Mitochondrial Allotopic Gene Therapy Approaches Using a *Drosophila* Model with an Endogenous *ATP6* Mutation

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University of Pittsburgh, 2014

Mitochondrial Encephalomyopathies are a group of disorders with common symptoms such as neurological, cardiac, and muscular dysfunctions. Mutations in *ATP6*, a protein-coding gene in the mitochondria genome, can lead to NARP, MILS, or FBSN diseases. *ATP6* encodes a protein subunit of the ATP synthase, also known as complex V. Currently, there is no cure for patients with *ATP6* mutations, and pharmacotherapies provide limited benefits. Because manipulation of mitochondrial genome is extremely difficult, allotopic expression of *ATP6* – specifically, expressing the mitochondrially-encoded *ATP6* gene in the nucleus – has been championed as a potential gene therapy strategy. Efficacies of allotopic rescue in *in vitro* systems have been controversial, with some studies showing the restoration of ATP synthase activity and some showing the lack of any rescue effects. We have isolated a *Drosophila* strain with a missense mutation in *ATP6*. The phenotypes of this mutant *ATP6* strain have been characterized and are very similar to those of human patients, making it an excellent model for diseases caused by *ATP6* mutation. The overarching goal of this dissertation is to import nucleus-encoded ATP6 protein into the mitochondria. This work examines the efficacies of multiple strategies in enhancing the functional outcomes of the first animal model with a stable and endogenous *ATP6* mutation and shows that algal ATP6 protein provides the most promising rescue results.

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PREFACE

Foremost, I would like to thank my thesis advisor Dr. Michael Palladino. Prior to coming to this laboratory, I was trained in immunology and in *in vitro* systems and had little experience with genetics and *Drosophila*. I am grateful that Dr. Palladino gave me a very exciting project with great clinical relevance. I was also given the task to train four undergraduates (Bryan Seelnatcht, Kris Burner, Aaron Hein, and Julie McConnell) and one first-year graduate student (Tianyu He), which was a challenging but ultimately rewarding experience as I learned firsthand the importance of time and talent management and resource allocations, which were not taught routinely in graduate school or medical school but are of immeasurable value. I also want to thank Dr. Alicia Celotto as she had spearheaded the allotopic ATP6 project for many years before I came on board, and she has offered invaluable advice and technical support. I want to thank everyone on the "ATP6" team, especially Atif Towheed, Desiree Markantone, Aaron Hein, and Julie McConnell. Atif has been a great co-worker and a great friend to discuss science and various topics; Aaron has helped generated transgenic flies with translational inhibitors and performed functional tests; and Julie has worked on various experiments such as ATP synthase assays and Western Blots, etc. I also want to thank all past and present members of this lab, especially Aaron Talsma and Atif Towheed for critically revising this dissertation and presentation. Thank you for the advice and assistance from my thesis committee members (Dr. Ferruccio Galbiati, Dr. Don DeFranco, Dr. Lin Zhang, Dr. Tim Greenamyre), the Department of

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1.0 INTRODUCTION

Mitochondrial Encephalomyopathy (ME) is a relatively common large group of syndromes that is currently incurable. The type of ME that we focus on is caused by *ATP6* mutation. ATP6 is a subunit of ATP synthase, also known as complex V, which is involved in generating the energy molecule ATP in oxidative phosphorylation. Mutations in *ATP6* commonly manifest as neurological, cardiac, and muscular dysfunctions, and variations in mutant mitochondrial DNA (mtDNA) loads lead to a range of severity in symptoms and age of disease onset. Studying *ATP6* has proven challenging due to the lack of a rigorous animal model. The Palladino lab has isolated a *Drosophila* strain *ATP6[1]* that has a stable, endogenous *ATP6* mutation. Previous characterization of this mutant has shown that *ATP6[1]* expresses disease phenotypes that are very similar to those in human patients and serves as an excellent model to study disease pathogenesis and develop gene therapy approaches for *ATP6* mutation. For my dissertation, I examined the efficacy of allotopic expression – expressing mitochondrial-DNA-encoded *ATP6* gene in the nucleus – and import of the engineered, functional ATP6 protein into the mitochondria to rescue the *ATP6[1]* mutant flies. The functional readouts of such rescue include measures of longevity and locomotor function, specifically recovery time after mechanical stress test. We have utilized different strategies including expressing *Chlamydomonas reinhardtii ATP6* gene, examining different mitochondrial targeting sequences (MTSs), attaching *SOD2* 3' untranslated region (3'UTR) or *OXA1* 3'UTR to mRNA, utilizing suboptimally-encoded codons,

employing translational inhibitors (TLIs), and overexpressing factors that are involved in mitochondrial biogenesis (AMPK, PGC1-α, and DSP1), mitophagy (ATG1), mitochondrial protein folding/degradation (mtHSP70), and mitochondrial protein import (OXA1).

1.1 MITOCHONDRIAL OVERVIEW

1.1.1 Endosymbiotic Theory and the Mitochondrial Genome

Endosymbiotic Theory proposes that mitochondria evolved from free-living bacteria and developed a symbiotic relationship within the host eukaryotic cells [1]. The theory is supported by the fact that mitochondria have their own genomes and a distinct translation system [2]. Many essential proteins for mitochondria and chloroplasts are encoded by the nucleus [3, 4], suggesting that during evolution many genes have translocated from ancestral organelles (mitochondria and chloroplasts) to the nucleus [5, 6]. mtDNA sequences have also been discovered in the nuclear genome [7]. The detailed mechanism of such transfer remains unclear, but there are two major theories. The “direct DNA transfer” theory suggests that recombination occurs between escaped organelle DNA and nuclear DNA [5]. The most impressive evidence supporting this theory points to the entire mitochondrial genome showing up in the second chromosome of the *Arabidopsis* nuclear genome [8]. The “cDNA transfer” suggests that cDNAs of organelle mRNAs are transferred to the nucleus. Genes present in the mitochondrial genomes of some plants are often found in the nuclear genomes of related species but without the introns [9-12]. Also, functional copies of mitochondrial genes are found in the nuclear genome of related species [13, 14]. Indeed, with the outflow of genetic content, human mtDNA 16.5-kilobase (kb)

genome is exceedingly small, comparing to the 3 million-kb nuclear genome. The double-stranded mitochondrial genome is circular and has only 13 established protein-coding genes, all of which are involved in the electron transport chain complexes supporting OXPHOS (oxidative phosphorylation). There are multiple mitochondria in a cell and multiple mitochondrial genomes within a mitochondrion [15], ranging from 2-10 mtDNA genomes in each mitochondrion and up to hundreds of mitochondrial genomes in muscle and nerve cells where energy demand is high [16].

1.1.2 Maternal Inheritance

mtDNA is maternally inherited [17, 18]. Uniparental inheritance is conserved in many species, including *Drosophila melanogaster* [19]. There are many theories regarding the prevalence of uniparental inheritance [20]. It has been suggested that by limiting the spread of potentially deleterious mutant genes, common in mitochondria due to oxidative damage, from parent to offspring, a wider spread throughout the population can be prevented [20, 21]. However, theoretical evolutionary advantages do not necessarily explain the prevalence of uniparental inheritance. It is suggested that uniparental inheritance may have little value to organisms, and the prevalence of uniparental inheritance is a result of transition between biparental and uniparental species, and that selection of other traits such as oogamy may lead to uniparental inheritance as a secondary result [20].

Regarding maternal inheritance specifically, it has been suggested that paternal mitochondria within sperms do enter zygotes and co-exist with maternal mitochondria briefly, but they are eliminated and not transmitted to the offspring [22]. The dilution model asserts that

the mitochondrial copy number is so low that it cannot be detected in the offspring with low-resolution experiments [23], while active degradation model suggests that paternal mitochondria are selectively eliminated to prevent transmission to the offspring [24]. Ubiquitin-mediated degradation of mitochondria has been demonstrated in monkeys and cows [25, 26], while fertilization can trigger paternal mtDNA autophagy in *Caenorhabditis elegans* [27, 28]. However, autophagy is not involved in sperm degradation in mice [29], suggesting species specificity. In *Drosophila*, mitochondria in sperm undergo physical transformation during spermatogenesis and endonuclease G is responsible for the mtDNA degradation during sperm fusion/elongation [30]. A second mechanism is also utilized that involves cellular remodeling to trim the mitochondrial mass, which ultimately is extruded as waste in the apical end of the sperm tail to remove residual sperm mtDNA [30]. The practical implication of maternal inheritance is that the offspring will not be affected if the paternal parent has a mitochondrial disease (symptomatic) or carries a mutation in his mitochondrial genome (asymptomatic). However, the offspring may or may not be affected if the maternal parent has a mitochondrial disease or carries a mutation in her mitochondrial genome (Figure 1), depending on the percentage of mitochondrial genome with the mutation the offspring has inherited (See Section 1.1.3).

1.1.3 Heteroplasmy and the Threshold Effect

Heteroplasmy describes a mixture of mtDNA sequences within a cell. When a single mtDNA sequence exists within a cell, and this state is called homoplasmy. When two or more mtDNA sequences exist in the cell, it is called heteroplasmy. Interestingly, a transgenic mouse strain with similar proportions of NZB and 129S6 “normal” mtDNA in a nuclear C57BL/6J background experiences reduced proportion of NZB mtDNA in successive maternal generations [31].

Incompatibility between C57BL/6J nuclear DNA and NZB mtDNA has been ruled out as measured by fertility and survivorship, and it is unclear why a particular mtDNA is selected against [31]. Mouse strain with heteroplasmic NZB and 129S6 mtDNA experiences reduced food intake and activity, increased anxiety-related behavior, and learning/memory impairment, but these phenotypes are not observed in mouse strains with either homoplasmic NZB mtDNA or homoplasmic 129S6 mtDNA [31]. This suggests that there is a mechanism to eliminate heteroplasmy, even if both of the mtDNA types are non-pathogenic. It is unclear why and how it is performed.

When heteroplasmy exists in a patient and one of the mtDNA types carries a pathogenic mutation, the percentage of heteroplasmy is an important determinant as to whether a patient becomes symptomatic and disease severity. The minimum percentage of mutant mitochondrial genomes required to cause dysfunction in a specific tissue is known as the threshold [15, 32]. The percentage of the mitochondria with mutant mtDNA, also known as the mutant load, is positively correlated to the severity of the symptoms and negatively with the age of disease onset. For example, patients with 70-90% heteroplasmy of mutant *ATP6* usually develop Neuropathy, Ataxia, Retinitis Pigmentosa (NARP) while patients with >90% heteroplasmy usually develop Maternally Inherited Leigh Syndrome (MILS), a much more severe phenotype [33-37]. NARP has a more moderate course and patients usually survive into adulthood, while MILS progresses more rapidly and childhood deaths are common. However, patients with less than 70% heteroplasmy are often asymptomatic and lead normal lives (Figure 2). Threshold to clinical symptoms is not fixed but depends on the tissue type and the energy demand [15]. This

dramatic threshold effect suggests that modifying the ratio of normal to mutant mtDNA may be sufficient to provide functional cures to many mitochondrial diseases.

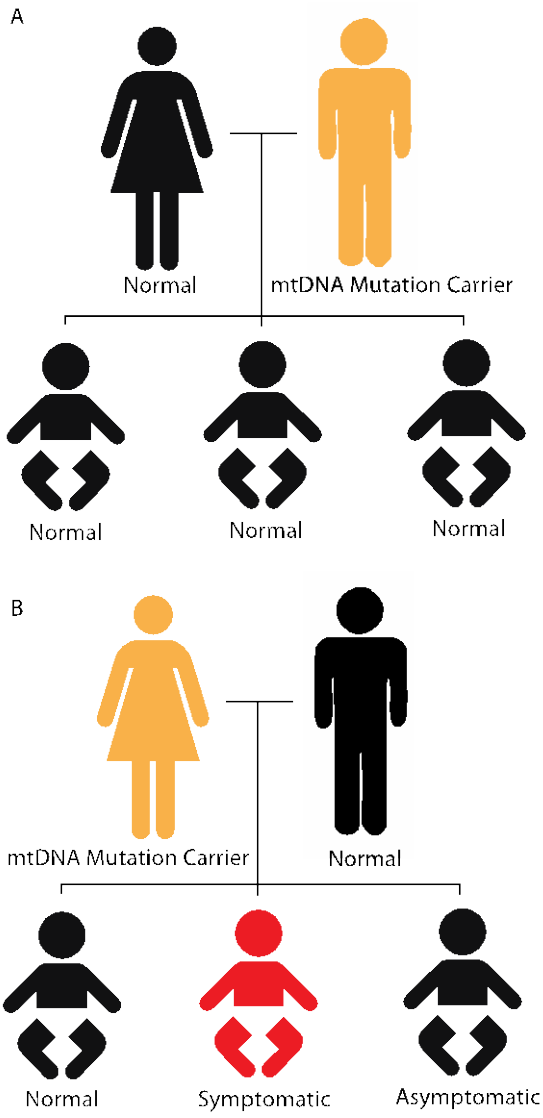

Figure 1. Maternal Inheritance in Mitochondrial Diseases.

(A). The paternal parent carries a mutation in his mitochondrial genome; however, the offspring will not be affected because they inherit mitochondria from the maternal parent only. **(B).** The maternal parent carries a mutation in her mitochondrial genome. The offspring may develop a mitochondrial disease, depending on the percentage of mitochondrial genome carrying the mutation he/she has inherited.

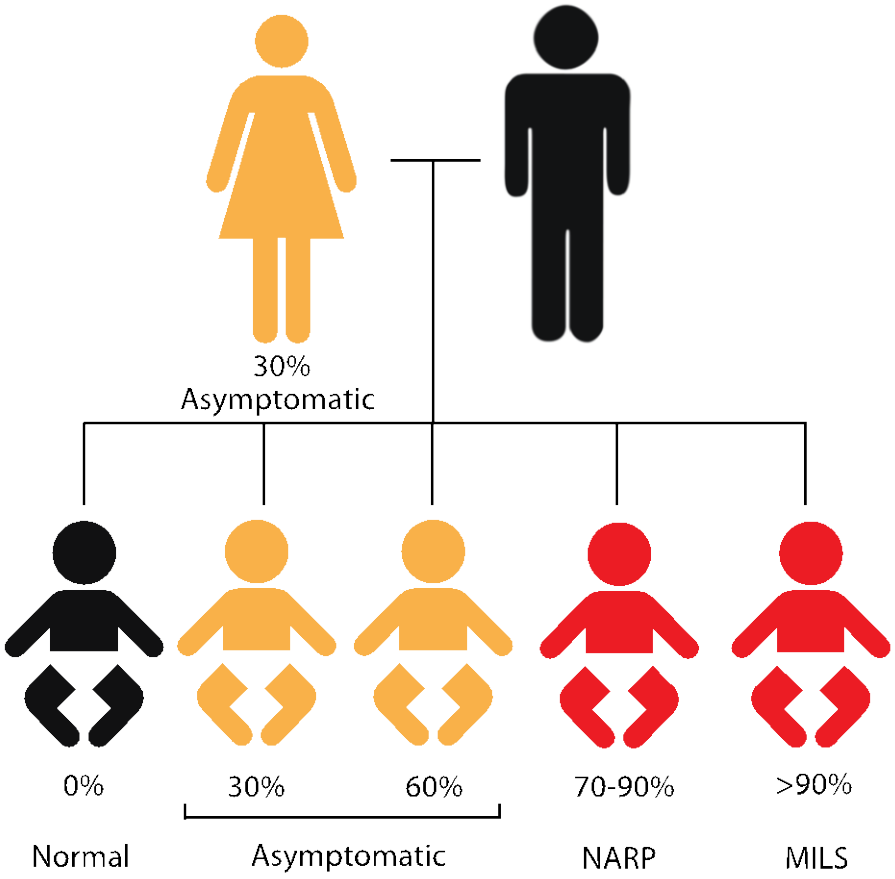

Figure 2. Heteroplasmy Determines the Severity of Mitochondrial Diseases.

In this example, the maternal parent carries a 30% heteroplasmy and is asymptomatic. Whether the offspring develops mitochondrial diseases depends on the percentage of mitochondrial genome with the mutation he/she has inherited. If the offspring has inherited more than 70% of the mitochondrial genome with an *ATP6* mutation, he/she will develop NARP. If an offspring has inherited more than 90% of the mitochondrial genome with an *ATP6* mutation, he/she will develop MILS.

1.1.4 Mitotic Segregation

During cell division, the daughter cells may experience a shift in the percentage of mutant mtDNA [15]. This shift can occur within an individual or between generations. Patients diagnosed with ME, lactic acidosis, and stroke-like episodes (MELAS) show a decline in mutant load in blood samples, suggesting that mutant mtDNA is selected against in rapidly dividing red blood cells during aging [38]. One clinical example of mitotic segregation is children surviving Pearson's syndrome may develop Kearns-Sayre-syndrome (KSS) in early adulthood [39]. Pearson's syndrome and KSS are characterized by mtDNA deletions [40-42]. Hallmark of Pearson's syndrome is that the patient, usually at infancy, will develop sideroblastic anemia [43]. If the patient survives this blood disorder, the patient's status usually improves with decreasing mtDNA deletion probably due to the rapid turnover of red blood cells. However, the patient may develop KSS later in life because mtDNA deletion in their muscles, which are post-mitotic tissues, can increase with time [39]. These examples demonstrate how the symptoms of mitochondrial disorders can change depending on the heteroplasmy and the tissues affected.

1.1.5 Mitochondrial Genetic Code

Mitochondria not only have their own genomic DNA but also utilize codons that are different from the mammalian universal nuclear genetic code [44], probably reflecting their origins as independent entities. In the universal code, AGA and AGG are translated as arginine, and it was thought that these two codons had become mitochondrial stop codons during vertebrate evolution

[45]. However, it has been shown that at least in human mitochondrial ribosomes -1 frameshift occurs at the AGA and AGC codons to terminate the two open reading frames (ORFs) in *MTCO1* and *MTND6*, respectively, so both ORFs terminate using the standard UAG codon [46]. However, it is not known if this applies to *Drosophila*. The practical implication of different genetic codes between mitochondria and nucleus is that "recoding" - modification of mitochondrial gene sequence - is necessary if the mitochondrial gene is to be expressed in the nucleus for gene therapy purpose [15].

1.2 MITOCHONDRIAL PROTEIN IMPORT OVERVIEW

1.2.1 Mitochondrial Targeting Sequences (MTSs)

Also known as Mitochondrial Targeting Signals, these sequences direct the cytosolic precursors of mitochondrial proteins to the mitochondria [47]. MTSs are generally 20-50 amino acids long, with a range from 10-80 residues [48]. The primary structures of MTSs are not conserved among closely related orthologs [49] and no homology is found among proteins that are targeted towards the same mitochondrial compartment [50], but MTSs tend to have mostly positive charges and very few if any negative charges [47]. Notably, they have the potential to form amphipathic helices in membranes, with one hydrophobic face and one positively charged face [51]. Most of these signals identified are located in the N-termini [52], but there are many internal targeting signals as well and their nature remains unclear [49] due to the intrinsic difficulties in isolating such signals without compromising the entire protein conformations [47]. There is at least one protein, yeast DNA helicase Hmi1, that has a naturally encoded C-termini

MTS and the protein is translocated from the C to N orientation [53]. Attaching a N-terminal MTS to the C-terminus of a protein artificially is sufficient to translocate the protein into the mitochondria in a C to N fashion [54]. Once the protein arrives at its destination, the MTS will be cleaved by a specific peptidase. There are four major types of MTS peptidase, with mitochondrial processing peptidase (MPP) cleaving most of the precursor proteins [55]. MPP resides in matrix and is a heterodimer consists of the larger α-MPP (Mas2) and the smaller β-MPP (Mas1) [55]. The catalytic activity of the protease resides in a zinc-binding motif of a subunit in β-MPP, and any mutation in the zinc-binding motif abolishes the catalytic activity [55]. Effort has been made to identify cleavage sites on the mitochondrial proteins; however, there is a high degree of degeneracy among cleavage sites of the proteins that have been analyzed, making reliable prediction difficult [56]. Inner membrane peptidase (IMP) is a heterodimer, consisting of two catalytic units, Imp1 and Imp2, and an accessory protein Som1 that has no enzymatic activity [57-59]. Imp1 and Imp2 have nonoverlapping substrate specificities and Imp2 is needed for stable expression of Imp1 protein [60]. IMP is responsible for cleaving the MTS from proteins that have their N-terminal MTS exposed in the inner membrane space [61]. It can cleave MTS of Cox2 independently of MPP [62] but can also process protein precursor further after the precursor has been processed by MPP [63]. However, only four substrates for Imp1 and one substrate for Imp2 have been identified [56]. Octapeptidyl aminopeptidase 1 (Oct1), formerly known as mitochondrial intermediate peptidase (MIP), is a metalloprotease that exists in monomeric form in the matrix [56]. It cleaves its substrate by removing an additional octapeptide only after the substrate has been processed by MPP [64]. Intermediate cleaving peptidase 55 (Icp55), like Oct1, resides in the matrix and cleaves residues after MPP processing [65]. However, instead of removing ocatpeptide, Icp55 cleaves single

phenylalanine, leucine, and tyrosine. By comparing Icp55 and Oct1 substrates at intermediate stages and upon full maturation, it is shown that there is an increase in half-life for mature proteins while there is an increase in turnover in the intermediate forms [65, 66]. Thus, the roles of Oct1 and Icp55 are to stabilize the N-termini of the proteins post-MPP processing, which can produce intermediate, unstable N-termini [65, 66]. Figure 3 is a simplified illustration to show that MTSs direct proteins from the cytosol to their final destinations. The MTSs are cleaved off from the proteins by various proteases to yield the final, mature forms.

