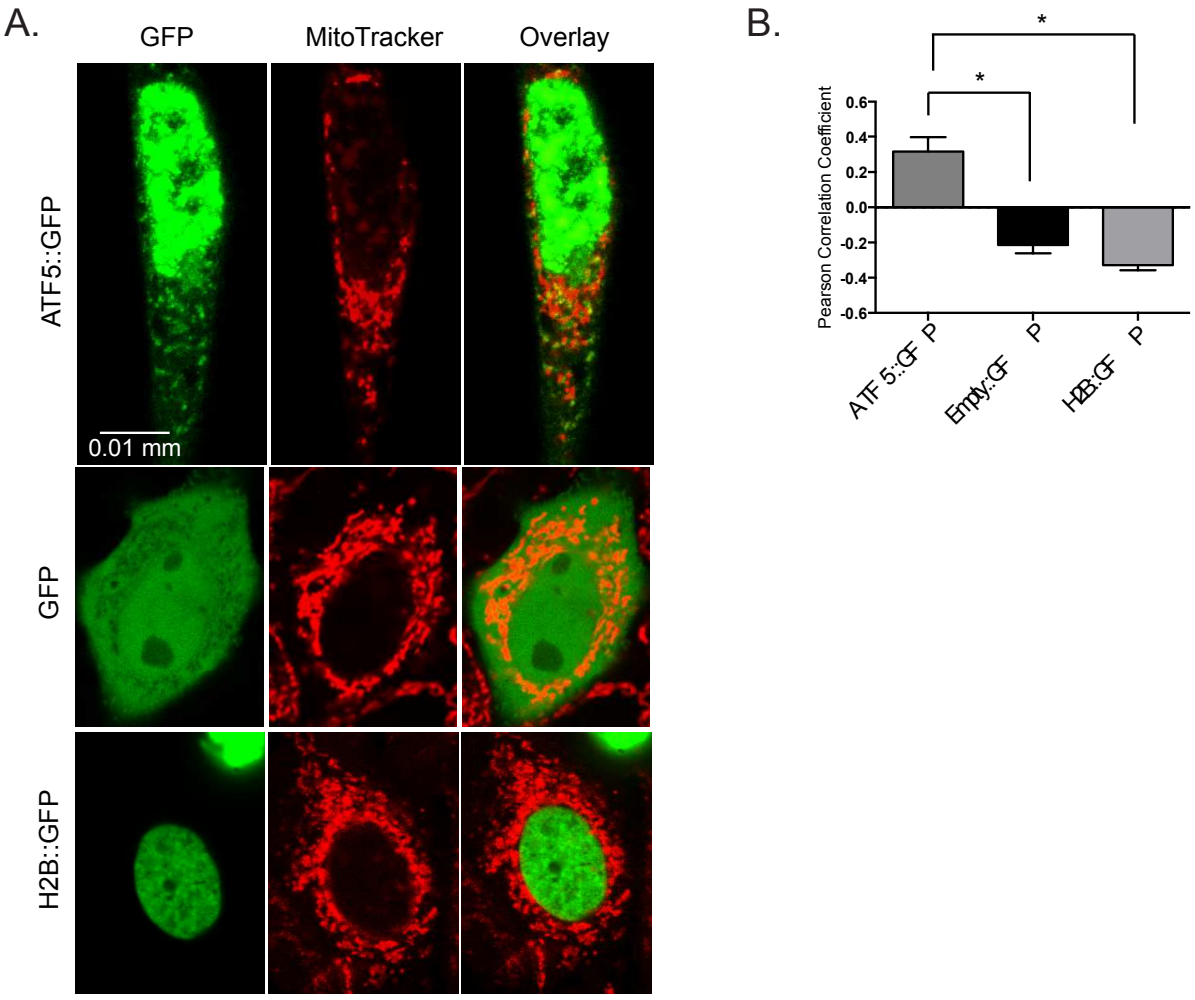

Figure 3.6 ATF5 is localized to mitochondria and nucleus

(A) Photomicrographs of HeLa cells expressing either ATF5::GFP, Histone 2B::GFP (H2B::GFP), or GFP and stained with Mitotracker. Scale bar, 0.01 mm.