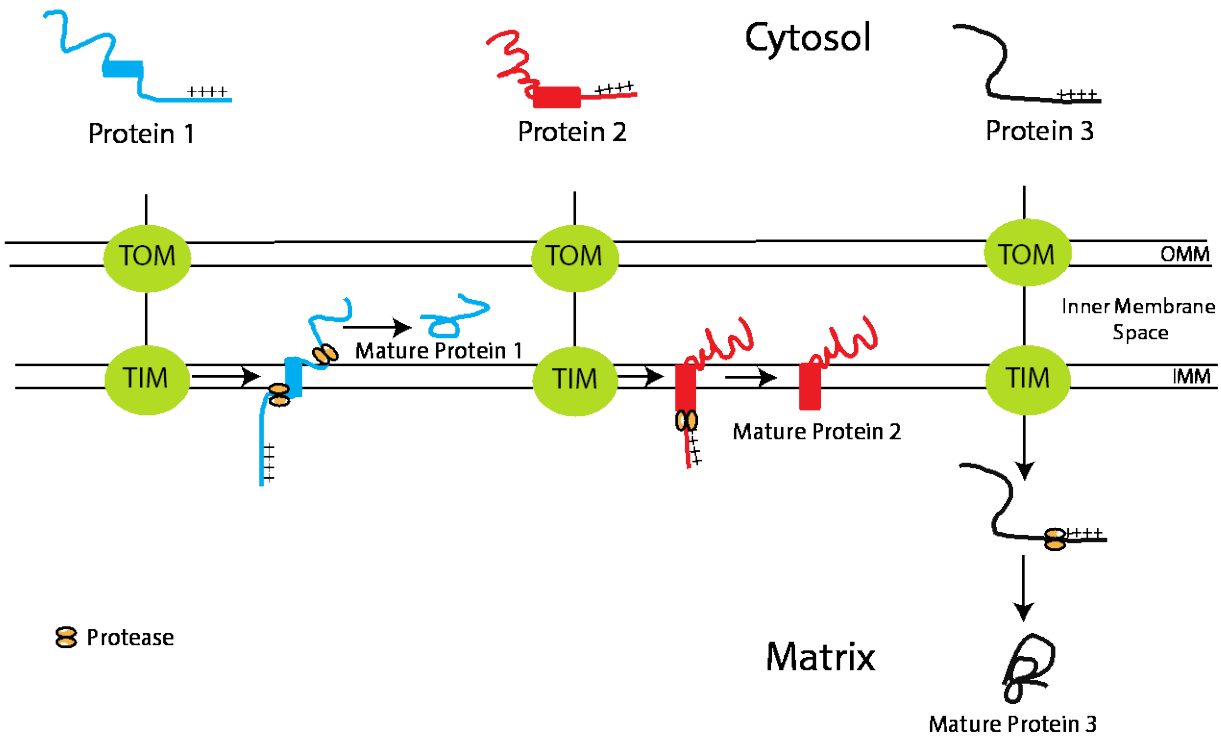

Figure 3. MTSs Direct Proteins from the Cytosol to Their Final Destinations.

Protein 1 passes through the TOM Complex and is laterally translocated to the IMM. Different proteases from the matrix and from the inner membrane space cleave off the MTS and the transmembrane region to yield a mature protein 1 in the inner membrane space. Protein 2 passes through the TOM Complex and once the transmembrane domain reaches the TIM Complex the protein is laterally translocated to the IMM. Protease from the matrix cleaves the N-terminal MTS to yield a mature protein 2 localized to the IMM. Protein 3 passes through the TIM-TOM Complex and the N-terminal MTS is cleaved to yield a mature protein 3 residing in the matrix. OMM is outer mitochondrial membrane. IMM is inner mitochondrial membrane.

1.2.2 Mitochondrial Protein Import Pathways

It is estimated that there are about 1000 proteins in the mitochondria of yeast *Saccharomyces cerevisiae* [67] and up to 1500 proteins in human mitochondria [68]. However, more than 98% of mitochondrial proteins are translated in the cytosol [48]. Mitochondrial proteins translated in the cytosol can be trafficked to the matrix, the inner membrane, the inner membrane space, or the outer membrane.

1.2.2.1 Protein Import to Matrix

Translocase of the outer membrane (TOM) complex and Translocase of the inner membrane (TIM) complex are the major components in transporting mitochondrial proteins from the cytosol into the matrix. TOM complex is the first component of the mitochondrial protein import machinery; virtually all mitochondrial proteins pass through TOM complex [49]. Most of the knowledge regarding TOM complex comes from *Neurospora crassa* and *Saccharomyces cerevisiae*, but the structure and function of the TOM complex in those organisms are comparable to those in animals and plants as well [69-71]. TOM complex has a molecular mass of 490-600kDa [72, 73], and seven components constitute the TOM complex, with TOM20, TOM22, and TOM70 being the surface receptors and TOM5, TOM6, TOM7, and TOM 40 as translocation pores [49]. TOM20 and TOM70 recognize protein precursors but have different substrate specificity. TOM 20 is the main receptor for protein precursors with N-terminal MTS [74], and TOM70 is important for the import of hydrophobic proteins, including those without N-terminal MTS [75]. TOM22 is responsible for connecting TOM20 to the translocation pore

and maintaining the integrity of the entire TOM complex [76]. It is also involved in recognition of protein precursors with TOM20 [77]. TOM40 is the central component of the translocation pore [78] with a β-barrel structure and TOM5, TOM6, and TOM7 are the accessory units. These accessory structures seem to stabilize the TOM complex, but their functions depend heavily on the model system. TOM6 in both *Saccharomyces cerevisiae* and *Neurospora crassa* promote stable interaction between TOM22 and TOM40 [79], but the loss of TOM7 in *Neurospora crassa* seems to destabilize the TOM complex [80] while in *Saccharomyces cerevisiae* the presence of TOM7 promotes a destabilizing effect [81]. Yeast TOM5 is responsible for mediating the insertion of protein precursor into the translocation pore [82] while TOM5 plays a minor role in *Neurospora crassa* [80].

TIM complex consists of 9 components, with TIM17, TIM21, TIM23 and TIM50 forming the protein transporting channel and TIM14, TIM16, TIM44, mtHSP70, and Mge1 being the import motor that transports protein into the matrix space. Of the protein transporting channel sector, TIM17, TIM23, and TIM50 are essential but TIM21 is not [83]. The short N-terminus of TIM17 has conserved negative charges, critical for precursor protein import [49, 84]. TIM17 is proposed to be important in gating the TIM23 pore [49, 84]. It is also a suppressor of mtDNA instability as overexpression of TIM17 leads to long-term mtDNA stabilization in a human cancer cell line that is prone to frequent mtDNA loss after the introduction of a mitochondrial missense mutation [85]. TIM21 interacts with the TOM complex by binding to the inner membrane space domain of TOM22 [83]. It also recruits complex III and complex IV of the respiratory chain and promotes preprotein insertion into the inner membrane [86]. TIM23 is the translocation channel [87] and the N-terminus of TIM23 consists of two parts: The first part

spanned the outer membrane and might help link TIM23 between the inner and the outer membrane [88]. It also facilitates the transfer of precursor protein from TOM to TIM and is essential for the survival of yeast at elevated temperature [88]. The second part, which spans from residue 50-100, is a coiled-coil domain that is essential for substrate binding and dimerization [89]. TIM50 is characterized by having a major domain exposed to the inner membrane space, and it is responsible for directing matrix-targeting precursor protein to TIM23 [90], recognizing precursor proteins that are destined for inner membrane [91], and linking the outer and inner mitochondrial membrane [92].

TIM14, TIM16, TIM44, mtHSP70, and Mge1 constitute the import motor. They are essential to translocate the rest of the precursor protein as the protein transporting channel sector can only transport the MTS of precursor protein with the energy derived from membrane potential. TIM14 is a DnaJ protein family member, with an essential J-domain facing the matrix and a single transmembrane domain embedding in the inner membrane [93]. It interacts with mtHSP70 [94] and TIM44 and such interaction is ATP-dependent [95]. It has been determined that TIM14 is required for efficient binding between mtHSP70 and the precursor protein and for the release of the mtHSP70-precursor complex from TIM44 [95]. TIM16 is another DnaJ homolog and interacts with TIM14 [96] for the formation of mtHSP70-precursor complex [97]. TIM16 is required for precursor proteins that are destined to the matrix but dispensable for those inserted into the inner mitochondrial membrane [97]. TIM44 interacts with TIM23, one of the membrane components of the TIM complex [98, 99]. TIM44 is capable of recruiting ATP-bound mtHSP70, which, like other HSP70 chaperones, has an ATPase domain at the N-terminus and a precursor protein-binding domain at the C-terminus [100, 101]. When mtHSP70 is bound to

ATP, it has an open pocket conformation that allows it to grasp the precursor protein. Once ATP is hydrolyzed, the ADP-bound mtHSP70 will develop low affinity for TIM44, and mtHSP70 is released from the import site [102]. The precursor protein will be imported into the matrix as mtHSP70 prevents it from sliding backwards [103]. Mge1 is essential in promoting the release of ADP from mtHSP70 [102].

1.2.2.2 Protein Import to Inner Membrane

Precursor proteins that are destined to the inner membrane utilized the TIM-TOM complex similar to the ones that are destined to the matrix. However, additional proteins are required to help process and insert them into the inner membrane. Three pathways have been identified: the TIM22 Pathway, the Stop-Transfer Pathway, and the Conservative Sorting Pathway.

The TIM22 pathway

The TIM22 pathway utilizes the TOM complex, small TIM proteins, and the TIM22 complex. Precursor proteins pass through the TOM complex and are then escorted by small TIM proteins to the TIM22 complex at the inner membrane [104]. Small TIM proteins include TIM9, TIM10, and TIM12. TIM9 stabilizes the T9-T10 complex, while the N-terminal of TIM10 serves as a substrate sensor and the C-terminal plays an essential role for complex formation [105]. TIM12 works downstream of TIM9-TIM10 complex, and it functions as a linker subunit between the soluble translocases and the membrane-bound translocases [106]. The TIM22 complex consists of SDH3, TIM18, TIM22, and TIM54. TIM22 is similar to TIM17 and TIM23 in sequence [107] and is the only essential protein of this complex, combining the functions of energy transduction, signal recognition, and channel formation in one component [108]. SDH3-TIM18 complex has roles in biogenesis and assembly of the TIM22 complex [109]. TIM54 is

involved in scaffolding and stability of the TIM22 complex and the assembly of a proteolytic complex, Yme1p [110]. Precursor proteins that utilize the TIM22 pathway include members of the solute carrier family and TIM17, TIM22, and TIM23. All of these precursor proteins share the characteristics of having even number transmembrane segments with both the N-terminus and the C-terminus exposed to the inner membrane space [49].

Stop-Transfer Pathway

The Stop-Transfer Pathway utilizes the TOM Complex and TIM23. Precursor proteins that utilize the Stop-Transfer Pathway generally have only one transmembrane segment and the N-terminus usually stays inside the matrix while the C-terminus stays in the inner membrane space [49]. The sorting signal is embedded in the transmembrane domain and it arrests the precursor protein during import and inserts the protein to the lipid bilayer laterally, and hence the name “stop-transfer”. Deletion of the transmembrane segment will lead to the precursor protein being transported into the matrix [111]. The general characteristics of the internal signal for the Stop-Transfer Pathway have been elucidated. The transmembrane segment of the precursor protein tends to lack proline residues [112], have a strong hydrophobic character [112], and the C-termini of the precursor proteins often have clusters of charged amino acids [113]. Proteins that appear to utilize the stop-transfer pathway include Cox5a, Cox11, Dld1, Tim50, Yme1, and Yme2.

The Conservative Pathway

Precursor proteins go through the TIM-TOM Complex and are targeted to the matrix, just like the precursor proteins that are destined to be in the matrix. However, these precursor

proteins are reinserted back into the inner membrane [49, 114]. Import into the matrix and the subsequent reinsertion are two distinct events; the N-terminus of a protein cannot be transported out of the matrix if the MTS is not processed [115]. Delta pH is required for the N-terminal translocation and matrix ATP hydrolysis is essential for the export of the protein into the inner membrane and the release of the mature protein from mtHSP70 [115]. OXA1 is a crucial component in inserting not only imported proteins from the cytosol but also nascent proteins synthesized inside mitochondria into the inner membrane from the matrix [116, 117]. Proteins that utilize the conservative sorting pathway tend to have proline residues in the transmembrane domain, lower hydrophobicity score [112], and multiple transmembrane segments [49]. Proteins that utilize the conservative sorting pathway include ATP9 from *Neurospora crassa* [115], Oxa1 [114], Oxa2 [118], Mrs2, and Yta10 [119]. Figure 4 illustrates the three pathways utilized by proteins destined to the inner mitochondrial membrane.

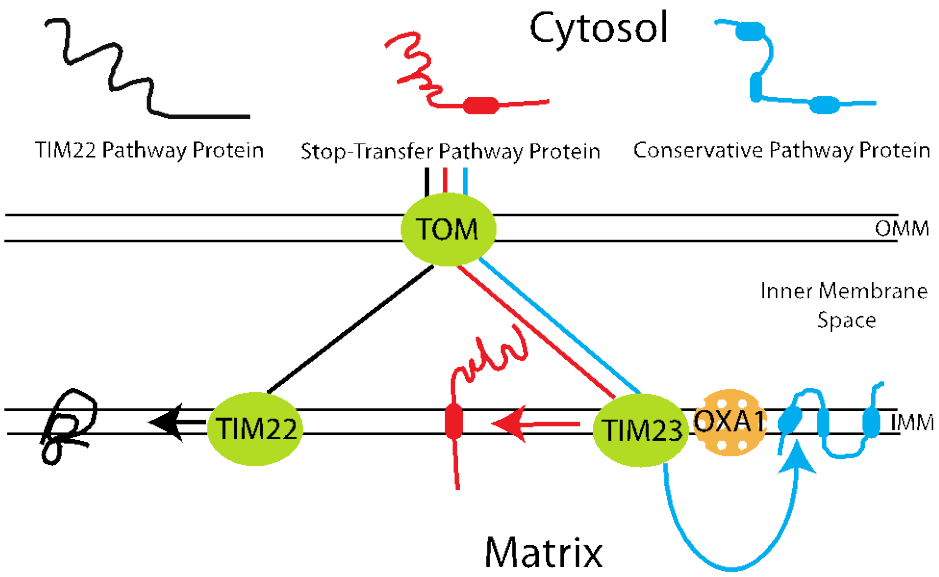

Figure 4. TIM22, Stop-Transfer, and Conservative Pathway for Proteins Destined to the Inner Mitochondrial Membrane.

Proteins that utilize the TIM22 Pathway pass through TOM complex and TIM22 (black). This pathway is utilized by solute carrier family proteins. Proteins that utilize the Stop-Transfer Pathway usually have a single transmembrane domain (red) and are translocated laterally from TIM23 to the IMM. Proteins that utilize the Conservative Pathway usually have multiple transmembrane domains (blue). They are translocated to the matrix and are reinserted into the IMM by OXA1 protein. OMM is outer mitochondrial membrane. IMM is inner mitochondrial membrane.

1.2.2.3 Protein Import to Inner Membrane Space

There are three classes of proteins destined to the inner membrane space, depending on their import mechanisms. The first class of protein has a two-part presequence - a classical N-terminal MTS and a hydrophobic segment. These precursor proteins go through the TOM Complex and interact with the TIM23 Complex. Generally, the N-terminus of the precursor protein goes into the matrix and the hydrophobic segment is arrested at the inner membrane and translocate laterally. The MPP will cleave the N-terminal MTS while the IMP complex in the inner membrane space will cleave the transmembrane portion to yield the mature protein [49, 120]. However, many different proteases have been identified to mediate cleavage and release of these inner membrane space proteins. For example, the yeast protein cytochrome c peroxidase (Ccp1) requires the m-AAA protease and a rhomboid peptidase Pcp1 to yield the final, mature Ccp1 protein [121]. The second class of proteins that are destined to the inner membrane space are usually small, and once they pass through the TOM complex they often are bound by an enzyme and are folded into stable forms by metal cofactors or disulfide bridges [49]. Apocytochrome c (without heme) is transported through the TOM complex [122] into the inner membrane space where it is bound by cytochrome c heme lyase [123, 124]. Cytochrome c is formed when heme is inserted. Its folded state prevents it from migrating out of the inner membrane space [49]. Precursor small TIM proteins interact with inner membrane protein Mia40 and are trapped in the inner membrane space by disulfide bridges [125, 126]. Copper-zinc-superoxide dismutase (SOD1) requires both metal cofactors and disulfide bridges in the inner membrane space for its uptake into the mitochondria [127]. The third class of inner membrane space proteins is membrane-bound. A classic example is cytochrome c heme lyase. It is imported without utilizing

ATP hydrolysis or electrochemical potential [128]. It is believed that binding to the affinity sites in the inner membrane space drives the translocation [49].

1.2.2.4 Protein Import to Outer Membrane

Proteins that are destined to the outer membrane utilize the TOM complex and the translocase of outer membrane β-barrel proteins (TOB) complex [129, 130], also known as the sorting and assembly machinery (SAM) complex [131]. β-barrel proteins are found only in the chloroplasts or the outer membrane of mitochondria in eukaryotic cells [132]. β-barrel proteins have multiple membrane-spanning segments that are 9-11 amino acid long [133], and require both TOM complex and TOB complex. There are three components to the TOB/SAM complex. TOM50, also known as SAM50 or TOB55, consists of two domains. The N-terminus is hydrophilic and has a novel structure, polypeptide translocation associated (POTRA) domain. It is involved in the release of precursor protein from the TOB/SAM complex [134]. MAS37, also known as SAM37 or TOM37, and TOB38, also known as SAM35 or TOM38, are the hydrophilic components of the TOB/SAM complex that reside on the cytosolic surface [135-137]. Both TOM50 and TOB38 are essential for yeast survival, but MAS37 is dispensable [137]. MAS37 is, however, essential for growth at elevated temperature [138], and it has a genetic interaction with TOM6 [139].

1.3 ATP SYNTHASE OVERVIEW

1.3.1 Types of ATP Synthase

There are three types of ATPases: F-type ATPases, also known as F_1F_o ($_o$ stands for oligomycin-sensitive) ATPases, V-type ATPases, and A-type ATPases [140, 141]. They are related in structures and mechanisms but have different functions and origins. F-type ATPases and V-type ATPases exist in eukaryotes, while A-type ATPases exist in archaea [141]. F-type ATPases synthesize ATP and hydrolyze ATP by reverse reaction, while V-type ATPases utilizes ATPs to pump proton across membranes. They are seen in intracellular membranes to maintain acidified compartments or in plasma membranes to pump protons out of cells [142, 143]. V-type ATPases are found in osteoclasts [144], epididymal lumen [145], distal nephron [146], renal proximal tubules [147], and inner ear [148], A-ATPases, not surprisingly, are structurally simpler [141]. Even though all three of the ATPases are related in structures, A-ATPases and V-ATPases are evolutionarily closer, with A-ATPases having two peripheral stalks and V-ATPases having three peripheral stalks and forming E-G heterodimer [141]. In this dissertation, we will focus solely on F-type or F_1F_o ATPases.

1.3.2 Structure and Functions of ATP Synthase

ATP synthase is an extremely complex structure. Attempts have been made to describe the structure using the mitochondria isolated from *E. coli*, yeast, bovine heart, and human cells. The subunit compositions, stoichiometry, and genetic specifications of F_1F_o ATP synthases of *E. coli*, yeast, bovine, and human are detailed in Table 1. The X-ray crystallographic structure for the

catalytic F_1 portion was first partially solved by Walker and his group in 1994 using bovine heart mitochondria [149] and more structural details have followed since [150-156]. Human ATP synthase, also known as Complex V, utilizes the proton electrochemical gradient to generate energy to phosphorylate ADP to ATP. The ATP synthase has two domains: F_1 and F_o. F_1 is the "central stalk" portion that extends into the matrix and has 5 different subunit types (3α, 3β, 1γ, 1δ, and 1ε) [149, 154]. F_o is the hydrophobic portion that is bound to the inner mitochondrial membrane and consists of main subunits a, b, c (8 identical units), d, F6, oligomycin sensitivity-conferring protein (OSCP) and accessory subunits e, f, g, and A6L [150, 157-159]. When protons cross from the intermembrane space to matrix through the inner mitochondrial membrane (IMM), a proton gradient is formed to establish a proton-motive force. This energy turns the c-subunit ring in F_o and the subunits γ, δ, ε from F_1, which sit on top of the c-subunit ring. Rotation of γ within the stationary $\alpha 3\beta 3$ hexamer leads to ATP synthesis [160, 161]. A stylized model of ATP synthesis is shown in Figure 5. Notably, only the F_o subunits a (ATP6) and A6L (ATP8) are encoded by the mtDNA [162].

Table 1. Stochiometry and Subunits of *E.coli*, Yeast, and Human ATP Synthase.

t.b.d. is to be determined. Table adapted from Rodenburg, 2012 [163].