(B) Pearson Correlation Coefficient (Dunn, Kamocka et al. 2011) of co-localization of ATF5::GFP, Histone 2B::GFP (H2B::GFP), or GFP and MitoTracker (Figure 3C) (n=5, mean ± SEM, *p<0.05).

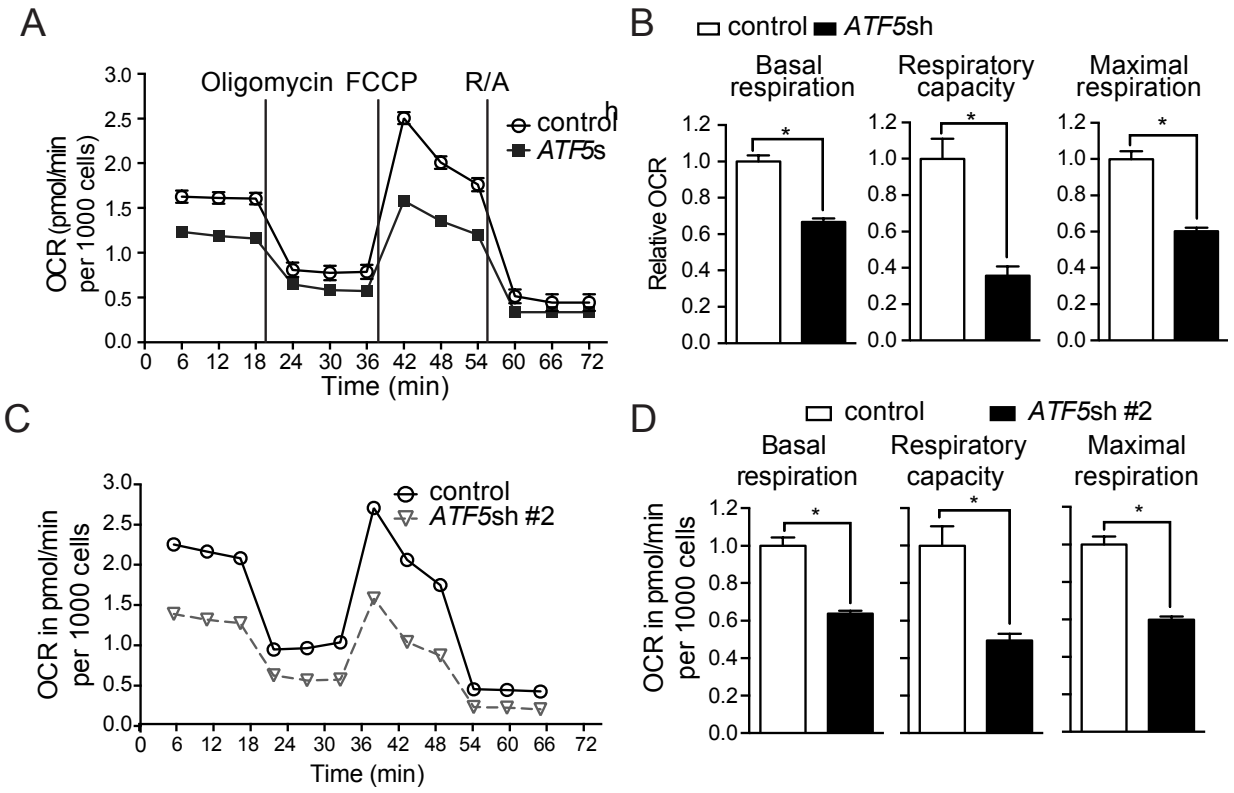

Figure 3.7 ATF5 promotes mitochondrial function

(A-B) Oxygen consumption rates (OCR) in control or *ATF5* shRNA HEK 293T cells (n=15, mean ± SEM,*p<0.05).

(C-D) Oxygen consumption rates (OCR) of control or *ATF5* shRNA #2 HEK 293T cells (n=15 (control) and n=16 (*ATF5*sh), mean ± SEM, *p<0.05).

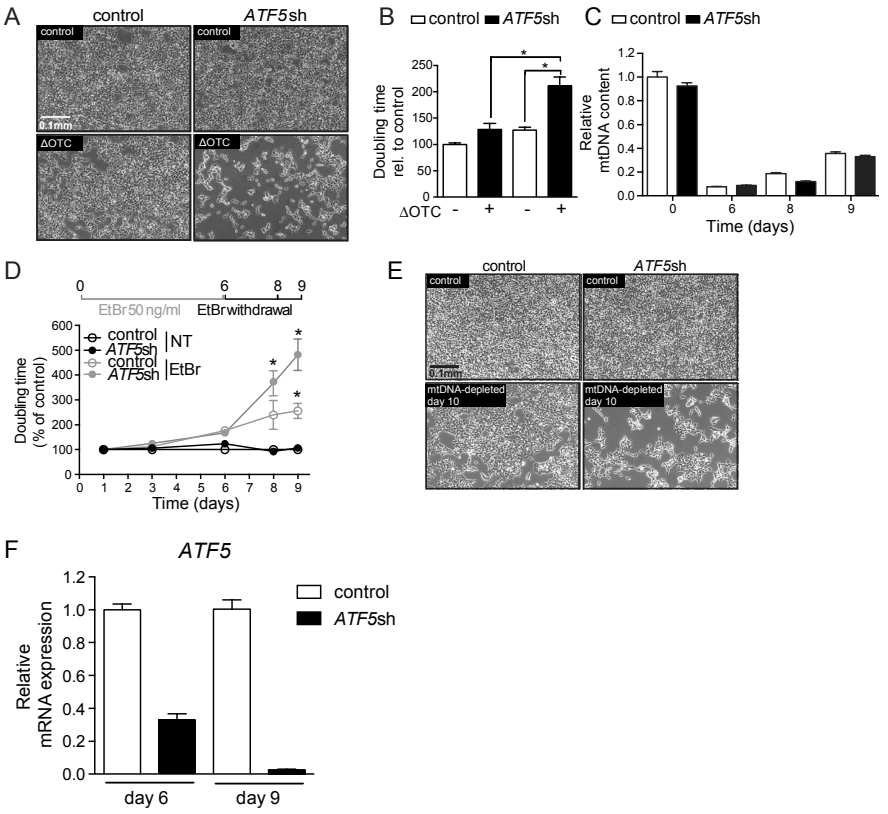

Figure 3.8 ATF5 promotes mitochondrial function during stress and the recovery from stress

A-B) Photomicrographs (A) and doubling times (B) of control or *ATF5* shRNA HEK 293T cells, with or without ΔOTC expression. Scale bar, 0.1 mm (n=3-7, mean ± SEM *p<0.05).

(C) mtDNA quantification of control and *ATF5* shRNA after 6 days of EtBr treatment, 4 days of withdrawal (n=3, mean ± SEM).

(D) Time course of doubling times of control and *ATF5* shRNA cells following 6 days of mtDNA depletion by EtBr treatment (n=3, mean ± SEM, *p<0.05).

(E) Photomicrographs of control and *ATF5* shRNA cells following 6 days of mtDNA depletion by EtBr treatment. Scale bar, 0.1 mm.

(F) Expression levels of *ATF5* mRNA in control or *ATF5* shRNA cells following EtBr