	Stoichiometry	Prokaryotes	Eukaryotes	
		E.coli	*S.cerevisiae*	*H.sapiens*
F_1	3	α	α	α
	3	β	β	β
	1	γ	γ	γ
	1	ε	δ	δ
	1	-	ε	ε
	1	δ	OSCP	OSCP
F_o	1	a	6	A
	1	-	8	A6L
	10-15	C_{10-12}	9_{10}	c_8
	1-2	b_2	4	B
	1	-	d	D
	1	-	h	F6
	1	-	f	F
	t.b.d.	-	e	E
	t.b.d.	-	g	G
	1	-	i	-
	1	-	k	-
Regulators	1	-	Inh1p	IF_1
	t.b.d.	-	Stf1p	-
	t.b.d.	-	Stf2p	-
Total Subunits		22	33	27

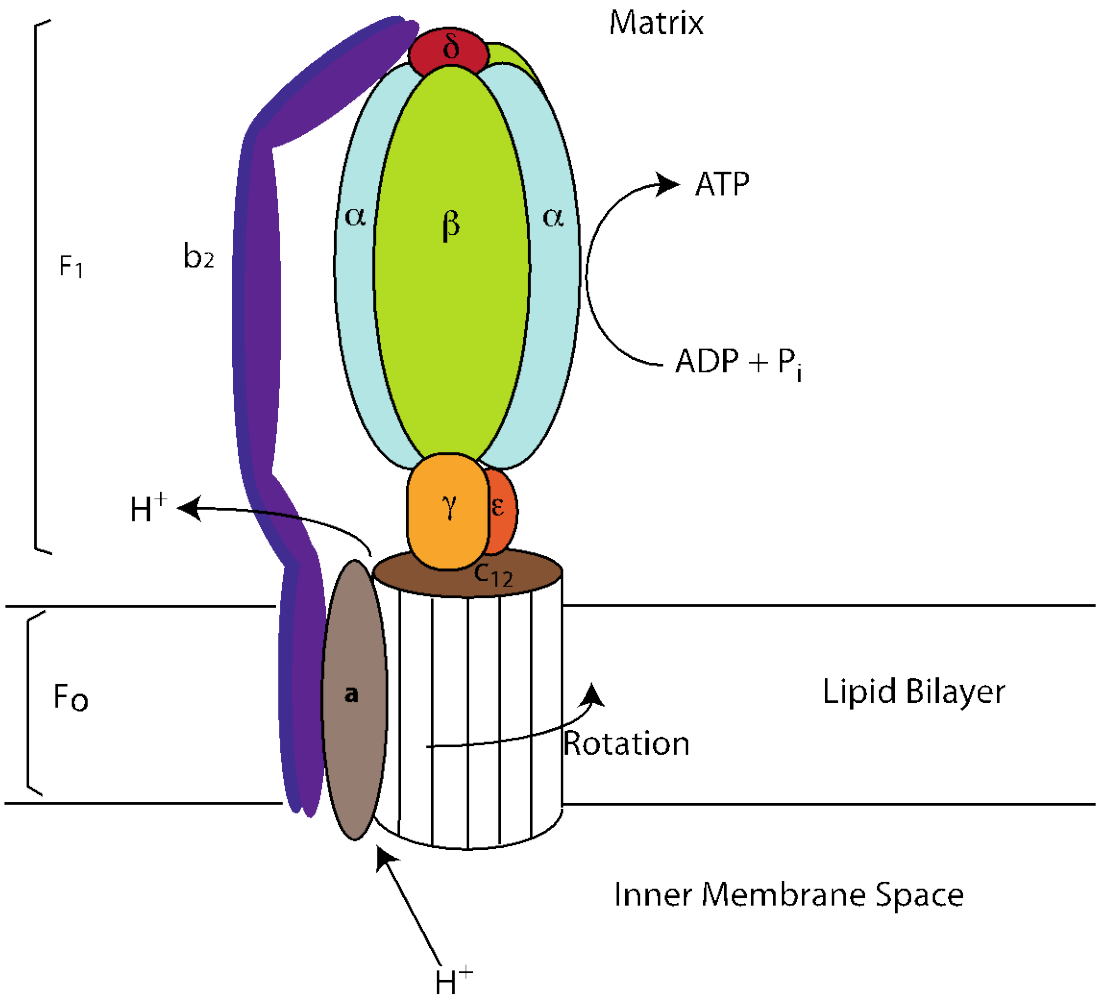

Figure 5. Model of *Escherichia coli* ATP Synthase (Complex V).

Complex V is comprised of F_1 and F_o. The a subunit, also known as ATP6 in yeast and *Drosophila*, in the F_o portion will be the focus of this thesis. This diagram is modified from *Weber J. Biochim Biophys Acta. 2006 Sep-Oct;1757(9-10):1162-70* [164].

1.3.3 Assembly of ATP Synthase

ATP synthase contains multiple subunits that require stepwise assembly. In a detailed paper that utilizes extensive radioactive labeling in yeast, the c-ring and F_1 are formed independently and are then assembled [165]. F_1 transactivates ATP6 and ATP8, the two mitochondrially-encoded proteins, which form into a complex [165]. It is still unclear at what stage the peripheral stalk (stator) joins the complex. However, it is consistent with previous reports that ATP6/ATP8 complex are connected with the rest of the complex at a much later stage [165]. Therefore, the assembly is not a single linear process but a parallel, separate and well-coordinated event. In human cells, the assembly process is slightly different. It is suggested that, like yeast, the F_1 complex is formed first. The formation of F_1 complex directs the formation of subunit c complex. F_1 complex and subunit c complex attach to each other, and the incorporation of other subunits follow [166]. By utilizing doxycycline to halt translation [166], examining the ATP synthase structure in patients with *ATP6* mutation [167, 168], and decreasing the *ATP6* mRNA transcripts [169], different researchers have identified intermediate, partially-assembled complexes and concurred that the incorporation of ATP6 protein takes place at the late stage of assembly.

2.0 MITOCHONDRIAL ENCEPHALOMYOPATHIES AND *ATP6* MUTATIONS

2.1 EPIDEMIOLOGY OF MITOCHONDRIAL ENCEPHALOMYOPATHIES

Mitochondrial Encephalomyopathies (MEs) consist of large groups of syndromes that have an overall prevalence of 10 patients with clinically relevant disease per 100,000 in the adult population [170] and affect 1 in 5000 newborns [171]. One study, utilizing randomly ascertained neonatal-cord-blood samples instead of clinically affected subjects, detects a pathogenic mutation in 1/200 live births [172]. There are two classes of MEs: class I MEs directly affect mtDNA or nuclear genes that encode OXPHOS proteins [173], and class II MEs involve nucleus-encoded mitochondrial proteins that are not OXPHOS-related [174]. ME syndromes are typified by characteristic neurological manifestations including seizures, encephalopathy, and stroke-like episodes, as well as other frequent secondary manifestations including cognitive impairment, migraines, cardiomyopathy, and diabetes mellitus [175]. Currently, there is no consensus criterion for treating mitochondrial diseases [176]. There are more than 250 mutations in class I ME (http://www.mitomap.org; accessed on July 17 2013) [177], and the most severe forms are the ones with mutations in the mtDNA. This is especially true of mutations that affect tRNAs and protein-coding genes such as *ATP6*. ATP6 is a subunit of the mitochondrial F_1F_o – ATP synthase. This enzyme, also known as complex V of the electron transport chain, is responsible for the final step of oxidative phosphorylation. 23 mutations have been identified in

the *ATP6* gene, of which 7 are associated with NARP [178-182], MILS [183-191], and Familial Bilateral Striatal Necrosis (FBSN) [192, 193]. Of these 7 mutations, all are missense mutations (http://www.mitomap.org; accessed on July 17 2013) [177].

2.2 PATHOGENESIS OF *ATP6* MUTATIONS

2.2.1 Heterogeneity of ATP6 Phenotypes

Patients and models with *ATP6* mutations present vastly different phenotypes and pathogeneses. *ATP6* mutations are usually heteroplasmic and lack tissue- and age-related variations [194], and family members with identical T8993G mutations can present with NARP or MILS [195, 196]. This is not surprising because the severity of presentation is correlated to mutant load. However, oligosymptomatic children can have similar mutant loads as their symptomatic siblings [197], and it has been reported that patients with high mutational load of T8993G do not always exhibit typical NARP/MILS phenotypes [198]. More interestingly, two point mutations at the same nucleotide can have very different pathogenic components [199]. For example, both 8993T>G and 8993T>C can lead to energy deprivation and ROS overproduction, but pathogenesis of 8993T>G (L156R) is caused by energy deficiency and has a more severe phenotype, while 8993T>C (L156P) leads to ROS overproduction and has a milder phenotype with a slight decrease of maximal ATP production [190, 200, 201]. In lymphocytes obtained from patients, a 20% ATP synthesis decrease is observed in cells with 8993T>C mutation, but a 65% ATP synthesis decrease is observed in cells with 8993T>G mutation [199]. A similar phenotypic presentation is also observed with point mutation at nucleotide 9176, with a T>G (L217R)

mutation being more clinically severe than the T>C mutation (L217P) [183, 184, 193, 202]. It is important to note that both mutation pairs occur at the same nucleotide (8993 or 9176) and change the same amino acid (Leu to Arg or Leu to Pro).

2.2.2 Defects in Proton Flow

It has been suggested that *ATP6* pathogenesis is due to a change in formation that impedes proton flow and interaction between subunits a and c [37]. A model of how proton passes through *E.coli* ATP synthase has been proposed [203]. Initially, positively-charged Arg-210 on subunit a interacts with the deprotonated, negatively charged Asp-61 on subunit c. When proton passes through the channel, Asp61 becomes protonated and causes the subunit c to rotate. This rotation puts Arg-210 into an altered position and in order to release the strain it snaps forward and becomes opposite to the next subunit c. When the proton exits, the next subunit c becomes deprotonated so the initial conformation is re-established but with the subunit c having rotated by 30 degree [203]. It is suggested that mutation of Leu (a highly conserved neutral amino acid) to Arg (a basic amino acid) reduces the ATP synthesis efficiency due to inappropriate interactions. In the case of Leu to Pro mutation, proline, being a classic helix breaker, distorts the proton channel to impede the flow and interferes with A6 → c contacts [37]. This hypothesis is supported by increased matrix pH (i.e. fewer protons flow through) in T8993G cell cybrids [204].

2.2.3 Inefficient Coupling of Proton Transport and ATP Synthesis

In contrast to the hypothesis that the *ATP6* pathogenesis is due to impeded proton flow, it has been suggested that the defect in ATP synthesis lies in the inefficient coupling between proton transport and ATP synthesis and there is no defect in proton flow [205, 206]. ATP is added to submitochondrial particles to allow ATP hydrolysis to occur as proton-pumping activity requires ATP hydrolysis. Proton-pumping activity is indicated by acidification of vesicles and measured by fluorescence. It has been shown that platelets obtained from patients and those obtained from control have essentially the same proton-pumping profiles, suggesting that the reduction of ATP synthesis is not due to impeded proton flow [205]. Fluorescent dye Rhodamine-123 is also used to determine passive proton flux, but there is no significant difference between cells from patients and cells from control [206], reiterating the point that protein flow is intact.

2.2.4 Defects in Bioenergetics

Because ATP6 is a subunit of the ATP synthase, changes in bioenergetics have been investigated. Cell cybrid systems are used to compare cell lines with different mutations (T8993G, T9176G, T8993C) and it is shown that all of them have variable degrees of ATP synthesis defects, even though all of them have 100% of the mutations. More surprisingly, variations exist among cell lines with the same mutations [207]. Cell lines with severe ATP synthesis defects show reduced mitochondrial respiration and reduced Complex I and IV activities, suggesting electron transport chain defects. In yeast, a strain without ATP6 has been developed [208]. Not surprisingly, neither oligomycin-sensitive ATP synthase activity nor ATP-driven proton translocation is detected. However, the lack of ATP6 also leads to a substantial

decrease in cytochrome c oxidase [208]. It has been shown that bioenergetics is unlikely to be the culprit of *ATP6* pathogenesis in an *in vivo* model [209]. Because the *ATP6[1]* mutants show a progressive nature of neurodysfunction, their levels of phosphoarginine (P-Arg), arginine (Arg), and adenylate pool (ATP, ADP, and AMP) have been examined at different time points of their adult lives. When the *ATP6[1]* mutants are young and asymptomatic, they have decreased P-Arg/Arg ratios and decreased ATP/ADP ratios. However, the P-Arg/Arg ratios and ATP/ADP ratios of the *ATP6[1]* mutants are not different from those of the age-matched controls when the mutants become symptomatic [209]. This suggests that bioenergetics changes do not cause age-related pathogenesis.

2.2.5 Defects in Assembly

Other explanations to describe the underlying *ATP6* pathogenesis have been sought besides defects in bioenergetics. In the T8993G mutation model, the ATP synthesis is drastically reduced in both humans [167, 205, 210, 211] and yeast [212] but the ATP synthase assembly and stability is not affected [213]. However, this issue is controversial as ATP synthase assembly is impaired in T8993G mutation cell line [168]. In T9176G mutation, ATP synthase assembly is significantly disrupted in yeast [214]. Subcomplexes of ATP synthase are detected and the few fully assembled F_1F_o complexes are very fragile and can easily dissociate into subcomplexes during detergent extraction [208]. However, the same mutation is shown to have a fully assembled yet inactive ATP synthase in *Escherichia coli* [215]. For T9176C mutation in yeast, the assembly of ATP synthase is mostly unaffected but ATP synthesis is decreased by 30-50% [216]. It is suggested that the proline residue affects the interaction between the ATP6 protein and the subunit c ring [216].

2.2.6 Defects in Membrane Fusion

A yeast model shows that membrane fusion is inhibited in ATP6 defective cells [217]. Utilizing yeast mutants lacking the entire *ATP6* gene and mutants having missense mutations leading to L183R or L247R alterations (homologous to human T8993G/L156R and human T9176G/L217R, respectively), it has been shown that the majority of the zygotes have mixed fusion profiles when the mutants are incubated with wildtype (WT) yeast, suggesting that there is a dominant inhibition in membrane fusion. Moreover, the defect is in inner membrane fusion while the outer membrane fusion remains intact as fluorescent protein attached to mitochondrial outer membrane shows similar kinetics to those of the WT [217].

2.2.7 Increase in ROS Production

ROS production has been shown to increase in both *in vitro* and *in vivo* models. In lymphocytes with 8993T→G/C mutation, there are increased ROS production and ROS-neutralizing enzymes activities [199]. Measurement of ROS production for this experiment has been made by 5-(and -6) chloromethyl-2.7-dichlorofluorescein diacetate (CM-DCFDA) fluorescence dye. It is noteworthy that a weakness of this dye is that it readily detects hydroxyl radical and peroxynitrite but not hydrogen peroxide or superoxide [218]. Mitochondrial Mn-SOD and cytosolic CuZn-SOD activities increase drastically in 8993T→ G cells but not in 8993T→ C cells, and catalase activity is not increased in either cell type [199]. The Palladino lab has generated a *Drosophila* strain that utilizes genetically encoded redox sensors (roGFPs) to monitor mitochondrial redox changes [219]. This probe is advantageous to exogenous probes because real-time detection of redox status in live cells is possible and the need to permeate and

incubate tissues with dye is minimized. They have shown that there is a progressive, age-related increase in redox change in *ATP6[1]* mutants comparing to age-matched controls and that no dysfunction is detectable in young mutants. Moreover, treatment with mitochondrial-targeted antioxidants improves the lifespan of *ATP6[1]* mutants and reduces mitochondrial oxidative stress in those animals [219].

2.2.8 The Effects of Inflammation on the Pathogenesis of *ATP6* Mutation

The role of inflammation in seizures has increasingly been recognized. However, seizure pathogenesis resulting from chronic mitochondrial dysfunction is not understood. Mutations in mtDNA that affect oxidative phosphorylation can lead to increased reactive oxygen species (ROS) production [220, 221]. Previous data from our lab has shown that mitochondrial reactive oxygen species (mtROS) is highly elevated in our *ATP6[1]* flies [219]. Studies have shown that mtROS can lead to the release of proinflammatory cytokines in mouse models and human cells *in vitro* [222-225]. Treatment with SOD mimetics, a reactive oxygen species (ROS) scavenger, inhibits proinflammatory cytokine production [226-228], suggesting that elevated ROS likely leads to inflammation activation. Furthermore, applying proinflammatory molecules, such as Interleukin (IL)-1β and Tumor Necrosis Factor (TNF)-α, to the brain exacerbated or provoked seizure activity, the mechanism of which is not known [229, 230]. Inflammation is defined as a homeostatic phenomenon induced by various types of properties by cells of the immune system, including both innate and adaptive immunity cell types [231]. Previously, it is shown that blockage of IL-1β and TNF-α [230, 232, 233], and Toll-like receptor signaling pathways [234] mediates powerful anti-convulsant effects. Moreover, in human febrile seizures, seizures

commence after inflammation (usually due to infections), so it is well established that inflammation can lead to seizure. A case report has also shown that febrile illness can exacerbate the symptoms of mitochondrial disease [235]. However, experimental evidence also suggests that inducing seizures in rats and mice can lead to the release of inflammatory mediators [236-239]. Therefore, in our aseptic fly model with a "built-in" seizure propensity, whether inflammation takes place and what effect it imposes on the seizure phenotype remain to be determined.

2.3 MODEL SYSTEMS TO STUDY *ATP6* MUTATIONS

2.3.1 *Escherichia coli*

Multiple *E.coli* models have been developed to model human ATP mutations. A T8993G mutation [240] and a T8993C mutation [241] have been constructed in *E.coli*. The *E. coli* model with a T8993G mutation fails to grow, suggesting that the mutation causes a substantial loss in oxidative phosphorylation. A follow-up fluorescence quenching experiment shows that the mutation leads to inhibition of proton translocation [240], suggesting that *E.coli* is very sensitive to the Leu → Arg change as this leads to a complete loss of ATP synthesis and proton pumping activities. The same group has also developed a *E.coli* strain with T8993C mutation, and the strain with a T8993C mutation has about 60% intact F_o subunit while the one with a T8993G mutation has less than 40% [241]. Strain that has T8993C mutation had about 30% of proton pumping capacity and close to 10% passive protein translocation upon F_1 depletion comparing to WT, which are both less severe than the T8993G phenotype. This is similar to what has been

observed in another model system cybrid. However, while the assembly of L156R ATP6 into the human ATP synthase complex is not affected [211, 213], the mutant L207R subunit a (the equivalent of L156R ATP6 in humans) cannot be inserted into the bacterial synthase complex [215]. Moreover, bacterial ATP synthase is missing at least 10 subunits that are present in the human ATP synthase [242]. For these reasons, other more suitable, eukaryotic models are sought after.

2.3.2 Yeast

Multiple *Saccharomyces cerevisiae* models with *ATP6* mutations have been developed and characterized [212, 214, 216, 243]. Yeast with NARP T8993G mutation grow very slowly possibly because the ATP synthesis rate is only 10% comparing to that of WT, although the ATP synthase is correctly assembled and is present at 80% WT level [212]. Unlike human NARP cells [37, 205], ATP hydrolysis in intact yeast mitochondria is significantly compromised. The oxygen consumption rate is also significantly lowered by 80% due to a decrease in the cytochrome c oxidase (complex IV) content [212]. Yeast with T8993C mutation has a 40-50% ATP synthesis rate comparing to that of WT, and although there is neither a deficit in ATP6 protein synthesis nor a deficit in proton translocation, the assembly of ATP6 into the ATP synthase complex is very unstable [243]. In yeast with 9176G mutation, the ATP synthesis rate is drastically reduced by >95%. The ATP6 protein is synthesized but incorporation of the ATP6 protein into the ATP synthase complex is not efficient [214]. In yeast with 9176C mutation, there is a 30% reduction in ATP production. However, a number of sub-complexes are detected, suggesting the decreased stability of ATP6 protein within the ATP synthase complex [216].

2.3.3 The Cybrid System

Cybrid, also known as transmitochondrial hybrid, is the fusion of an enucleated cell with mutant mtDNA and a mitochondrial-less cell (ρ° cell), usually from the immortal cell lines [244]. The protocol involves the fusion of platelets with mutant mtDNA and osteosarcoma cells with no mtDNA [210, 245]. Cybrid cell lines with different levels of mutant mtDNA can be made. The advantage of the cell cybrid system is that it allows comparisons among different individuals as the nuclear confounding variables are eliminated. However, there are many drawbacks to this approach. Cybrids can exhibit high degree of variability in pathogenesis and wide variations in biochemical defects among cell lines with the same mutations (see section 2.2.4) [207]. Cybrids are also fusion cells that do not resemble cells in the natural state, and the nuclear background of the ρ° cells will affect the heteroplasmic stability of the mtDNA [246, 247], the biochemical defects of the complexes [248], and the phenotypes [249, 250] of the models. The physiology of the mitochondria isolated from different tissues to make cybrids can be drastically different [251], and the nuclear DNA determines mitochondrial functions depending on the specific tissues [252]. The cybrid system requires a much higher mutation load than that seen in humans to alter biochemical parameters, suggesting that this approach may not reflect the human disease processes [253]. Also, the growth of cybrids is highly subjected to the medium conditions (e.g. galatose vs. glucose) [210] and may not reflect the complex metabolic demands in intact tissues. The ρ° cells are derived from tumor cells, of which often have chromosomal rearrangements. Because only 2 subunits of the ATP synthase complex are mitochondrially encoded and the rest are encoded in the nucleus, the abnormal chromosomal number/arrangement or altered nuclear-mitochondrial interactions may affect the stoichiometry of the complex (i.e. excessive or

depleted numbers of certain subunits), complicating the analysis of mitochondrial genotype-phenotype correlation [254]. Lastly, tumor cell lines are generally glycolysis dependent, which may not mirror myopathies that usually occur in more aerobic cells.

Besides being used to study pathogenesis, cellular cybrids and various mutant cell lines have also been widely used to determine the efficacies of a gene therapy approach known as allotopic expression. Allotopic expression involves expressing a gene that is normally encoded in the mitochondria in the nucleus and transporting the protein from the cytosol into mitochondria. Allotopic protein import has been examined not only for ATP6 [255-258] but also for ND4 [259-261], ND6 [262], COX3 [257], cytochrome b [261], and ATP8 [261]. However, the outcomes are controversial. ATP8 protein but not ND4 or cytochrome b has been shown to colocalize with mitotracker in mitochondria [261], and ND6 has been shown to colocalize to the mitochondria but it is incorrectly targeted to the outer mitochondrial membrane [262] (also see. Section 3.1 for ATP6-specific outcomes).