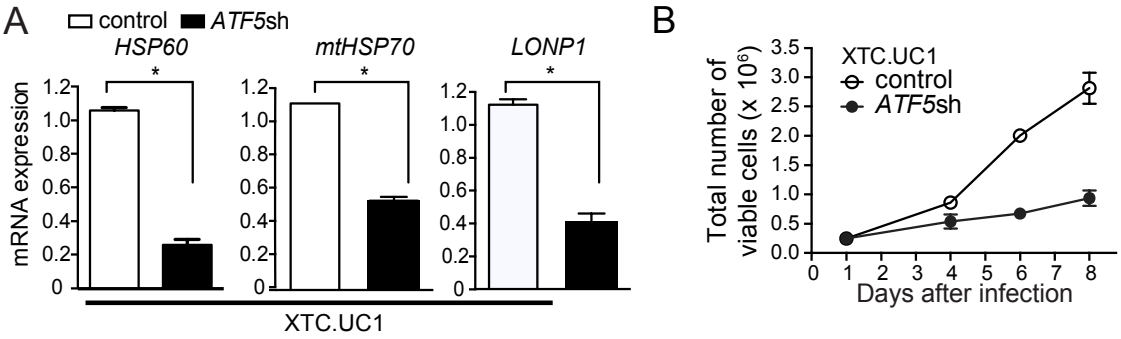

Figure 3.9 UPR(mt) is required for XTC.UC1 cell growth

(A) Expression levels of *HSP60*, *mtHSP70*, or *LONP1* mRNA in control or *ATF5* shRNA XTC.UC1 cells (n=3, mean ± SEM, *p<0.05).

(B) Growth curve of control or *ATF5* shRNA XTC.UC1 cells (n=2, mean ± SEM).

Chapter 4. Discussion

4.1 UPR^{mt} in propagation of deleterious mtDNAs

Many mitochondrial diseases are a result of mutations in mtDNA. Due to the heteroplasmy levels, one might think that these mutations may not manifest at all, but mutated mtDNA has been shown to proliferate more rapidly than would be expected given random segregation of mtDNA and often comes to be more abundant than the wild-type mtDNA in a cell (Stewart and Chinnery 2015). The mechanism of this faster-than-expected propagation of mutated mtDNA remains an unanswered question. Recently, our lab has shown that in *C. elegans* the UPR^{mt} plays a role in the expansion of mutated mtDNA in cells (Lin, Schulz et al. 2016). Worms containing mutated mtDNA activate the UPR^{mt}, and furthermore worms that lack *atfs-1* quickly lose copy numbers of the mutated mtDNA. The UPR^{mt} acts counter to the mitophagy pathway, which serves to clear mutated mtDNA and overexpression of the E3 Ubiquitin ligase Parkin has been shown to clear mutant mtDNA from cells (Suen, Narendra et al. 2010). Perturbing mitophagic turnover has also been shown to result in a rise in mutant mtDNA copy number (Lin, Schulz et al. 2016). This data shows that a possible way for mutated mtDNA to gain a proliferative advantage is to activate the UPR^{mt} in cells, increasing biogenesis and other reparative pathways that keep the damaged mitochondria, and the mutated mtDNA within, from being turned over.

A natural question is whether this mechanism of mutant mtDNA proliferation is present in the mammalian system. As shown in Chapter 3, the mammalian UPR^{mt} is regulated by the bZIP transcription factor ATF5. In order to study whether mutant mtDNA hijacks the UPR^{mt} in order to proliferate, I propose to use various models of cybrid cells that have mutated mtDNA to study the effect of the UPR^{mt} on mutant mtDNA propagation. One potential model is a fibrosarcoma cell line that has had mutant mtDNA inserted into it from a patient that has a single base-pair mutation in the COXI gene of mtDNA (Suen, Narendra et al. 2010). To study if the UPR^{mt} has any effect on the proliferation mutant mtDNA in these cells, short-hairpin RNAi of *ATF5* will be used to knockdown expression of that transcription factor and determine if ablation of UPR^{mt} signaling in these cells reduces copy number of the mutant COXI gene. Metabolic changes in the form of oxidative phosphorylation or glycolytic rate will also be monitored. Any metabolic changes caused by UPR^{mt} ablation would be studied using a Seahorse XF analyzer.

If UPR^{mt} inhibition causes a loss of mutant mtDNA copy number, several possibilities for follow-up experiments exist. Studies in the worm and elsewhere have shown that the fusion and fission machinery play a role in preventing proliferation of mutant mtDNA (Chen, Vermulst et al. 2010, Lin, Schulz et al. 2016). The interplay of the UPR^{mt} pathway and the fission and fusion machinery could be studied as it is currently unknown how those two pathways interact in mammals. Another potential area for study is the UPR^{mt} role in

counterbalancing the PINK1/Parkin mitophagy pathway, as it has been shown that mitophagy can clear mutant mtDNA in the mammalian system (Suen, Narendra et al. 2010). If UPR^{mt} inhibition causes altered metabolism in the cybrid cells, the interaction of the UPR^{mt} with various metabolic pathways could be interrogated.

To follow levels of mutated mtDNA in wild-type or *ATF5* knockdown, restriction length polymorphisms that are created by the COXI mutation will be followed. This method is verified and has been used to track the disappearance of mutant mtDNA after overexpression of Parkin in these cells (Suen, Narendra et al. 2010). One limitation of this method is that it is not quantitative, missing any changes in heteroplasmy that are not “all-or-nothing”. If the changes in mutant COXI copy number are more subtle than that, another potential method would be to use digital PCR to catch any change in mutant mtDNA levels (Belmonte, Martin et al. 2016). If no changes in copy number level are shown, it is also possible that a single base pair change mutant is simply too difficult to track. Another model of mutant mtDNA would then be used, preferably one that contains a large deletion that is easy to track. It is also a possibility that the small amount of ATF5 left after knockdown of expression is enough to maintain mutant mtDNA levels. In this case, CRISPR of the *ATF5* gene in these cells will be carried out to ensure a full knock-out.

4.2 UPR^{mt} and other Stress Response Pathways

Studies of the mammalian UPR^{mt} strongly suggest many interactions with other stress responses in the cell. Mitochondrial poisons are known to cause eIF2α phosphorylation and subsequent activation of the integrated stress response, a pathway that as its name suggests synthesizes responses to a wide array of cellular stresses (Pakos-Zebrucka, Koryga et al. 2016). We have also shown indirect activation of eIF2α through treatment with proteasome inhibitors in order to cause ATF5 accumulation and upregulation. Of particular interest would be to study the role of the endoplasmic reticulum and its unfolded protein response has in cross-communicating with the UPR^{mt} as we have shown that mitochondrial stress can also activate genes involved in the UPR^{ER} and others have shown that endoplasmic reticulum stress can activate mitochondrial genes associated with the UPR^{mt} such as LONP1 (Hori, Ichinoda et al. 2002). CHOP, a transcription factor proposed to play a role in UPR^{mt} signaling, also is activated by the UPR^{ER} (Wang, Lawson et al. 1996, Aldridge, Horibe et al. 2007).

PKR has already been shown to be a potential UPR^{mt} signaling protein (Rath, Berger et al. 2012), and it would be interesting to test whether any other eIF2α kinases also play a role in modulating UPR^{mt} signaling. Previous studies by us have already shown in worms that GCN-2 signaling works in parallel to the UPR^{mt} to slow-down translation of proteins in response to UPR^{mt} activation (Baker, Nargund et al. 2012). GCN-2 has been shown by others to control ATF5

expression via eIF2α phosphorylation, a potential difference between the worm and mammalian UPR^{mt} signaling pathway.

One prominent method of communication between the ER and mitochondria is calcium. ER is the primary store of Ca^{2+} in the cell, and ER release of Ca^{2+} is uptaken by the mitochondria. Changes in cytosolic Ca^{2+} levels are known to cause stress responses that affect the mitochondria, but it is unknown what, if any, role the UPR^{mt} plays in calcium signaling in the cell. Ca^{2+} levels could be a potential point of cross-talk between the ER and mitochondria, and enable coordination of the UPR^{mt} and UPR^{ER}.

In conclusion, the UPR^{mt} is a fascinating signaling pathway that has only begun to be elucidated. Mitochondria's central role in the evolution of eukaryotes speaks to the prominence of this organelle, evidenced by the great number of crucial functions carried out by the organelle besides its well-known role in energy production. The pleiotropic consequences of mitochondrial dysfunction also highlights how important the organelle is, and makes a thorough understanding of mitochondria biology and its many roles necessary for a greater understanding of all disease. The more that is elucidated about the UPR^{mt} and its interactions with other processes in the cell, the more we will come to understand that the mitochondria is the thin-blue line between the order of a well-functioning cell, and the chaos of cellular dysfunction.