2.3.4 *ATP6* Mutant Mice

Pinkert and his group reported the first transgenic mouse model for ATP6 allotopic expression [263]. It is shown that cytoplasmically-translated ATP6 proteins can be localized to mitochondria. However, it is doubtful if it would make a good model for ATP6 mitochondrial encephalomyopathy. The A6M mice (mice that express mutant *ATP6* gene in the nucleus) actually performed better in rota-rod test than the control, which did not reflect mitochondrial encephalomyopathy phenotype progression as seen in human patients. Moreover, there is no significant difference in mitochondrial function between the transgenic mice and the controls.

More importantly, the idea of expressing an exogenous mutant *ATP6* gene in the nucleus to model a disease that had its origin/ mutation in the mitochondria could be problematic. Any phenotype that is observed in the mice cannot be conclusively attributed to the *ATP6* mutation; in fact, it can be due to various factors such as mutant protein accumulation in the cytosol at a toxic level.

3.0 GENE THERAPY APPROACHES TO MITOCHONDRIAL ENCEPHALOMYOPATHIES

ATP6 synthase subunit a, also known as ATP6 in *Drosophila melanogaster* and in *Saccharomyces cerevisiae* (yeast), is encoded by the mtDNA *ATP6* gene [162]. Several approaches to mitochondrial gene therapies have been proposed. Because the presence and severity of clinical symptoms depend on the percentage of heteroplasmy, inhibiting the replication of mutant mtDNA by antisense oligonucleotides may allow WT mtDNA in the same cell to accumulate [264]. However, this might promote mtDNA depletion, where mitochondria with deletions in their mtDNA are created [265]. A similar idea of decreasing the ratio of mutant mtDNA to WT mtDNA but utilizing a drastically different method has been described recently [266]. Mitochondrially-targeted transcription activator-like effector nucleases (mitoTALENs), enzymes that enter mitochondria to bind specific sequences of interest, are utilized to differentially cleave the mutant mtDNA harboring a ND6 point mutation while sparing the WT mtDNA, successfully skewing the mutant mtDNA to WT mtDNA ratio [266]. Another approach involves importing WT DNA directly into the mitochondria. However, it is unclear if the very different transcription and translation systems of mitochondria, which specialize in handling circular DNA molecules, will be able to recognize exogenous DNA, which presumably have a very different structure [265]. The approach that is utilized in my dissertation involves expressing desired mitochondrial gene in the nucleus (allotopic expression), translating the

protein in the cytosol, and importing the protein back into mitochondria. The advantages of this approach are that the vast majority of mitochondrial proteins are indeed translated in the cytosol and subsequently imported into the mitochondria, and the protein import mechanism by translocases of the inner and outer membrane (TIM-TOM complex) is fairly well characterized. Such approach has already been successful in yeast. In 1988, Nagley and his group successfully rescued an ATP8 mutation phenotypically [267]. However, such approach has yet to be shown successful in any animal model and some allotopic expression results remain very controversial. Another distinct advantage of this approach is that even inefficient allotopic gene therapy might be efficacious to many affected individuals due to the mitochondrial threshold effect. Therefore, we rigorously test allotopic expression in vivo and explore the challenges and limitations to this approach, as well as design strategies for possible solutions and improvements in efficacy.

3.1 ALLOTOPIC EXPRESSION OF *ATP6*

Because *ATP6* is expressed in mitochondria and currently there is no way to introduce foreign genes into the mitochondrial genome in higher eukaryotic cells [261], allotopic expression, which involves expressing a mtDNA-encoded *ATP6* in the nucleus and transporting the ATP6 protein from the cytosol into the mitochondria, has been suggested as a viable therapy possibility [268]. It has been demonstrated successfully in yeast [267, 269]; however, the results from mammalian cells are mixed. NARP cybrids expressing oligomycin-resistant *ATP6* gene in the nucleus can tolerate 1000 folds higher concentration of oligomycin (1000ng/mL vs. 1ng/mL), suggesting that the nucleus-encoded oligomycin-resistant ATP6 protein has incorporated into the mitochondrial ATP synthase complex [258]. Allotopic expression of ATP6 [255] and

specifically algal *Chlamydomonas reinhardtii* ATP6 [256] have been successful in rescuing a human cell line with an *ATP6* mutation. However, the result is controversial as it has also been shown by another group that the algal protein does not incorporate successfully into Complex V [270]. This concern is echoed by a group that utilized mass spectrometry and failed to detect algal ATP6 incorporation into the ATP synthase [257].

3.2 THE *ATP6[1] DROSOPHILA* MUTANT MODEL

The Palladino lab has isolated a *Drosophila* strain with a missense glycine to glutamate mutation at position 116 in the *ATP6* gene [271]. This fly strain, *ATP6[1]*, exhibits phenotypes that include reduced longevity, progressive locomotor impairment, seizures, and neural dysfunction, which are very similar to the human disease progression [271]. More specifically, seizures can be induced in aged *ATP6[1]* flies by strobe light, suggesting that sensory hyperstimulation is sufficient to induce this specific behavioral phenotype in an age-dependent manner [209], and it is documented that strobe light (photostimulation) can induce seizures in susceptible humans [272]. This further highlights the suitability of using the *ATP6[1] Drosophila* as a model for mitochondrial encephalomyopathy. Notably, this *ATP6[1]* mutant model has a 98% *ATP6[1]* heteroplasmy, which is higher than any other heteroplasmy documented in viable organisms. Thus, this model provides a rigorous test of efficacy of various allotopic expression strategies and takes full advantage of this model's tractable genetics. However, this also leads to serious challenges in demonstrating rescue effects through functional assays (see discussion).

3.3 ENGINEERED *ATP6* GENE

Because mitochondrial codons and nuclear codons are different, recoding is necessary so that the correct amino acid sequences will be translated [255]. There are three basic elements to the engineered *ATP6* in the nucleus: MTS, *ATP6* gene, and 3'UTR. A number of transgenic strains has been generated and tested by combining different MTSs, recoded *ATP6* gene, and 3'UTRs to determine the optimal components of an allotopic construct used *in vivo* (Figure 6).

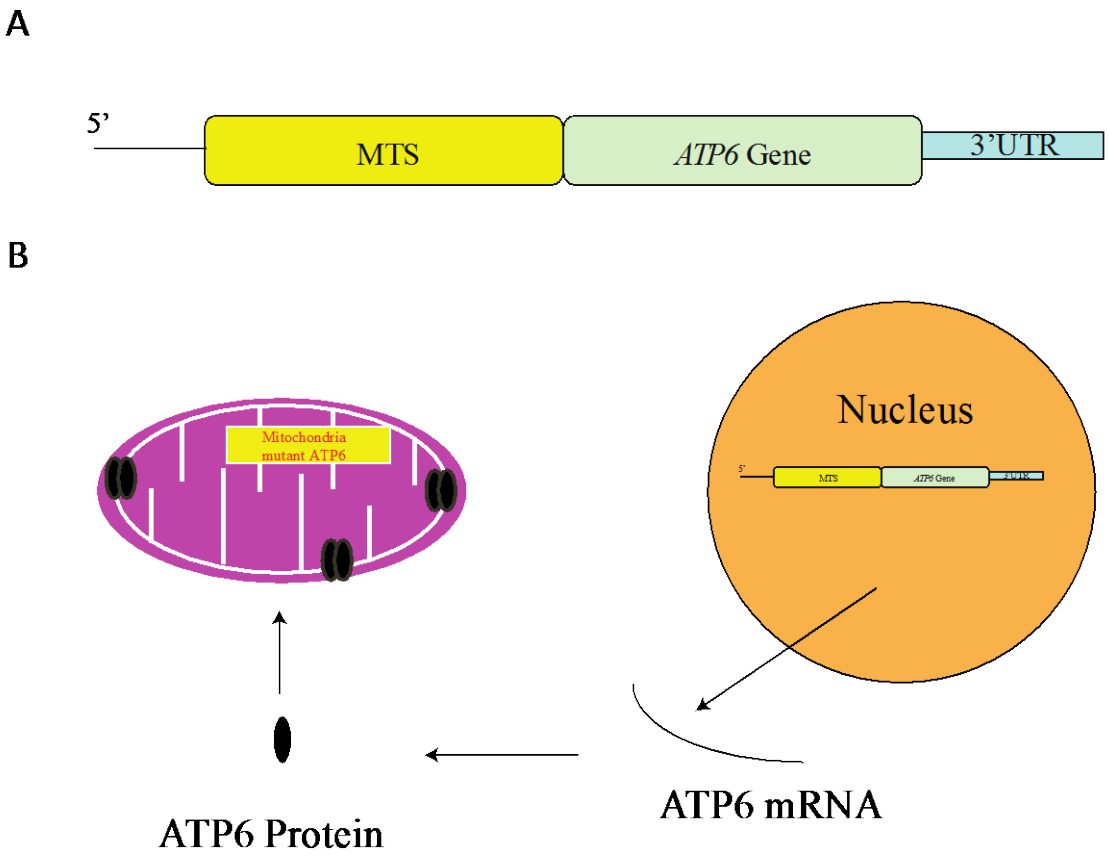

Figure 6. Allotopic Gene Therapy.

(A) Basic construct of a nucleus-encoded *ATP6* gene. The first part is a mitochondrial targeting sequence (MTS), which directs the protein into the mitochondria. The second part is the recoded *ATP6* gene. Recoding is necessary for proper translation in the nucleus. The third part is the 3' untranslated region (3'UTR). **(B)** Nucleus-encoded *ATP6* gene is transcribed into mRNA in the nucleus and translated into protein in the cytosol and import into the mitochondria.

3.4 UAS-GAL4 SYSTEM

The bipartite UAS/GAL4 system is the most widely used method in *Drosophila* to achieve spatially- controlled gene expression. GAL4 is a yeast transcription activator protein, and upstream activating sequences (UAS) is followed by a target transgene cloned downstream. The GAL4 strain and the UAS strain are different parental lines combined in the F1 to activate the system. Transcription of GAL4 is controlled by promoters with characterized temporal spatial patterns. Selected offspring of the mating between the GAL4 and UAS strains will have the target gene expressed at specific locations determined by the promoter of GAL4 (Figure 7). There are 35717 transgenic GAL4 constructs and 67168 transgenic UAS constructs (Flybase.org, accessed on January 13 2014), allowing tremendous combinations. However, there are some limitations to the UAS-GAL4 system. For example, it is generally not simple to induce gene expression, and systems utilizing ligand-induced GAL4 chimeras have been developed to remediate this deficiency. Another potential issue is that many GAL4 lines have dynamic expression, so the transcription level of target gene may vary throughout the lifespan of the animals. In our studies we have focused on utilizing the daughterless-GAL4 and ubiquitous-GAL4, both broadly expressed in all tissues, to determine which transgenic combination provides the best rescue outcome.

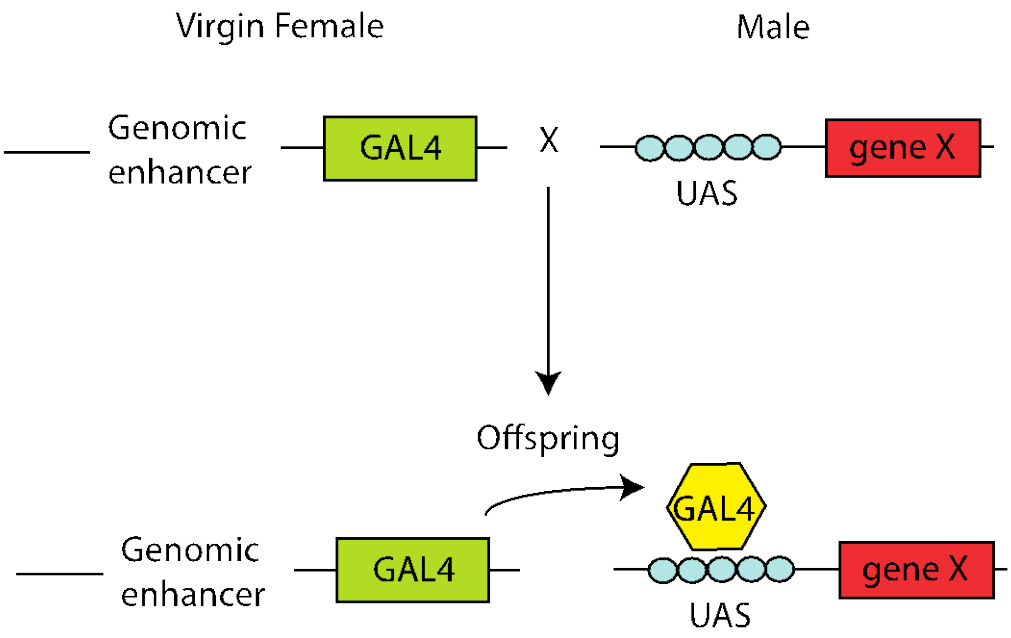

Figure 7. The UAS-GAL4 System

Virgin *ATP6[1]*; daughterless-GAL4/ daughterless-GAL4 females are mated to male flies with the desired transgene (UAS-TG). Female offspring have the maternally inherited *ATP6[1]* as well as one copy each of the UAS-TG and the daughterless-GAL4 and are used in functional assays. This figure is modified from St Johnston D. Nat Rev Genet. 2002 Mar; 3(3):176-88 [273].

3.5 SITE-SPECIFIC RECOMBINATIONS

Transgenesis utilizing standard P element-mediated transgeneis often encounters position effects as identical transgenes are inserted randomly into the genome. To avoid this issue, we utilized pUASTattB transgenesis using the PhiC31-mediated integrase. This allows site-specific integration into well-characterized target sites. This specific construct not only is highly efficient with 5-10% of the injected embryos producing transgenic animals that are targeted correctly but also eliminates the need to analyze multiple versions of each transgene as all transgenes and controls are inserted into the same location of the genome.

3.6 MAJOR HURDLES WITH ALLOTOPIC EXPRESSION

There are several major hurdles associated with allotopic expression. Hydrophobicity of the ATP6 protein and competition for incorporation into the ATP synthase complex between exogenous ATP6 and endogenous mutant ATP6 are two major challenges. Additionally, avoiding cytoplasmic aggregation, efficient import into a sufficient number of mitochondria, proper folding and localization of ATP6 within complex V are also necessary for allotopic expression of ATP6 to be an efficacious strategy. We have focused on tackling these two major challenges and strategies to overcome these obstacles, which have been detailed below.

3.6.1 Hydrophobicity

The mitochondrial genome has been shedding its genome since its primordial endosymbiont ancestor invaded the eukaryotic cell more than a billion years ago, and the gene content of mitochondria became stabilized about 800 million years ago [274]. The human mitochondrial genome now has a size of 16.5kb and contains 37 genes, of which 13 are protein-coding genes, 22 are transfer RNAs (tRNAs), and 2 are ribosomal RNAs [162]. Of the 13 protein coding genes, 7 code for subunits in complex I, 1 in complex III, 3 in complex IV, and 2 in complex V, with ATP6 being 1 of the 2 proteins in complex V (the other being ATP8). The fact that these 13 genes remain in the mitochondrial genome while most of the other genes have transferred to the nucleus suggests selective barriers preventing their migration. Several hypotheses have been postulated to explain the retention of a mitochondrial genome. It is possible that the nuclear genetic code and the mitochondrial genetic code have diverged enough that translating mitochondrially-encoded genes by cytoplasmic machinery has become infeasible [275, 276]. It is also possible that the 13 protein-coding genes are retained in mitochondria to ensure that the assembly of oxidative phosphorylation complex occurs in the inner membrane and prevent mistaken insertion of those proteins into other membranes that can lead to cellular damage through ROS production [277, 278]. However, it is certain that the polypeptides translated from these 13 genes are extremely hydrophobic and will be challenging to transport them from the cytosol into the mitochondria. Due to their hydrophobicity, it is likely that the proteins would aggregate in the cytoplasm and be degraded by the proteasome or authophagy. Thus, strategies that will allow the ATP6 protein to become more hydrophilic and soluble could be critical for efficient import.

3.6.1.1 Strategy 1: *Chlamydomonas reinhardtii* ATP6 Subunit

In eukaryotic organisms, all F_1 subunits and most F_o subunits are nucleus-encoded. In yeast, the exceptions of the F_o sector are *atp6*, *atp8*, and *atp9* [279]. These genes encode proteins that are highly hydrophobic, with five putative transmembrane regions for ATP6 [279]. However, it has been determined that genes that are usually present in the mitochondrial genome in most organisms, including *nad3*, *nad4L*, *cox2*, *cox3*, *atp6*, *atp8*, and *atp9*, are lacking in the mitochondrial genomes of some algae, such as *Chlamydomonas reinhardtii* [280, 281], *Chlamydomonas eugametos* [282], and *Chlorogonium elongatum* [283]. A study has shown that 1) calculating the highest average hydrophobicity of 60-80 amino acids in a protein and 2) measuring the maximum hydrophobicity of the transmembrane segments provide useful indication of the probability that a protein can be imported into mitochondria [284]. By establishing that ATP6 protein has 5 transmembrane regions after taking consideration of the conserved and essential amino acids and the topology of amino and carboxyl termini, Gonzalez-Haplhen and his group have quantified the mean hydrophobicity values of ATP6 for *Chlamydomonas reinhardtii* and other plant and algal groups. The three transmembrane segments that are presumably not directly involved in proton translocation or interaction with c-ring subunits have the biggest drop (>50%) in their local hydrophobicity values [279]. This might be one of the reasons why the algae *Chlamydomonas reinhardtii* are one of the few known organisms that are capable of expressing *ATP6* in the nucleus and transporting the polypeptides into the mitochondria. In fact, when we aligned the amino acid sequences of *Chlamydomonas reinhardtii*, *Drosophila melanogaster*, and human, it was obvious that algae had a very long and hydrophilic MTS (Figure 8). We hypothesized that this property was responsible for the importability of algal ATP6. In this project, we expressed *Chlamydomonas reinhardtii ATP6*

gene from the nucleus of *Drosophila* allowing algal ATP6 protein import into *Drosophila* mitochondria. Three transgenic strains have been generated: NoCrAV, NoCrAS and NoCrAO, in which No stands for the lack of exogenous MTS, CrA stands for ***C****hlamydomonas* ***r****einhardtii* ***A****TP6*, V stands for SV40 3'UTR, S stands for SOD2 3'UTR, and O stands for OXA1 3'UTR. Because the algal MTS had its own N-terminal MTS, we thought it was unnecessary to add an exogenous MTS to target it to the mitochondria. Three different 3'UTRs were attached to each individual strain to determine if it would further improve import (see strategy 3). A potential complication associated with this strategy is that the ATP synthase complex may not be functional as not all the subunits are derived from the same species. Other groups have attempted similar approaches *in vitro* but the results have been controversial, with one group showing that ATP synthase activity is significant improved upon algal allotopic expression [256] and another group reports that the algal ATP6 subunit is not assembled into the ATP synthase complex [270]. The mating scheme of transgenic strains used is shown in Figure 9.

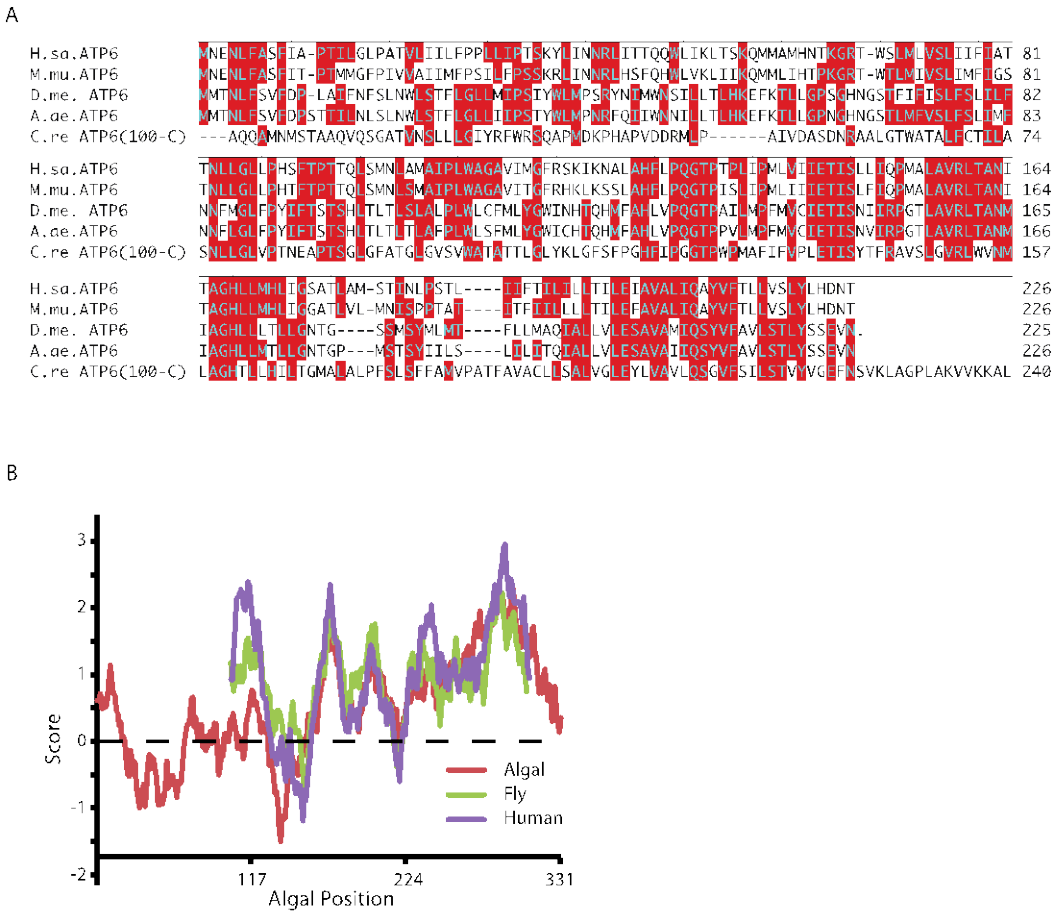

Figure 8. ATP6 Protein of *Chlamydomonas reinhardtii*.