Chapter 5. Methodology

Worm strains and plasmids

The reporter strains *hsp-60$_{pr}$::gfp(zcIs9)V*, *hsp-4$_{pr}$::gfp(zcIs4)V*, *atfs-1(tm4525)V* and *hsp-60$_{pr}$*::gfp* and RNAi feeding conditions have been described previously (Calfon, Zeng et al. 2002, Nargund, Pellegrino et al. 2012, Nargund, Fiorese et al. 2015). Induction of *hsp-4$_{pr}$::gfp* by heat shock was performed as in (Yoneda, Benedetti et al. 2004). Briefly, *hsp-4$_{pr}$::gfp* was incubated at 30°C for 1 hour to induce heat shock, then imaged.

To generate the ATFS-1$^{1\text{-}100}$::GFP mammalian expression plasmid, a PCR product corresponding to the first 100 amino acids of *C. elegans* ATFS-1 was amplified from cDNA and cloned into the pEGFP-N1 plasmid. The *C. elegans hsp-16$_{pr}$::atfs-1$^{\Delta 1\text{-}32.myc}$* expression plasmid was generated by PCR amplifying from N2 cDNA a fragment corresponding to amino acids 33-472 of ATFS-1 and cloning in frame and downstream of the Myc epitope sequences in the *hsp-16$_{pr}$:: ^{TAG} ubl-5* plasmid (10), replacing the ubl-5 open reading frame. The *hsp-16$_{pr}$::atfs-1$^{\Delta 1\text{-}32.myc}$::gfp* expression plasmid was generated by ligating an *ApaI-SalI* fragment containing the 3` end of atfs-1 and GFP from the *atfs-1$_{pr}$::atfs-1::gfp* expression plasmid (5) into similarly digested *hsp-16$_{pr}$::atfs-1$^{\Delta 1\text{-}32.myc}$*. The *hsp-16$_{pr}$::atfs-1^{FL}* expression plasmid was generated by PCR amplification of the *atfs-1* open reading frame from cDNA and ligating it into *NheI-EcoRV* digested *hsp-16$_{pr}$::atfs-1$^{\Delta 1\text{-}32.myc}$* which removes the Myc sequences placing the

atfs-1^{FL} sequence downstream of the hsp-16 promoter. To generate the *hsp-16*$_{pr}$*::ATF5* expression plasmid, *ATF5* cDNA was amplified and ligated into the *hsp-16*$_{pr}$*::atfs-1* plasmid replacing *atfs-1* (Nargund, Pellegrino et al. 2012). Generation of *hsp-16*$_{pr}$*::ATF4* was achieved by amplifying *ATF4* cDNA and ligating into *hsp-16*$_{pr}$*::ATF5* plasmid, replacing *ATF5*. The transgenic *C. elegans* lines were generated by co-injecting either the *hsp-16*$_{pr}$*::ATF5* or *hsp-16*$_{pr}$*::ATF4* plasmid (25 ng/µl) with a *myo-3*$_{pr}$*::mCherry* (60 ng/µl) marker plasmid, and pBluescript (65 ng/µl) into *hsp-60*$_{pr}$*::gfp; atfs-1(tm4525)* generating multiple stable extra-chromosomal arrays. The *hsp-16*$_{pr}$*::ATF5* transgene was crossed into the described reporter worms. The *myo-3*$_{pr}$*::ΔOTC* plasmid was generated by amplifying ΔOTC (Zhao, Wang et al. 2002) and ligating it into the *myo-3*$_{pr}$*::mCherry::ubl-5* plasmid by replacing the *ubl-5* open reading frame. The ATF5::GFP mammalian expression plasmid was generated by amplifying *ATF5* cDNA and ligating into the EGFP-N1 plasmid (Clontech). All plasmids were confirmed by sequencing.