(A) Amino acid alignment of ATP6 polypeptides of *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Aedes aegypti*, and *Chlamydomonas reinhardtii* using the Clustal W method. The first 100 amino acids of the N-terminus of the algae were removed due to its extended mitochondrial targeting sequence for better alignment of the actual ATP6 protein. **(B)** The hydrophobic plots of algal ATP6, *Drosophila* ATP6, and human ATP6. Algal ATP6 has a significantly longer MTS and is more hydrophilic than *Drosophila* ATP6 and human ATP6.

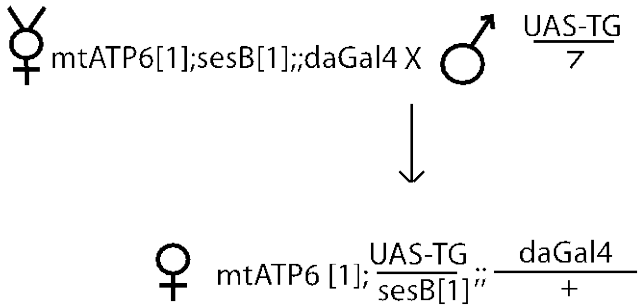

Figure 9. Generation of Transgenic Fly Strain with the Expression of Nucleus-Encoded *ATP6* transgene (UAS-TG).

Virgin *ATP6[1]* flies homozygous for *sesB[1]* and *daughterless-Gal4* (*daGal4*) were maintained as a stable stock. They were mated to male flies carrying the transgenic nucleus-encoded *ATP6* gene. Because mitochondria are maternally inherited, all the offspring will carry the *ATP6[1]* mutation. However, only the female offspring are used in functional assays to avoid behavioral and physiological differences. mt is mitochondria.

3.6.1.2 Strategy 2: Mitochondrial Targeting Sequence (MTS)

Because the *Drosophila ATP6* gene is naturally encoded in mitochondria and the protein is localized in the inner mitochondrial membrane, it does not normally rely on an MTS to direct it into the mitochondria. We have to predict which pathway it would utilize if *ATP6* **were** a nucleus-encoded gene and ATP6 protein would require translation and import from the cytosol into mitochondria. We have identified a number of MTSs that utilizes different import pathways to transport proteins into mitochondria. TIM22, stop-transfer, and conservative pathways are the three known pathways that transport proteins into the inner membrane. Of the three pathways, stop-transfer and conservative pathways are more likely to be used by *Chlamydomonas reinhardtii* ATP6 because it has an N-terminal MTS [279]. TIM22 is typically utilized by membrane-bound TIM subunits and by solute carrier family, such as ADP/ATP carrier proteins [49]. One important characteristic of the ADP/ATP carrier proteins is that they typically do not have N-terminal MTS [285], Algal ATP6, however, is predicted to have a 107 amino-acid N-terminal MTS [279], making the TIM22 pathway a less likely candidate to be utilized by both algal ATP6 and our engineered fly ATP6 protein. Proteins that utilize the stop-transfer pathway tend to have a single transmembrane domain while those that utilize the conservative pathway tend to have multiple transmembrane domains. Algal ATP6 is predicted to have five transmembrane domains, thus, the conservative pathway is most likely to be used by both the algal ATP6 and ATP6 engineered for allotopic expression. The MTSs used in our experiments include *P1* MTS [255, 286], *OXA1* MTS [287], *SOD2* MTS [288, 289], *DLD* MTS [113], and *COX8* MTS [255, 290]. Their accession numbers and sequences used are listed in Table 2. Transgene with no exogenous MTS were also tested. Transgenic strains with one of three 3’

UTRs (see strategy 3) were also used with the MTSs to determine the best functional combination.

Table 2. Mitochondrial Targeting Sequences (MTSs)

The parentheses in the first column indicate the origins of the MTSs. The accession number of each MTS and the numbers in the parentheses indicate the start and end nucleotides. P1 MTS is originated from humans but has been recoded for optimal expression in *Drosophila*.

MTS	Accession Number	Recoded?
P1 (human)	AK311848.1 (34-216)	Yes (for fly expression)
sOxa1 (yeast)	BK006939.2 (475020-475080)	No
Sod2 (human)	AK313082.1 (1-216)	No
Dld (yeast)	NM_001180234.1 (1-216)	No
Cox8	CU687634.1 (17-90)	No

3.6.1.3 Strategy 3: *SOD2* 3'UTR and *OXA1* 3'UTR

The vast majority of mitochondrial proteins are encoded in the nucleus and translated in the cytosol; therefore, protein transportation from the site of synthesis mitochondria and import are critically important for normal mitochondrial function. Considering that protein transport can take longer than the protein's half-life in some cases [291], mRNA localization is a relevant factor for ensuring that proteins are translated near the site of import [292]. In fact, more than 70% of the mRNA present in *Drosophila* embryos are found in spatially distinct patterns [293], suggesting that mRNA localization is a widely adopted strategy for the majority of eukaryotic transcripts [294]. 47% of yeast mRNAs encoding mitochondrial proteins are transported to the mitochondrial surface [295]. When one of those proteins, ATP2, which encodes the beta subunit of ATP synthase, loses its 3'UTR, a severe respiratory deficiency associated with inefficient import of the precursor protein is observed, suggesting the importance of 3'UTR in targeting the polypeptides to the mitochondrial surface and facilitating the import process [296]. These and other findings suggest the likelihood of co-translational mitochondrial import. In HELA cells with nucleus-encoded ATP6, more recoded *ATP6* RNAs are isolated from the mitochondrion-bound polysomes *in vitro* when *SOD2* 3'UTR is attached to the recoded *ATP6* gene in the nucleus [288]. Moreover, the recoded ATP6 and endogenous ATP alpha show significant colocalization, suggesting that the nucleus-encoded ATP6 protein is translocated into the mitochondria. This is further confirmed as a significant amount of the mature form ATP6 protein is resistant to protease K digestion and Triton X-100, suggesting that the recoded ATP6, which normally resides in the transmembrane region, has been assembled into complex V [288]. Attaching a *SOD2* 3'UTR to ATP6 and ND4 significantly improved fibroblasts' ability to grow

in galactose, which forces the cells to utilize OXPHOS to produce ATP. The growth rates are significant higher in the NARP fibroblasts that have both *SOD2* MTS and *SOD2* 3'UTR than those with *SOD2* MTS and SV40 3'UTR, suggesting that *SOD2* 3'UTR by itself can significant improve functional outcome (cell growth rate). There is also a significant increase in complex V activity in NARP flbroblasts with the allotopic expression of ATP6 [289]. We will examine these 3'UTRs that reportedly allow co-translational import in an *in vivo* setting. The 3'UTRs and the accession numbers are listed in Table 3. The transgenic fly strains generated to test strategies 2 and 3 are listed in Table 4.

Table 3. 3' Untranslated Region (3'UTR)

The parentheses in the first column indicate the origins of the 3’UTRs. The accession number of each 3’UTR is shown in the second column. The numbers in the parentheses in the second column indicate the start and end nucleotides.

3’UTR	Accession Number	Recoded?
SV40	EF362409.1 (5286-5985)	No
Sod2 (fly)	NM_057577.3 (802-862)	No
Oxa1 (yeast)	BK006939.2 (475992-476228)	No

Table 4. Codes of Transgenic Fly strains for Strategy 2 (MTS) and Strategy 3 (3'UTR)

The code is an abbreviation for the MTS, the *ATP6* gene, and the 3'UTR used in each construct.. The parentheses indicate the origins of the MTS and the 3'UTR. The ATP6 gene is recoded to optimize translation in cytosol following nuclear transcription.

Code	MTS	Gene	3'UTR
PAV	P1 (human)	recoded fly ATP6	SV40
sOAV	sOxa1 (yeast)	recoded fly ATP6	SV40
SAV	Sod2 (human)	recoded fly ATP6	SV40
DAV	Dld (yeast)	recoded fly ATP6	SV40
8AV	Cox8	recoded fly ATP6	SV40
noAV	No MTS	recoded fly ATP6	SV40
PAS	P1 (human)	recoded fly ATP6	Sod2 (fly)
sOAS	sOxa1 (yeast)	recoded fly ATP6	Sod2 (fly)
SAS	Sod2 (human)	recoded fly ATP6	Sod2 (fly)
DAS	Dld (yeast)	recoded fly ATP6	Sod2 (fly)
8AS	Cox8	recoded fly ATP6	Sod2 (fly)
noAS	No MTS	recoded fly ATP6	Sod2 (fly)
PAO	P1 (human)	recoded fly ATP6	Oxa1 (yeast)
sOAO	sOxa1 (yeast)	recoded fly ATP6	Oxa1 (yeast)
SAO	Sod2 (human)	recoded fly ATP6	Oxa1 (yeast)
DAO	Dld (yeast)	recoded fly ATP6	Oxa1 (yeast)
8AO	Cox8	recoded fly ATP6	Oxa1 (yeast)
noAO	No MTS	recoded fly ATP6	Oxa1 (yeast)

3.6.1.4 Strategy 4: Suboptimally-Encoded Codons

Protein transport into mitochondria has been studied for more than three decades [297-300] with a strong interest in whether post-translational or co-translational mechanism are used [301]. Tagging a mitochondrial targeting sequence (MTS) to a nonmitochondrial protein and adding it to purified and isolated mitochondria leads to efficient import of the nonmitochondrial protein [302], suggesting a post-translational mechanism. However, several lines of evidence suggests that co-translational mechanism is plausible as well. When cyclohexamide is added to yeast culture to arrest translation, a large number of ribosomes are found attached to the mitochondrial surface [297]. Once the mitochondrion-bound ribosomes are isolated, they are found to have more mRNAs for mitochondrial proteins attached to them than the free cytosolic ribosomes [303]. Further research shows that ribosomes bind at specific sites on mitochondria [300], and strongly suggests that ribosomes binds specifically at protein import sites [304, 305]. Therefore, it is possible that co-translational import and post-translational import mechanisms are used for different proteins. In fact, co-translational import and post-translational mechanisms might be used by the same protein [306]. According to this model, during the early phase translation has already been commenced on ribosomes and by the time the ribosomes reach the TOM complex the precursor protein translation is completed and the import process is assisted by a molecular chaperone. However, during the late phase when the ribosomes have already docked at the TOM complex the process switches to co-translational import [306]. If the algal ATP6 and the engineered ATP6 utilize co-translational import, a potential complication arises as translation is believed to be much more efficient than import [307], utilizing suboptimally-encoded codons may slow down translation and allow better synchronization of translation and import. The

sequence alignments of the recoded *ATP6* and suboptimally-recoded *ATP6* are shown in Figure 10. We have designed a number of transgenes to test this strategy (Table 5).

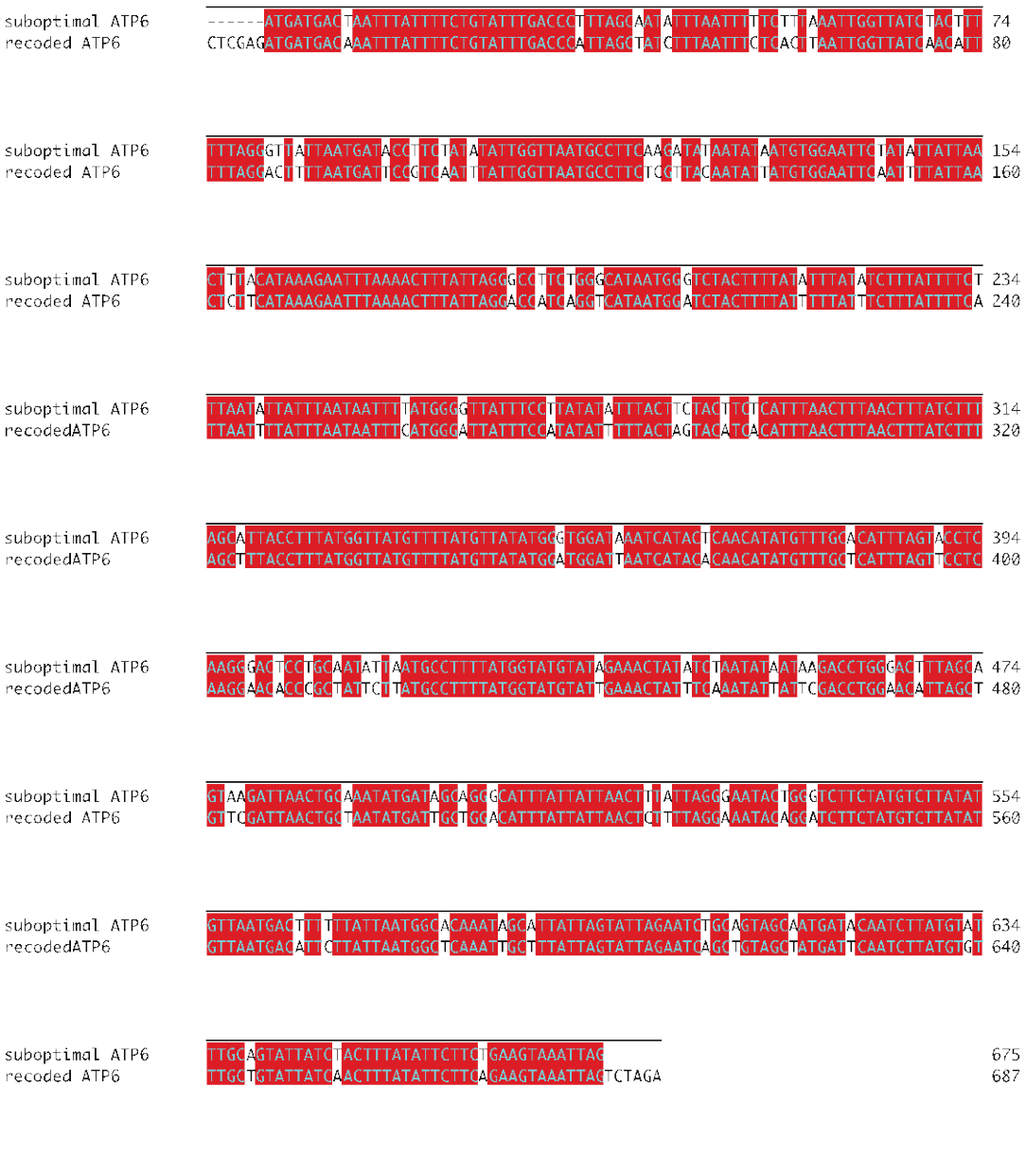

Figure 10. Nucleotide Sequence Alignments of Suboptimally-Encoded *ATP6* and Recoded *ATP6*.

The nucleotides that matched were shown in red. Notice that the amino acid translations for both sequences are identical.

Table 5. Codes of Transgenic Fly Strains for Strategy 4 (Suboptimally-Encoded Codons).

s.o.-recoded is suboptimally-encoded.

Code	MTS	Gene	3'UTR
SsoAS	SOD2 (human)	s.o.-recoded ATP6	SOD2 (fly)
NosoAS	No MTS	s.o.-recoded ATP6	SOD2 (fly)
DsoAS	DLD (yeast)	s.o.-recoded ATP6	SOD2 (fly)
SsoAO	SOD2 (human)	s.o.-recoded ATP6	OXA1 (yeast)
DsoAO	DLD (yeast)	s.o.-recoded ATP6	OXA1 (yeast)

3.6.1.5 Strategy 5: Mitochondrial Biogenesis Factors –PGC1-α, AMPK, and DSP1

Peroxisome proliferator-activated receptor (PGC1-α) was the first mitochondria biogenesis regulator to be discovered [308]. Cells overexpressing PGC1-α have increased expression of Cox II, Cox IV, ATP Synthase, and increased mtDNA content [308]. AMP-activated protein kinase (AMPK) plays a major role in mitochondrial biogenesis in chronic energy depletion [309, 310]. Rats fed with β-guuandinopropionic acid, a pharmacological AMPK activator, for 8 weeks have shown increased cytochrome c protein expression and mitochondrial content, suggesting that AMPK is involved in mitochondrial biogenesis [311]. Data argue that AMPK works upstream of PGC1-α and activates PGC1-α through direct phosphorylation [312]. AMPK also phosphorylates ULK1, a human ATG1 homolog, which is responsible for mitophagy [313] (see strategy 7). Therefore, AMPK controls mitochondrial homeostasis by increasing the production of new mitochondria and removing the old and defective mitochondria [314] The hypothesis for this strategy is that overexpressing AMPK or PGC1-α will increase the total number of mitochondria, which may help overcome the energy deficits caused by the *ATP6* mutation and improve the functional status of *ATP6[1]* mutant flies. DSP1 is a *Drosophila* gene that belongs to the High-Mobility Group Box (HMGB) family, and it has been shown that HMGB1 regulates heat shock protein beta-1 (HSPB1), which in turn regulated mitophagy after mitochondrial injury *in vitro* [315]. The mating scheme and transgenic strains are listed in Figure 11, Figure 12, and Figure 13 for PGC1-α, AMPK, and DSP1, respectively.

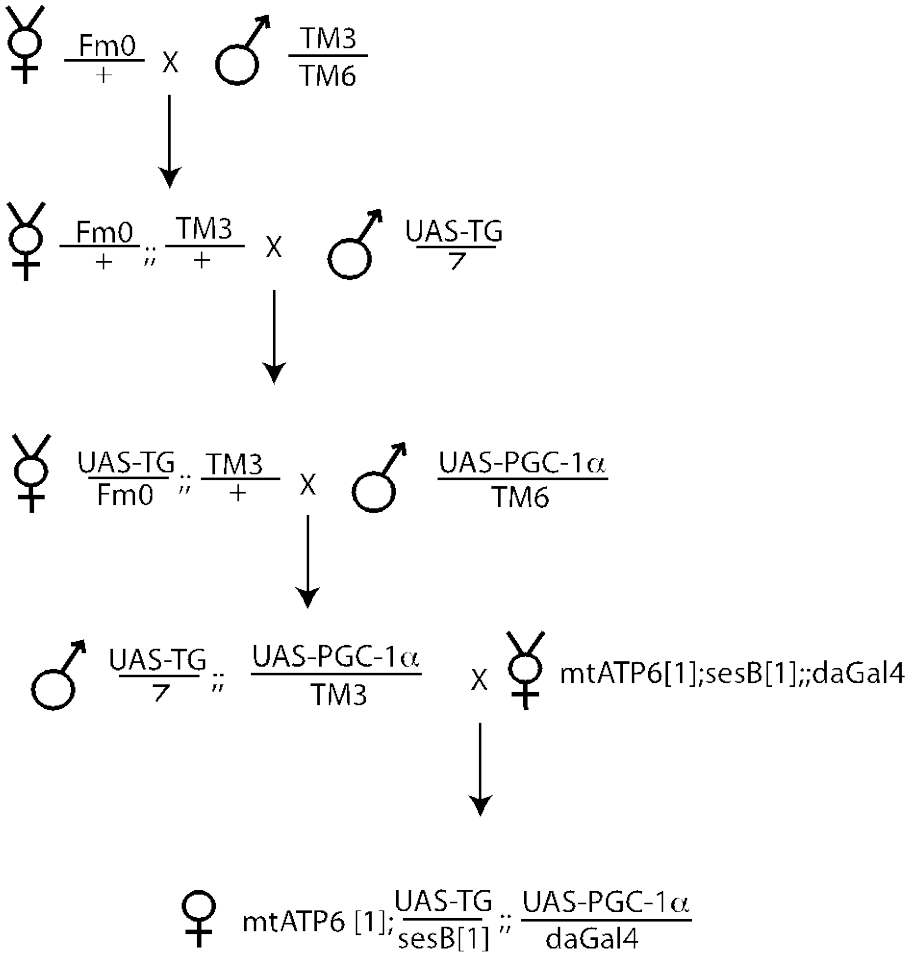

Figure 11. Generation of Transgenic Fly Strain with Nucleus-Encoded ATP6 and PGC-1α Overexpression.

Mating scheme to generate experimental group animals. FM0, TM3, and TM6 are balancers and served as phenotypic markers for screening flies.

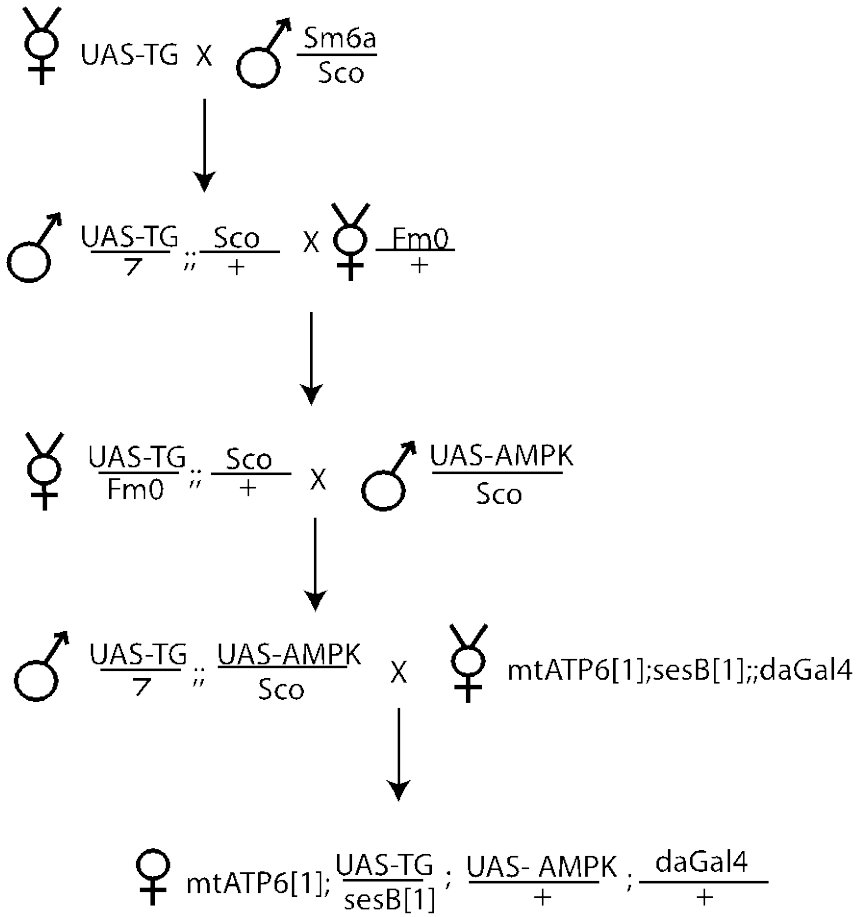

Figure 12. Generation of Transgenic Fly Strain with Nucleus-Encoded ATP6 and AMPK Overexpression.

Mating scheme to generate experimental group animals. SM6a, FM0, Sco, are balancers and served as phenotypic markers for screening flies.

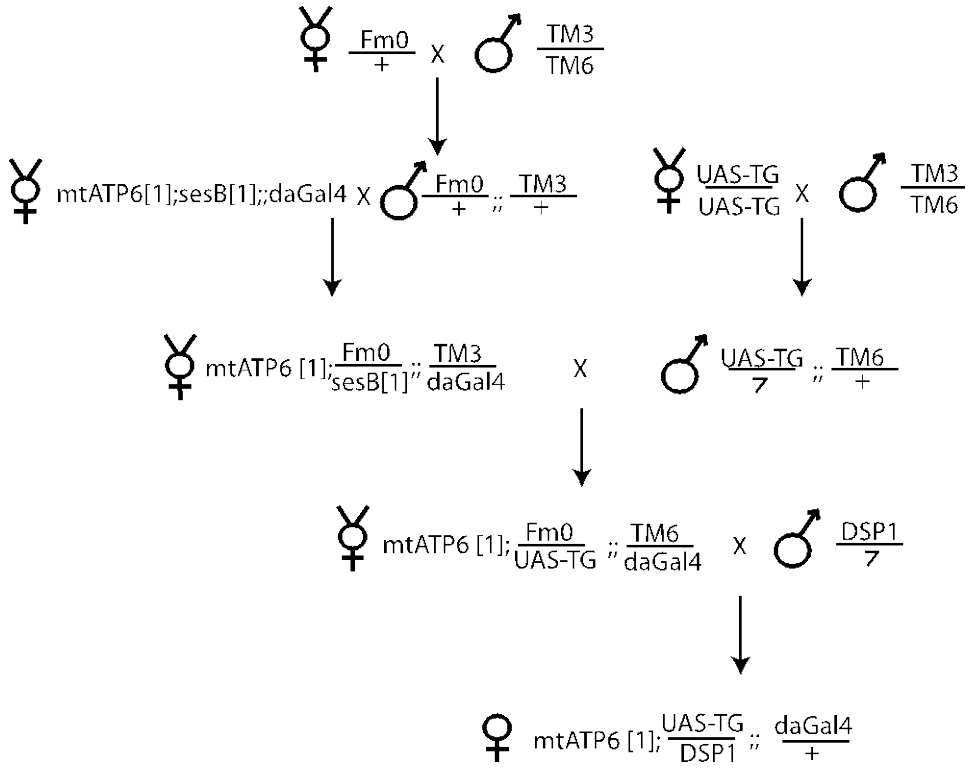

Figure 13. Generation of Transgenic Fly Strain with Nucleus-Encoded ATP6 and DSP1 Overexpression.

Mating scheme to generate experimental group animals. FM0, TM3, and TM6 are balancers and served as phenotypic markers for screening animals.

3.6.1.6 Strategy 6: Mitochondrial Autophagy Factor – ATG1

ATG1 is required for autophagy [316], a process that is important for survival, regulation, and development. It not only degrades old and misfolded proteins and aged or dysfunctioning organelles but also provides nutrients and energy when the cell experiences various stressors [317]. The hypothesis for this strategy is that overexpressing ATG1 may decrease the number of defective mitochondria and increase mitochondria turnover to compensate for the degradation. Such overexpression may improve the functional status of *ATP6[1]* mutant flies. The mating scheme is shown in Figure 14.

3.6.1.7 Strategy 7: Mitochondrial Protein Folding Factor – mtHSP70

As described in the previous section "Mitochondrial Protein Import", mtHSP70 is a member of the import motor component in the TIM complex [49]. mtHSP70 plays a dual role in precursor protein translocation and ATPase activity [318]. ATPase activity and binding of the precursor protein to mtHSP70 are essential for the unfolding of the precursor protein and complete protein translocation [319]. The hypothesis is that overexpressing mtHSP70 may help with the exogenous ATP6 protein translocation and this, in turn, will help improve the functional outcomes of the *ATP6[1]* mutant flies. The mating scheme and transgenic strains used are shown in Figure 15.

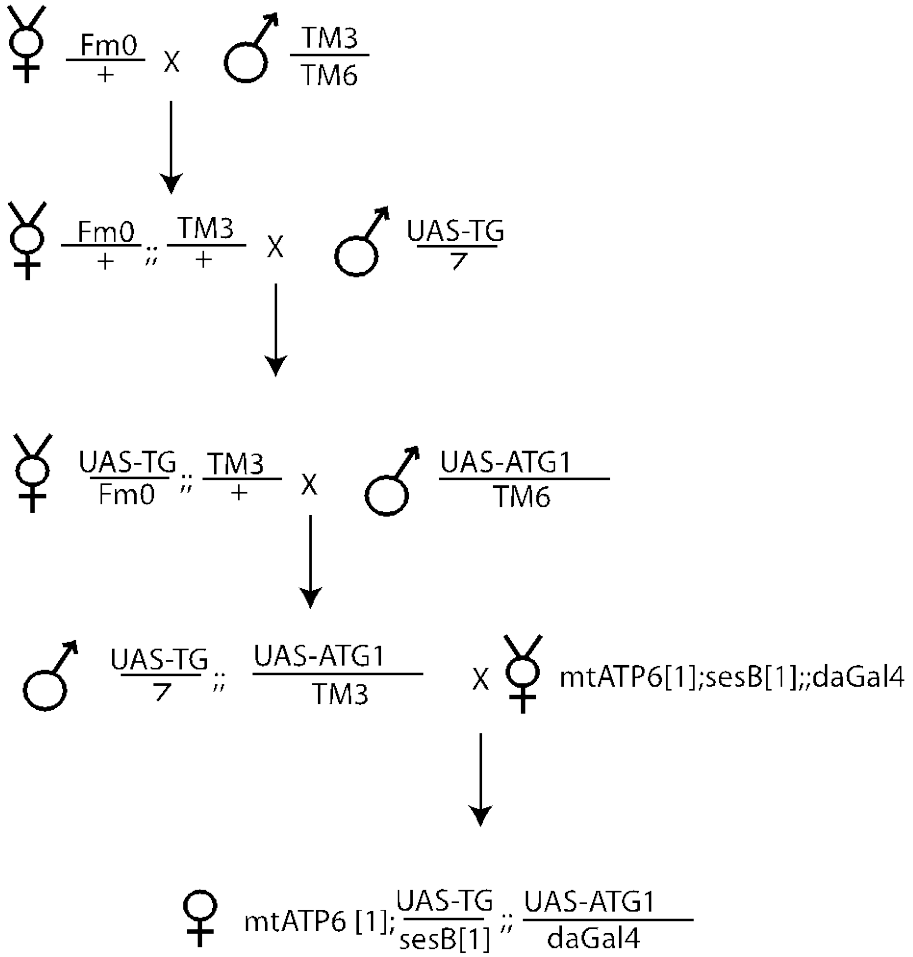

Figure 14. Generation of Transgenic Fly Strain with Nucleus-Encoded ATP6 and ATG1 Overexpression.

Mating scheme to generate experimental group animals. FM0, TM3, and TM6 are balancers and served as phenotypic markers for screening animals.

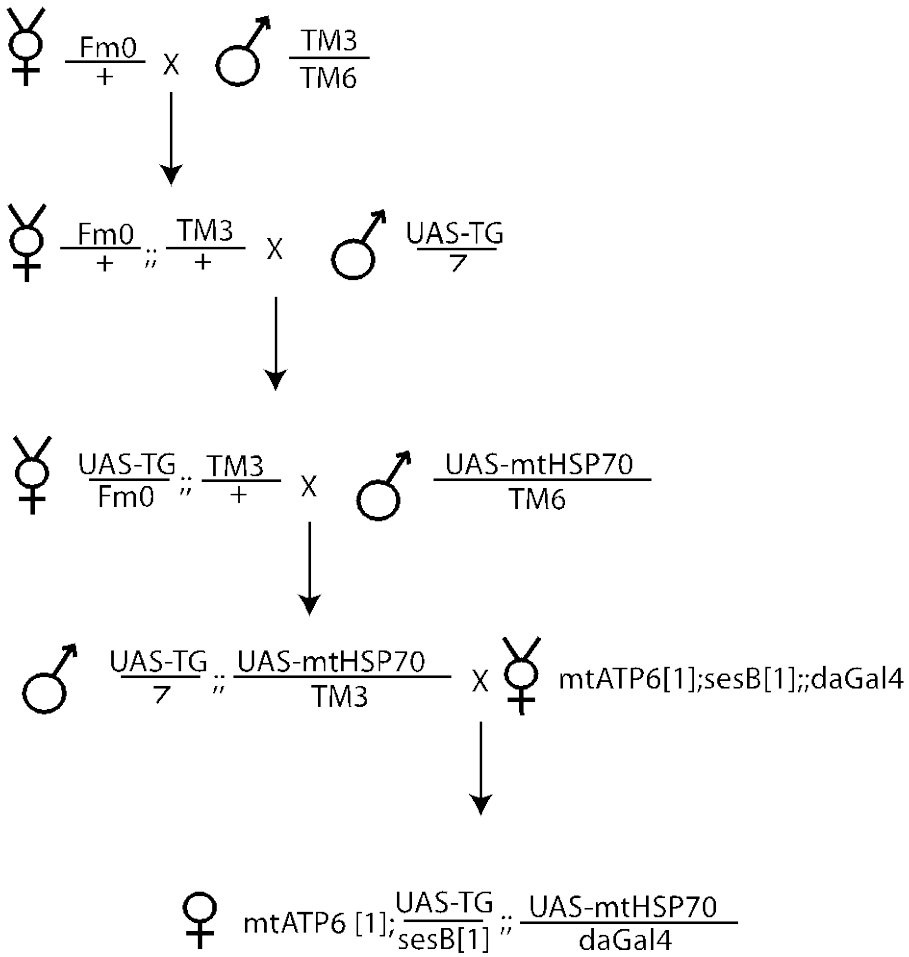

Figure 15. Generation of Transgenic Fly Strain with Nucleus-Encoded ATP6 and mtHSP70 Overexpression.

Mating scheme to generate experimental group animals. FM0, TM3, and TM6 are balancers and served as phenotypic markers for screening animals.

3.6.1.8 Strategy 8: Mitochondrial Protein Insertion Factor – OXA1

OXA1 is essential for inserting proteins from the matrix into the inner mitochondrial membrane [116, 117]. Given the characteristics of ATP6, it is hypothesized that nucleus-encoded ATP6 would utilize the conservative pathway, which requires OXA1. The hypothesis for this particular strategy is that overexpressing OXA1 may improve the efficiency of exogenous ATP6 protein insertion into the inner mitochondrial membrane, facilitate ATP synthase complex formation, and this will improve the functional outcome of the mutant flies. The mating scheme and transgenic strains used are shown in Figure 16.

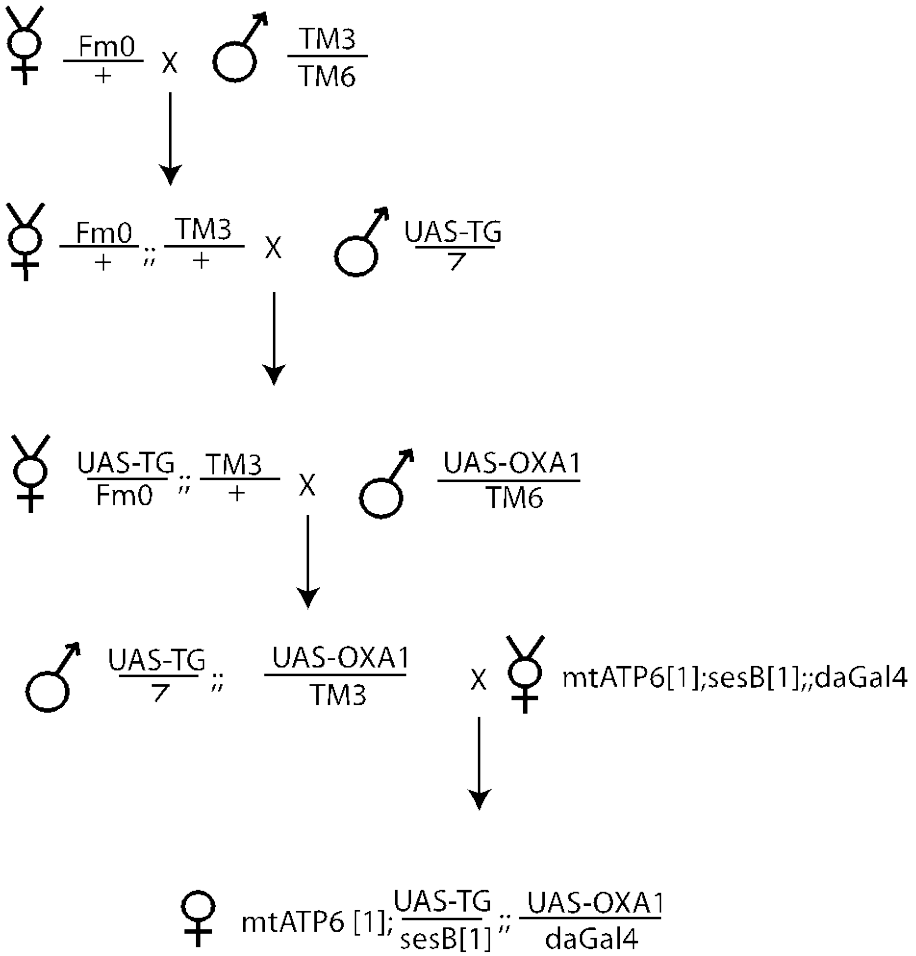

Figure 16. Generation of Transgenic Fly Strain with Nucleus-Encoded ATP6 and OXA1 Overexpression.

Mating scheme to generate experimental group animals. FM0, TM3, and TM6 are balancers and served as phenotypic markers for screening animals.

3.6.2 Challenge 2: Endogenous Mutant ATP6 Competition

Although the ATP synthase complex is dysfunctional, mutant ATP6 protein is still being translated in the mitochondria, suggesting there will be competition between the endogenous ATP6 protein and the exogenous ATP6 protein (Figure 17). Because the amount of endogenous ATP6 protein present will most likely be much higher than that of the amount of exogenous ATP6 protein imported, the exogenous ATP6 protein may not be incorporated into the ATP synthase complex successfully and in sufficient quantity to improve the lifespan and the locomotor function. In addition to competition, toxicity from total ATP6 protein overexpression could also be problematic. Therefore, a strategy to decrease the amount of endogenous ATP6 mutant protein may be necessary.

3.6.2.1 Strategy 9: Utilizing Translational Inhibitors (TLIs)

The Palladino lab has recently designed and developed a mitochondrial-targeted RNA expression system (*mtTRES*) that expresses small chimeric RNAs that function as translational inhibitors (TLIs). It consists four components: 1) RNA Polymerase (RNAP) III promoter; 2) Non-coding leader sequence (NCL), a structured RNA or portion thereof that is expressed in the nucleus but is found readily within the mitochondria (5S rRNA, MRP or RNP); 3) a sequence that is complementary to the endogenous mRNA to prevent the docking of the mitochondrial ribosome; and 4) the RNAP III termination element (Figure 18). This system has been tested with three different NCLs (5s, MRP, RNP) and two different target sequences (*ATP6* and *CoxII*) and effectively decreases the translation of endogenous proteins (Towheed et al., submitted). We will

be utilizing the *mtTRES ATP6*-TLI system in conjunction with our allotopic ATP6 expression to determine whether this will improve the function of our allotopic constructs. The mating scheme and transgenic strains used are shown in Figure 19.

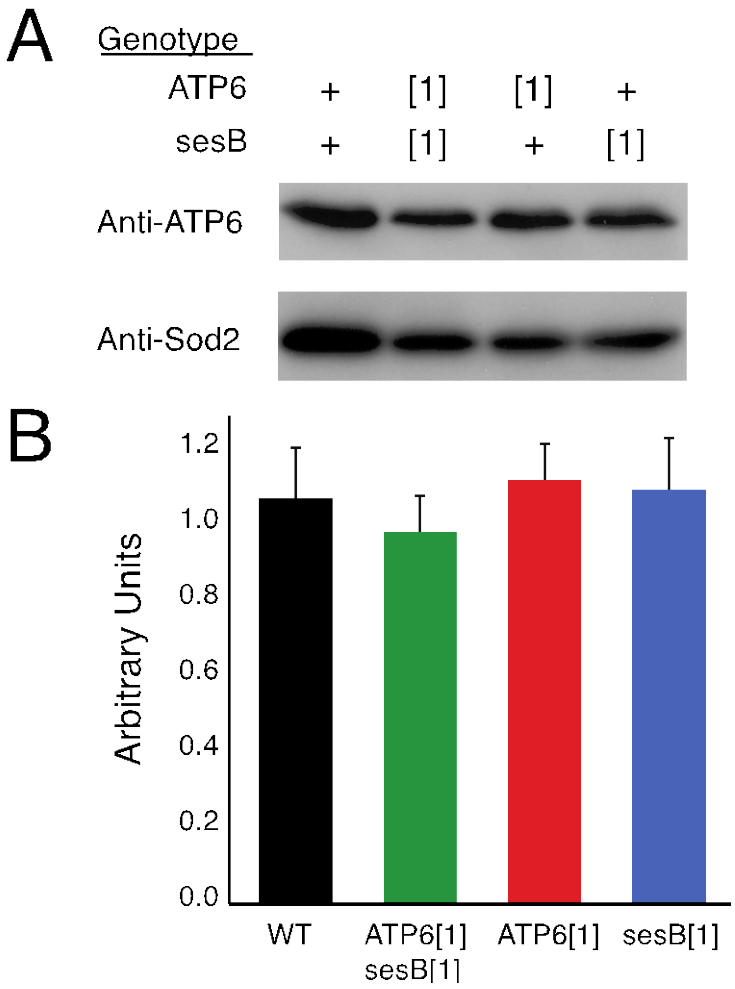

Figure 17. Endogenous Mutant ATP6 Protein Expression Levels.

ATP6 protein levels measured in WT flies (denoted as *ATP6+* and *sesB+*), *ATP6[1];sesB[1]*, *ATP6[1]*, and *sesB[1]* flies by Western Blot. ATP6 protein level was normalized to Sod2, a mitochondrial protein, as loading control. **(A)** Representative Western Blot. **(B)** Quantitative analysis of normalized ATP6 expression levels from WT and *ATP6* mutant flies. Figure was produced by Dr. Alicia Celotto and permission was granted to be used in this dissertation.

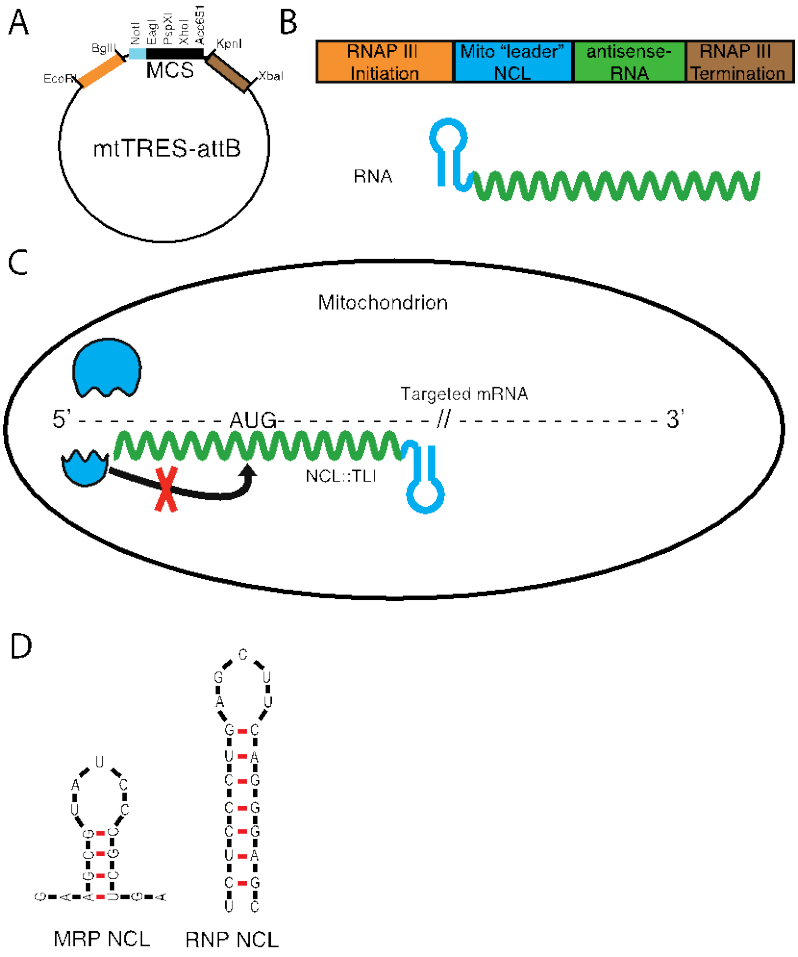

Figure 18. RNA-based mitochondrial Translational Inhibitor (TLI) approach.