Cell culture and plasmids

HeLa cells were transfected with 4µg of GFP or ATFS-11-100::GFP expressing plasmid via Lipofectamine. The cells were imaged six hours after transfection. While mitochondrial localization was observed 4-10 hours following transfection, the cells began dying 12-15 hours posttransfection.

Expression of dominant-negative AFG3L2 was induced in stable HEK293 cells by the addition of 1μg/ml tetracycline (Ehses, Raschke et al. 2009) and the cells were harvested 48 hours later. The ΔOTC expression plasmid (Zhao, Wang et al. 2002) was transfected into Hela cells via Lipofectamine and the cells were harvested after 72 hours. HeLa and HEK 293T cells were cultured in DMEM supplemented with 10 % FBS, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin. *ATF5*-knockdown cells were generated through lentiviral-driven gene silencing using two different shRNAs (ATF5sh #1(Thermo Scientific #TRCN0000017638): 5'-AAGTCTTCCATCTGTTCCAGC-3'; ATF5sh #2(Sigma): 5'-TGTCTTGGATACTCTGGACTT-3') expressed by the pLKO.1 vector, while pLKO.1 was used as a control (Sigma). To induce mitochondrial stress, cells were either transfected with ΔOTC plasmid (Zhao, Wang et al. 2002), or treated with 400 μM paraquat for 48 hours unless stated otherwise. Treatment of HEK 293T cells with antimycin (Sigma), piericidin (Santa Cruz), and/or oligomycin (Sigma) was for 24 hours before collecting cells for qPCR as described below. Cellular doubling times were calculated as described (Chanvorachote, Luanpitpong et al. 2012).

Electrophoretic Mobilty Shift Assay (EMSA)

GST-ATFS-1 was expressed in *E. coli* (BL21) as described (Nargund, Pellegrino et al. 2012). GST-ATFS-1 was affinity purified using GST-spin trap columns (GE Healthcare). The EMSA was performed as described (Sano, Ohyama et al. 2001). 3 µg of purified ATFS-1 was incubated with pre-annealed IRDye700 labeled oligonucleotides (Integrated DNA technologies) composed of the mtDNA non-coding region including the UPR^{mt}E. Identical duplexed DNA with a scrambled UPR^{mt}E was used to verify the specificity of the DNA-protein complex. The mixture was then separated on a 4% native polyacrylamide gel and imaged using an Odyssey Infrared Imager (Li-Cor Biosciences). EMSA was performed multiple times. EMSA wild type oligo F: 5'-ATTTATATTATAAATATGATGAAGTACTAAAAAAAAGATG-3' R: 5'-CATCTTTTTTTTAGTACTTCATCATATTTATAATATAAAT-3'. Scrambled UPR^{mt}E oligo F: 5'-ATTTATATTATAAATGAAGTAATGTACTAAAAAAAAGATG-3' R: 5'-CATCTTTTTTTTAGTACATTACTTCATTTATAATATAAAT-3'.

RNA Isolation, qRT-PCR and Western Blots

Total RNA was isolated using RNA Stat-60 (amsbio) or RNEasy Plus Mini-Kit (Qiagen). cDNA synthesis and qPCR was performed as described (Pellegrino, Nargund et al. 2014)using the following primers: hBD-2: forward GCCTCTTCCAGGTGTTTTTG and reverse GAGACCACAGGTGCCAATTT, hBD-4: forward ATGTGGTTATGGGACTGCCC and reverse AGCATGCATAGGTGTTGGGA, HD-5: forward TCCTTGCTGCCATTCTCCTG

and reverse ACTGCTTCTGGGTTGTAGCC, LL-37: forward GCTGGGTGATTTCTTCCGGA and reverse CCTGGGTACAAGATTCCGCA *ATF5* forward 5'-CTGGCTCCCTATGAGGTCCTTG-3' and reverse 5'-GAGCTGTGAAATCAACTCGCTCAG-3'; *HSP60* forward 5'-GATGCTGTGGCCGTTACAATG-3' and reverse 5'-GTCAATTGACTTTGCAACAGTCACAC-3'; *mtHSP70* forward 5'-CAAGCGACAGGCTGTCACCAAC-3' and reverse 5'-CAACCCAGGCATCACCATTGG-3'; *LONP1* forward 5'-CATTGCCTTGAACCCTCTC-3' and reverse 5'-ATGTCGCTCAGGTAGATGG-3'; *HD-5* forward 5' – ACCTTGCTATCTCCTTTGCAGG-3' and reverse 5' – CGGTTCGGCAATAGCAGGTG-3'; *HSP10* forward 5'-TGGCAGGACAAGCGTTTAG-3' and reverse 5'-GGTTACAGTTTCAGCAGCAC-3'; *PDI* forward 5'-TGAGAACATCGTCATCGCC -3 and reverse 5' – CGTTCCCCGTTGTAATCAATG – 3'; *ERO1A* forward 5' -CAAGGGACAAGTGAAGAGAAC -3' and reverse 5'-CCCCATTTCTTTTCTAACCAG -3'. And, *HPRT* forward 5'-CTTTGCTGACCTGCTGGATT-3' and reverse 5'-TCCCCTGTTGACTGGTCATT-3' was used as a reference gene.

SDS-Page, western blots and mouse liver sucrose fractionation were performed as described (Graham 2001, Nargund, Pellegrino et al. 2012) using the following antibodies: OTC (Santa Cruz), Tubulin (Santa Cruz) NDUFS3

(Abcam), GFP (Roche), ATF5 (Zhou, Palam et al. 2008), and KDEL (Stressgen) and imaged on an Odyssey infrared imager (LI-COR).

Microscopy

HeLa cells were transfected using Lipofectamine 2000 (Life Technologies) with 1 µg of GFP, Histone 2B::GFP, or ATF5::GFP expressing plasmids. The cells were treated with 10 µM MG-132 for 6 hours, stained with MitoTracker Red FM (Life) for 30 minutes prior to fixation with 4% paraformaldehyde (Fisher Chemicals) and incubated with primary GFP antibody (Roche) followed by incubation with secondary antibody AlexaFluor488 (Life). Samples were imaged on a Nikon Eclipse T*i*.

C. elegans images were acquired as previously described (Nargund, Pellegrino et al. 2012).

Respiration Analysis

Oxygen consumption was measured using a Seahorse Extracellular Flux Analyzer XF^e96 as described (Yoshida, Tsutsumi et al. 2013). Analysis plates were coated with Corning Cell-Tak. 64,000 cells were seeded per well in 30 µl of XF-Media by centrifugation. After a 25 minute incubation at 37°C in an incubator w/o CO_2, 145 µl of XF-Media was added to each well followed by another incubation period of 20 minutes, and then the plates were subjected to

analysis using 1 μM oligomycin, 500 nM FCCP and 1 μM rotenone/antimycin as indicated. Data were normalized to cell number.

mtDNA Content

DNA was extracted from $2x10^6$ cells using phenol/chlorophorm/isoamyl alcohol (25:24:1) (Guo, Jiang et al. 2009) and mtDNA quantitation by qPCR was performed as described (Venegas and Halberg 2012) using the following primers: mtDNA 16S rRNA forward 5'-GCCTTCCCCCGTAAATGATA-3' and reverse 5'-TTATGCGATTACCGGGCTCT-3'; nDNA β2-M forward 5'-TGCTGTCTCCATGTTTGATGTATCT-3' and reverse 5'-TCTCTGCTCCCCACCTCTAAGT-3'.

Bioinformatics

Promoter search was performed using the Signal Search Analysis Server program FindM using the UPR^{mt} Element previously described (Nargund, Fiorese et al. 2015). BLAST analysis against the whole sequence of ATFS-1 was done using the BLASTP 2.3.1+ program (Altschul, Wootton et al. 2005).

Statistics

Unless stated otherwise, experiments were performed at least three times, or in triplicates and expressed as mean ± SEM. Bar graphs of qPCR and Seahorse data show one representative experiment. Group differences were assayed using two-tailed Student's t test. Significance was considered when $p \leq 0.05$. Pearson correlation coefficient was performed as described in (Dunn, Kamocka et al. 2011).

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