(A) Mitochondria-targeted RNA expression TLI system (*mTRES*) vector used to knock down endogenous mutant ATP6 expression. **(B)** The four components of the TLI construct. RNAP III initiation (orange) and termination sequences (brown), non-coding leader sequence (NCL) (blue), and anti-sense RNA (green), which is complementary to the target mRNA. **(C)** TLI functions by preventing the docking of mitochondrial ribosomes and decreasing the rate of translation. **(D)** The structures of the two NCLs, MRP and RNP, used in this experiment. Figure used with permission from Towheed et al. (submitted).

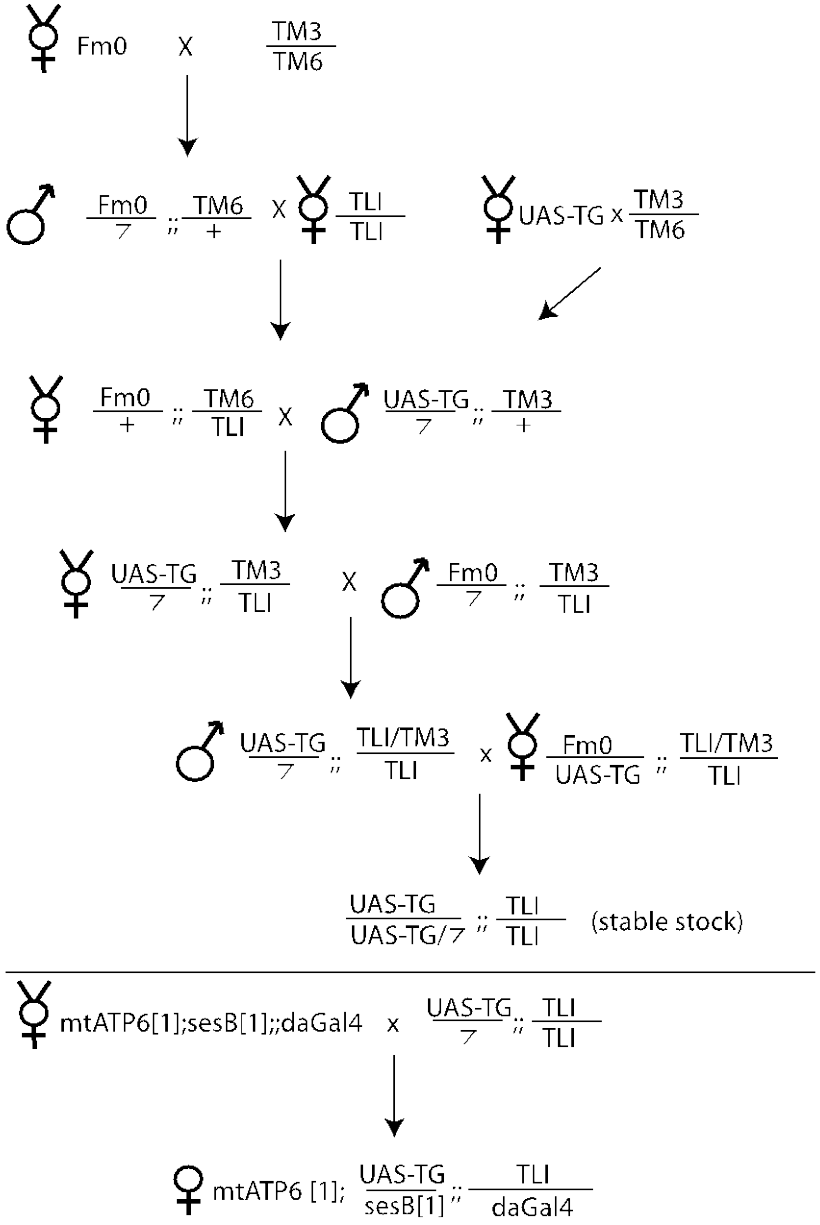

Figure 19. Generation of Transgenic Fly Strain with Nucleus-Encoded ATP6 and Translational Inhibitor (TLI).

Mating scheme to generate experimental group animals. FM0, TM3, and TM6 are balancers and served as phenotypic markers for screening animals.

4.0 MATERIALS AND METHODS

***Drosophila melanogaster* Stocks and Maintenance** *Drosophila* stocks were maintained on standard cornmeal molasses fly media. *ATP6[1]* mutants were isolated from a *sesB[1]* background [271]. The absence of such mutation would be denoted as either *sesB[+]* or simply *[+]*. Virgin *ATP6[1];sesB[1]* was mated to male *daGal4* and homozygous animals *ATP6[1];sesB[1];;daGal4* were collected after sequence confirmation. *ATP6[1];sesB[1];;daGal4* was the main stock used throughout our experiments. All stocks were obtained from Bloomington Stock Center unless indicated otherwise. PGC-1α was obtained from Dr. David Walker, UCLA. Canton S flies are used as WT. All of the UASB-TGs were maintained in homozygous states and flipped on a regular schedule.

Engineering Transgenic Constructs The pUASTattB (UASB) transgenesis system was utilized [320]. A modified pUASTattB strain was generated so that all of the constructs could be generated from a few precursor clones (Figure 20). The accession numbers of all MTSs and UTRs were listed (Table 2-3). Full-length transgene cDNA was PCR amplified and cloned into pUASB to generate pUASB-TG at the attB site.

***Drosophila* Transgenesis** The vector bearing engineered *ATP6* construct underwent site-directed PhiC31 mediated attP/B transgenesis (Figure 21). For all of the transgenes, the *attp18* insertion

site was used except *OXA1*, which utilized *VK00027* attP, and *mtHSP70*, which utilized *attP2*. The *attp18* insertion site is located on the first chromosome. *VK00027* and *attp2* are located on the third chromosome. Genetic Services was used for DNA injection (Cambridge, MA, USA) and successfully transgenesis was identified through the presence of *white*$^{mc+}$.

Translational Inhibitor (TLI) Expression in Transgenic Flies. RNA complementary to the endogenous *ATP6* sequence was designated as TLI. TLI was expressed to prevent the docking of mitochondrial ribosomes and decrease the translation of endogenous ATP6 proteins. Two TLI constructs using *MRP* or *RNP* elements, were tested and each was expressed in transgenic flies in conjunction to the expression of algal *ATP6* gene. Both *MRP* and *RNP* were inserted at the *VK00027* attp site on chromosome 3. The *TLI-MRP::ATP6* sequence is AGAAGCGTATCCCGCTGAGCGAAAATAAATTTGTTATCATTTTCA and the *TLI-RNP::ATP6* sequence is TCTCCCTGAGCTTCAGGGAGGAAAATAAATTTGTTAT-CATTTTCA. The underlined sequence is the respective noncoding leader sequence (NCL) and the rest is the *ATP6* complementary sequence. *RNP* is noted as *RNaseP* in Figure 38. *VK00027* is abbreviated as *VK27* when used in genotypes and figures in this dissertation. *ATP6[1];sesB/+;;VK27/daGal4* and *ATP6[1];NoCrAO/sesB;;VK27/daGal4* were used as controls when compared to *ATP6[1];NoCrAO/sesB;;MRP/daGal4* and *ATP6[1];NoCrAO/sesB;;RnaseP/daGal4*, the test strains with expression of both algal *ATP6* transgene and the TLI.

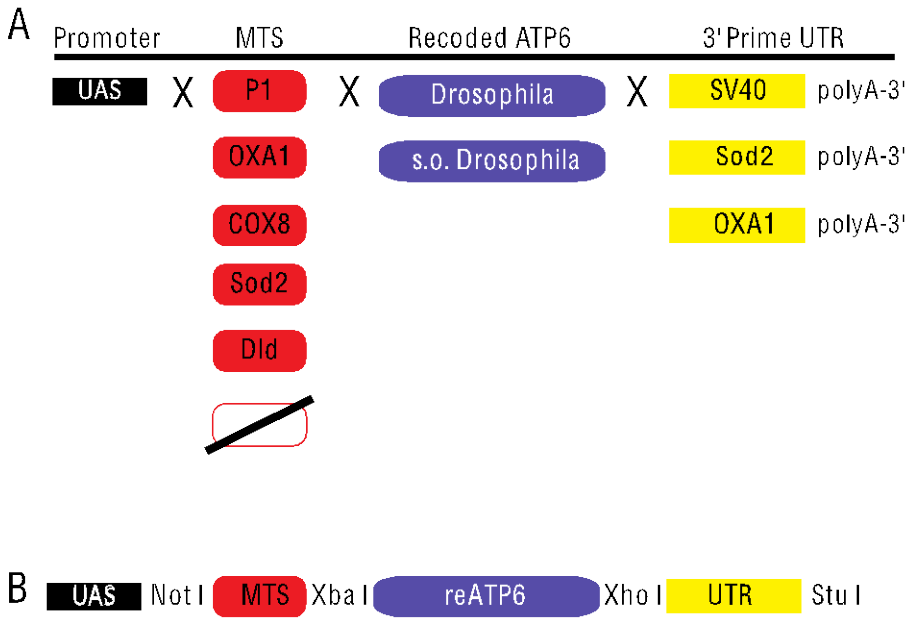

Figure 20. Generation of UAS Constructs.

Determining the optimal transgenic construct of MTS, recoded *ATP6*, and 3'UTR for allotopic expression. *pUAST-attB* transgenesis system and Phi31 integrase system was used to allow transgenes be inserted in identical sites (attp). The promoter UAS was activated by established Gal4 lines such as daughterless-Gal4 or ubiquitous-Gal4. **(A)** Different MTSs (red) combined with recoded *ATP6* (purple) and 3'UTR (yellow) to form different transgenic fly strains. s.o. is suboptimally-encoded. **(B)** Unique cloning sites were designed so all these different transgenes could be generated from a few initial clones. Figure usage was granted by Dr. Michael Palladino.

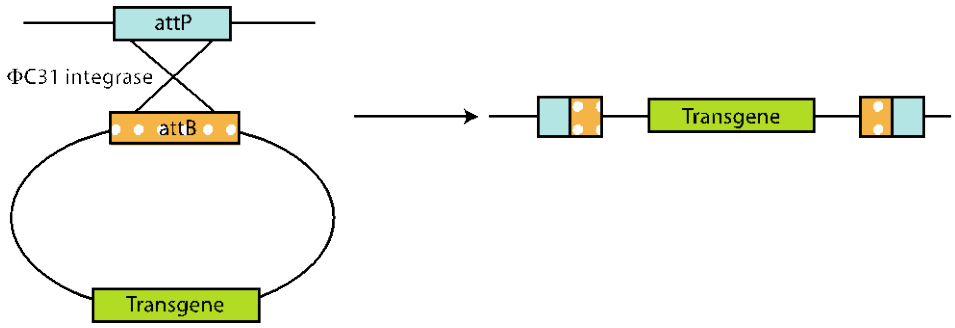

Figure 21. attP/B Transgenesis with PhiC31 Integrase.

Transgenesis involves integration using a specific attP site on chromosome. Once the transgene was integrated it remains at the site stably because the hybrid site cannot undergo integrase-catalyzed mobilization. Figure modified from Fish MP, Groth AC, Calos MP, Nusse R. Nat Protoc. 2007;2(10):2325-31 [321].

Analysis of ATP6 Polypeptide Sequences ATP6 protein sequences of *Homo sapiens, Mus musculus, Drosophila melanogaster, Aedes aegypti,* and *Chlamydomonas reinhardtii* were obtained from NCBI Protein Database. The sequences were aligned with software DNASTAR MegAlign (Version 9.10 (109) Intel) by Clustal W method.

Lifespan and Behavioral Analysis Flies were collected under light carbon dioxide-induced anesthesia within 24 hours of eclosion and were kept on standard cornmeal molasses medium at a density of ~15 animals per vial. All flies were kept at a humidified, temperature-controlled incubator at either 25°C or 29°C. Flies were transferred to fresh vials every other day and scored for death. Lifespan was analyzed by Log-Rank Test (Prism V5.0b). ~ 45 animals per genotype were used to complete lifespan test. Mechanical sensitivity / stress test was assayed by vortexing the flies on a tabletop vortexer (Vortex Genie 2, Scientific Industries, Inc. New York, USA) in a standard vial for 20 seconds [322, 323]. The recovery time was measured as the time between the end of the vortexing treatment and the first normal movement (walking forward). The recovery time was capped at a maximum of 300 seconds. Stress tests were performed on Day 16 and Day 20 for animals housed at 25°C and on Day 8 and Day 12 for animals housed at 29°C. 1-way ANOVA (Kruskal-Wallis Test) was used for statistical analysis within group. Dunn's Multiple Comparison Test was used to determine the statistical significance of the recovery time of each genotype.

Strobe Light Test The protocol has been published in [209] and is excerpted here. Flies were recoded using PAX-it version 6 software (Midwest Information Systems, InC. Villa Park, IL)

through a PAXCAM (camera) mounted on a ZEISS dissection microscope (W.E.L. Instrument Co). Flies were transferred from food vials to new 35mm petri dish and were allowed to rest for 20 minutes before filming. Flies were recorded for 5 minutes before they were subjected to strobe lighting (SHIMPO, Itasca, IL) at a frequency of 1450 flashes per minute for 20 seconds. Filming continued for 5 minutes after strobe light treatment. Video analysis was performed using iMovie (Apple, Cupertino, CA). The recovery time was measured as the time between the end of the strobe light treatment and the first normal locomotor activity (i.e. walking forward or grooming). A two-tailed Mann-Whitney U test was used for statistical analysis at day 25.

Western Blot for Algal-*Drosophila* Chimeras Five thoraces of each genotype (CH1S, CH2S, CH3S, CH4S, CH5S, NoCrAO and Canton-S wild type) were collected and homogenized in 100ul denaturing SDS buffer. The homogenized solution was heated to 98°C for 10 minutes and 30ul were loaded onto a 12% SDS-page gel. The proteins were transferred to a 0.2um PVDF transfer membrane (Millipore, MA, USA) and then blocked with a 1% PBS-Tween-Milk solution for 2 hours. The membrane was then incubated with 1:2000 rabbit anti-fly ATP6 primary antibody, which was generated using purified HKEFKTLLGPSGHNGS peptide (NEOBioSci, MA, USA) overnight for 16 hours. The membrane was washed before incubating with 1:5000 goat-anti-rabbit secondary antibody (Biorad, USA) for two hours. The membrane was washed, incubated with ECL chemiluminescent substrate for detection of HRP (Pierce, Thermo Scientific, Illnois, USA), and developed. Mouse-anti-*Drosophila* β-tubulin primary antibody (1:4000) (Santa Cruz Biotechnology, Texas, USA) was used as a loading control.

Mitochondria Purification This procedure was modified from a published protocol [324]. Forty dissected thoraces per genotype were collected and homogenized with a plastic pestle in cold mitochondrial isolation medium (MIM: 250mM sucrose, 10mM Tris-HCl, 0.15mM MgCl2, pH 7.4) with protease inhibitor mix. The homogenized mixture was centrifuged at 500xg at 4°C for 5 minutes twice to remove debris. The supernatant was then centrifuged at 5000xg for 5 minutes to form a pellet containing mitochondria. The pellet was washed or resuspended in either a denaturing SDS buffer for Western Blot or an assay buffer for ATP synthase assay.

ATP Synthase Assay. The procedure was modified from the protocol as described in [325] and.[326]. Briefly, 200 whole flies were homogenized using 40ml glass dounce tissue grinders (Kimble Chase Kontes, Fisher Scientific). Cellular debris was removed by centrifuging at 1300g x 5 minutes. Mitochondria were pelleted by 20,000g x 10 minutes. Ficoll gradient (15%, 23%, and 50%) was used to isolate mitochondria and spinned at 30,700g for 6 minutes. The mitochondria were permeabilized by digitonin and energized by ADP, malate, and puryvate. A luceferin and luciferase-based assay was used to determine ATP synthase activity.

Statistical Analysis. Statistical software Prism Pad version 5 was used to conduct all statistical analyses. Lifespan was analyzed by Log-Ranked Test. Mechanical stress test was analyzed by one-way ANOVA (Kruskal-Wallis test). Dunn's Multiple Comparison Test was used to determine the statistical significance of the recovery time of each genotype.

5.0 RESULTS

5.1 PHENOTYPIC CHARACTERLIZATION OF *ATP6[1]* MUTANT ANIMALS

Our goal was to determine if allotopic expression of ATP6 would rescue the mutant *ATP6[1]* animals. To that end, we utilized functional outcomes such as lifespan and stress test recovery time to evaluate the efficacies of our strategies against hydrophobicity and endogenous mutant ATP6 competition. The lifespan of mutant female *ATP6[1];sesB[1]/+;;daGal4* and WT female *ATP6[+];sesB[1]/+;;daGal4* were examined (Figure 22). The average lifespan for *ATP6[1];sesB[1]/+;;daGal4* was 23 days and the average lifespan for *ATP6[+];sesB[1]/+;;daGal4* female was 89 days. Upon stress test, the mean recovery time for mutant female was 221 seconds on Day 16 and 294 seconds on Day 20 while the mean recovery time for WT female was 9 seconds on Day 16 and 5 seconds on Day 20 (Figure 23). To examine the effect of strobe light on the behaviors of *ATP6[1];sesB[1]/+;;daGal4* and *ATP6[+];sesB[1]/+;;daGal4*, the animals were subjected to strobe light stimulation for 20 seconds and the recovery time was measured. The average recovery time at Day 5, Day 13, and Day 20 was comparable between *ATP6[1];sesB[1]/+;;daGal4* and *ATP6[+];sesB[1]/+;;daGal4*, but a significant difference between the two genotypes was observed on Day 25 (Figure 24). Convulsive and paralytic behaviors were observed in *ATP6[1];sesB[1]/+;;daGal4* but not in *ATP6[+];sesB[1]/+;;daGal4* on Day 25. Videos of

behavioral analysis under strobe light stimulations are available at http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0025823

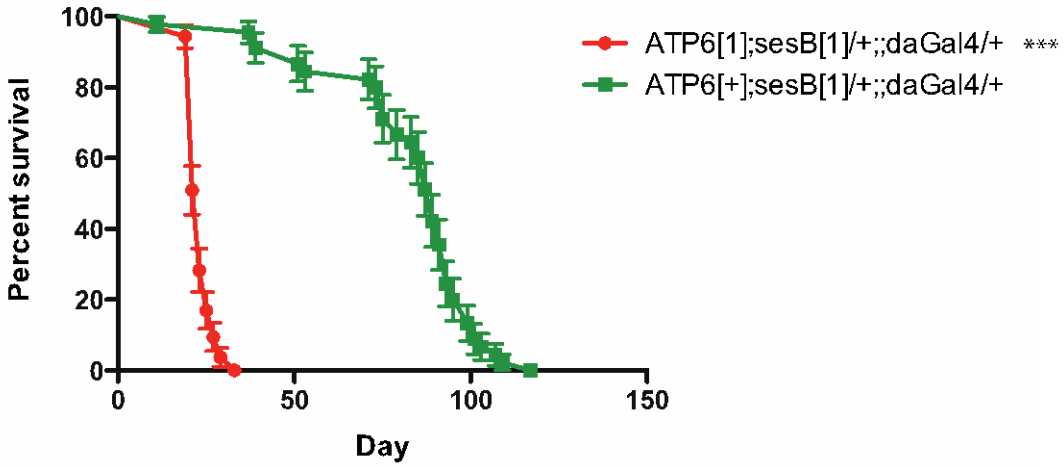

Figure 22. Lifespan of *ATP6[1]* Mutants and *ATP6[+]* WT Incubated at 25ºC.

The lifespan for mutants is shown in red and the lifespan for WT is shown in green. Median survival for *ATP6[1]* mutants = 23 days; n=53. Median survival for WT = 89 days; n=45. $p<0.0001$, Log-rank test.

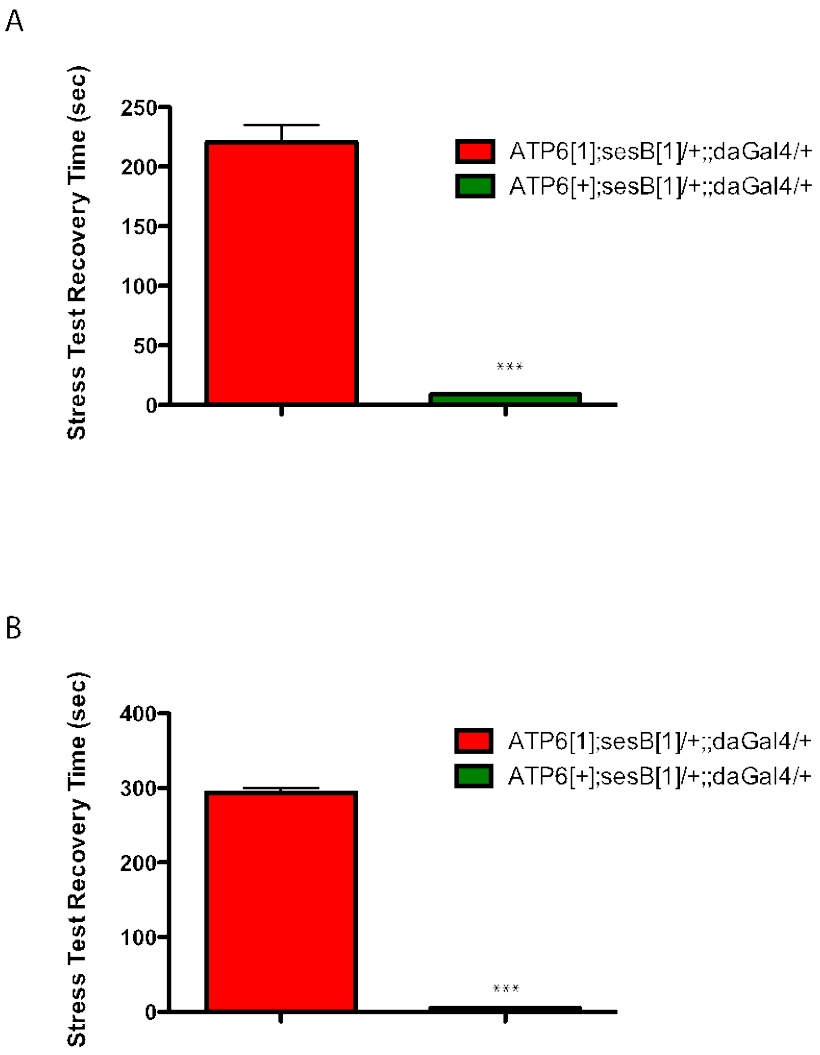

Figure 23. Mechanical Stress Test Recovery Time of *ATP6[1]* Mutants and *ATP6[+]* WT.

Flies were incubated at 25ºC and mechanical stress tests were conducted on **(A)** day 16 and **(B)** day 20. The mechanical stress test recovery time for mutants is shown in red and for WT shown in green. Average recovery time on day 16 was 221 seconds for *ATP6[1]* mutants (n= 16) and 9 seconds for WT (n=15); $p<0.0001$, unpaired t-test. Average recovery time on day 20 was 294 seconds for *ATP6[1]* mutants (n=10) and 5 seconds for WT (n=14); $p<0.0001$, unpaired t-test.

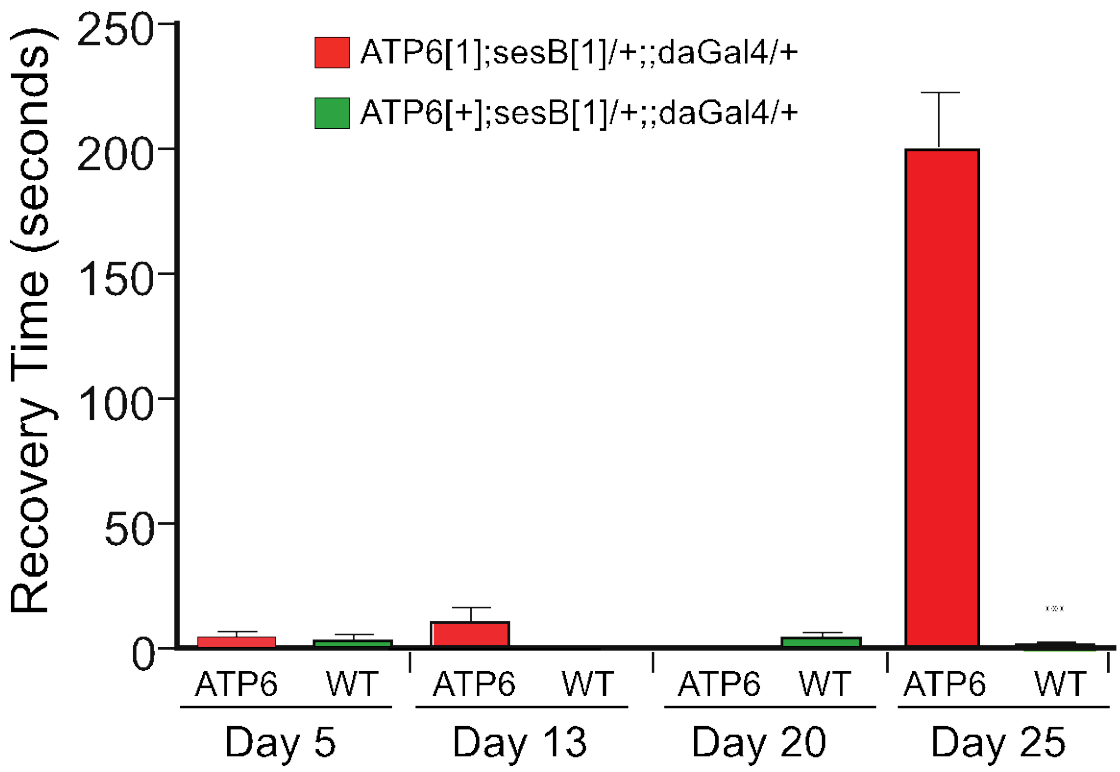

Figure 24. Strobe Light Recovery Time of *ATP6[1]* Mutants and *ATP6[+]* WT.

Flies were incubated at 25ºC and tested on the designated post-eclosion day. The strobe light recovery time of *ATP6[1]* mutants is shown in red and the strobe light recovery time of WT is shown in green. $p<0.0001$ on day 25, unpaired t-test.

5.2 STRATEGY 1: ALGAL ATP6 SUBUNIT

Functional Assays for Full-Length Algal Transgenic Flies

The hypothesis for strategy 1 was that expressing algal ATP6 protein would lead to improvement in the functional assays of *ATP6[1]* mutant flies because algal ATP6 protein was naturally imported from the cytosol to the mitochondria. Transgenic male flies with *NoCrAV*, *NoCrAS*, or *NoCrAO* were mated to either virgin female *ATP6[1];sesB[1];;daGal4* or virgin female *ATP6[1];ubiGal4.* Their female offspring were used for functional assays. *ATP6[1];attp18/sesB[1];;daGal4* and *ATP6[1];attp18;ubiGal4* were used as controls for flies driven by *daGal4* and flies driven by *ubiGal4*, respectively. The lifespan and stress test for transgenic flies driven with *daGal4* are shown in Figure 25 and Table 6-8. All three algal transgenic fly strains showed significant increase in lifespan at 25°C and improved recovery time on day 16 at 25°C. Transgenic strain NoCrAO performed the best as it also showed a significant increase in lifespan at 29°C and improved stress test recovery time on day 20 at 25°C and day 12 at 29°C. The lifespan and stress test for transgenic flies driven with *ubiGal4* are shown in Table 9-11. Transgenic flies driven by *ubiGal4* exhibited recurrent seizure-like activities after initial recovery post-mechanical stress test, making it difficult to ascertain accurate recovery time. Therefore, transgenic flies driven by *daGal4* were used for the follow-up experiments in strategy 1.

Functional Assays for Algal –*Drosophila* Chimera *ATP6* Flies

Because transgenic flies with algal transgenes displayed improved lifespan and stress test recovery time, we were interested in determining the crucial regions of the algal and fly ATP6 proteins that provide phenotypic improvements. We generated five algal-*Drosophila* chimera transgenic strains (Figure 26A). Functional assays were performed (Figure 26B-E, Table 12-15). All the algal-chimera transgenic strains had significantly improved lifespan at 25°C and 29°C. Importantly, flies with CH2S, CH4S, and especially CH5S algal-chimera transgenes had significantly longer lifespan comparing to full-length NoCrAS. Three of the five algal-chimera transgenic strains had significantly decreased stress test recovery time on day 16 at 25°C and all five had significantly decreased test recovery time on day 20 at 25°C.

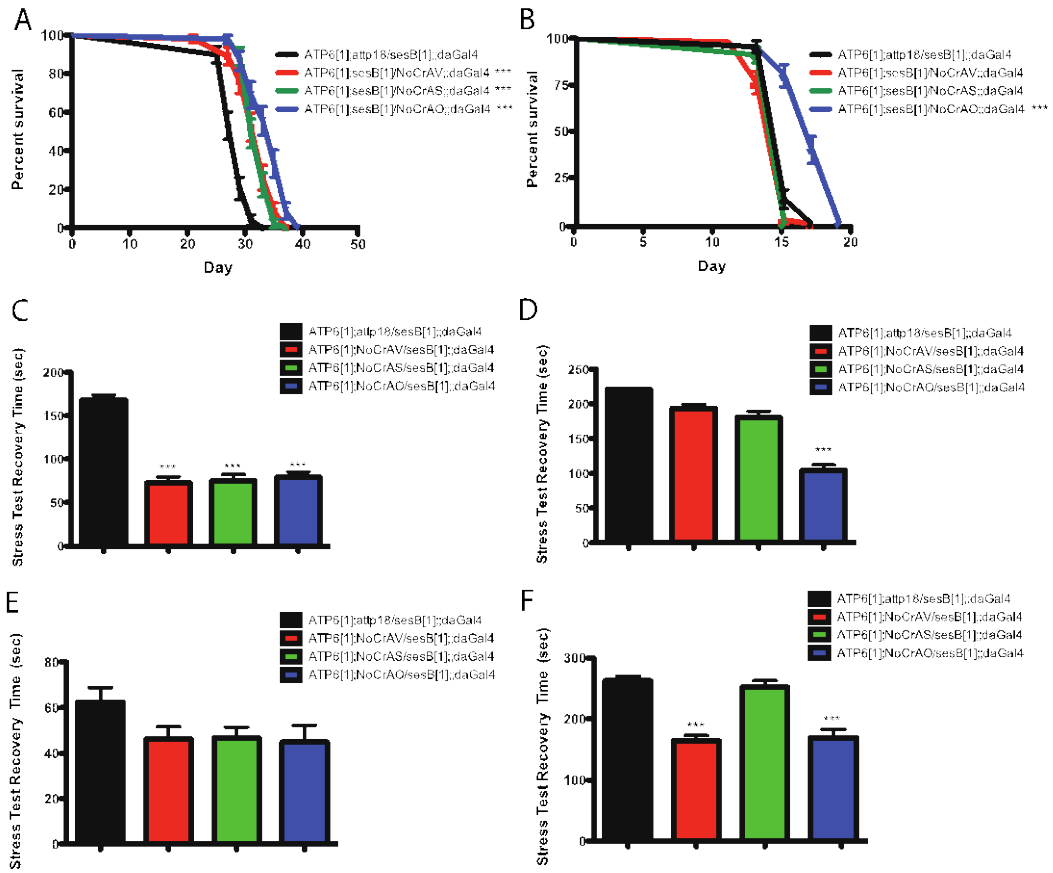

Figure 25. Functional Assays for Flies Expressing Full-Length Algal Transgenes.

Lifespan and mechanical stress test recovery time of *Drosophila* with nuclear-encoded algal *ATP6* transgenes driven by daughterless-Gal4 (daGal4). Lifespan of transgenic flies incubated at **(A)** 25ºC and **(B)** 29ºC. Mechanical stress test recovery time at 25ºC on **(C)** day 16 and **(D)** day 20. at 29ºC on **(E)** day 8 and **(F)** day 12.

Table 6. Lifespan of algal Transgenic Flies driven by daughterless-Gal4.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18/sesB[1];;daGal4* (control).

Code	Maximum Lifespan	Median Lifespan	% of Control	N	p-value
25°C					
attp18	33	29	-	49	-
NoCrAV	37	33	114%	46	<0.0001
NoCrAS	37	31	107%	45	<0.0001
NoCrAO	39	35	121%	45	<0.0001
29°C					
attp18	17	15	-	45	-
NoCrAV	17	15	0%	50	0.0013
NoCrAS	15	15	0%	45	0.0194
NoCrAO	19	17	113%	45	<0.0001

Table 7. Mechanical Stress Test Recovery Time of Algal Transgenic Flies Driven by daughterless-Gal4 at 25°C.

One-way ANOVA Kruskal-Wallis Test and Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Recovery Time	SE	% of Control	N	p-value <0.05?
Day 16 at 25°C					
attp18	168.2	5.8	-	17	-
NoCrAV	72.8	6.8	43%	16	Yes
NoCrAS	75.5	7.0	45%	15	Yes
NoCrAO	79.0	6.5	47%	15	Yes
Day 20 at 25°C					
attp18	221.0	0.17	-	9	-
NoCrAV	193.6	5.7	88%	16	No
NoCrAS	181.1	8.5	82%	15	No
NoCrAO	104.6	7.9	47%	15	Yes

Table 8. Mechanical Stress Test Recovery time of Algal Transgenic Flies Driven by daughterless-Gal4 at 29°C.

One-way ANOVA Kruskal-Wallis Test and Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Recovery Time	SE	% of Control	N	p-value <0.05
Day 8 at 29°C					
attp18	62.6	6.1	-	16	-
NoCrAV	46.1	5.6	74%	16	No
NoCrAS	46.6	5.0	74%	16	No
NoCrAO	44.8	7.5	72%	16	No
Day 12 at 29°C					
attp18	262.7	7.5	-	16	-
NoCrAV	164.1	8.7	62%	15	Yes
NoCrAS	251.9	10.8	96%	15	No
NoCrAO	169.7	13.3	65%	16	Yes

Table 9. Lifespan of Algal Transgenic Flies Driven by ubiquitous-Gal4.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18;ubiGal4* (control).

Code	Maximum Lifespan	Median Lifespan	% of Control	N	p-value
25°C					
attp18	39	37	-	47	-
NoCrAV	43	38	103%	46	0.0003
NoCrAS	41	37	0%	45	0.0093
NoCrAO	45	41	111%	47	<0.0001
29°C					
attp18	21	19	-	45	-
NoCrAV	21	17	89%	50	<0.0001
NoCrAS	19	17	89%	46	<0.0001
NoCrAO	21	19	100%	44	ns

Table 10. Mechanical Stress Test Recovery Time of Algal Transgenic Flies Driven by ubiquitous-Gal4 at 25°C.

One-way ANOVA Kruskal-Wallis Test and Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18;ubi-Gal4* control.

Code	Recovery Time	SE	% of Control	N	p-value <0.05?
Day 16 at 25°C					
attp18	101.4	12.1	-	16	-
NoCrAV	53.4	4.2	53%	16	Yes
NoCrAS	37.1	6.7	37%	16	Yes
NoCrAO	59.3	4.9	58%	16	No
Day 20 at 25°C					
attp18	146.2	4.0	-	16	-
NoCrAV	104.2	8.6	71%	16	Yes
NoCrAS	79.4	9.3	54%	16	Yes
NoCrAO	93.8	10.5	64%	16	Yes

Table 11. Mechanical Stress Test Recovery Time of Algal Transgenic Flies Driven by ubiquitous-Gal4 at 29°C.

One-way ANOVA Kruskal-Wallis Test and Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18;ubi-Gal4* control.

Code	Recovery Time	SE	% of Control	N	p-value <0.05?
Day 8 at 29°C					
attp18	15.3	2.9	-	16	-
NoCrAV	11.7	3.1	76%	16	No
NoCrAS	23.2	4.2	152%	16	No
NoCrAO	11.4	4.5	75%	16	No
Day 12 at 29°C					
attp18	167.7	17.7	-	10	-
NoCrAV	96.8	9.0	58%	16	Yes
NoCrAS	82.9	7.8	49%	16	Yes
NoCrAO	70.6	6.5	42%	16	Yes

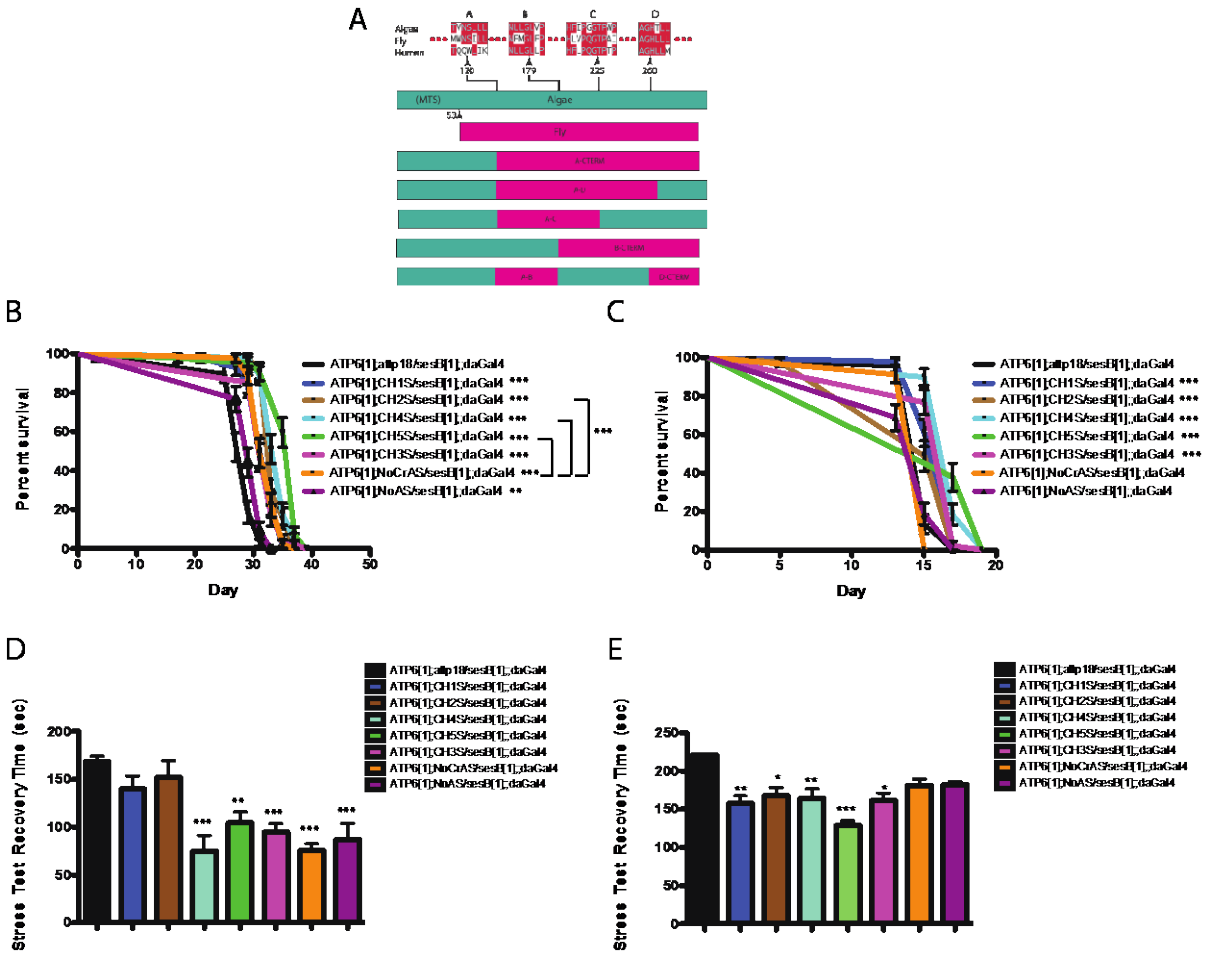

Figure 26. Constructs of Algal Chimeras and Functional Assays

(A) Five algal chimeric transgenes were generated to determine the crucial region that led to functional outcome improvements. Lifespan of transgenic flies at **(B)** 25ºC and **(C)** 29ºC. Mechanical stress test recovery time on **(D**) day 16 and **(E)** day 20 at 25ºC.

Table 12. Lifespan of Algal Chimera Transgenic Flies Driven by daughterless-Gal4 at 25°C.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18/sesB[1];;daGal4* (control).

Code	Maximum Lifespan	Median Lifespan	% of Control	N	p-value
attp18	33	29	-	48	
CH1S	35	32	110%	42	<0.0001
CH2S	37	33	114%	45	<0.0001
CH3S	39	31	107%	47	<0.0001
CH4S	39	35	121%	45	<0.0001
CH5S	39	37	128%	42	<0.0001
NoCrAS	37	31	107%	45	<0.0001
NoAS	33	29	100%	43	0.0041

Table 13. Lifespan of Algal Chimera Transgenic Flies Driven by daughterless-Gal4 at 29°C.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18/sesB[1];;daGal4* (control).

Code	Maximum Lifespan	Median Lifespan	% of Control	N	p-value
attp18	17	15	-	45	
CH1S	17	17	113%	46	<0.0001
CH2S	17	15	100%	41	0.0006
CH3S	19	17	113%	43	<0.0001
CH4S	19	17	113%	49	<0.0001
CH5S	19	17	113%	45	<0.0001
NoCrAS	15	15	100%	45	0.0194
NoAS	17	15	100%	48	0.1426

Table 14. Mechanical Stress Test Recovery Time of Algal Chimera Transgenic Flies Driven by daughterless-Gal4 on Day 16 at 25°C.

One-way ANOVA Kruskal-Wallis Test and Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Day 16	SE	% of Control	N	p-value <0.05?
attp18	168.2	5.8	-	17	-
CH1S	139.8	13.5	83%	15	ns
CH2S	152.1	17.2	90%	15	ns
CH3S	94.5	16.4	56%	15	Yes
CH4S	74.8	11.1	44%	16	Yes
CH5S	104.4	9.1	62%	16	Yes
NoCrAS	75.5	7.0	45%	15	Yes
NoAS	87.1	16.7	52%	15	Yes

Table 15. Mechanical Stress Test Recovery Time of Algal Chimera Transgenic Flies Driven by daughterless-Gal4 on Day 20 at 25°C.

One-way ANOVA Kruskal-Wallis Test and Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Day 20	SE	% of Control	N	p-value
attp18	221	0.17	-	9	-
CH1S	158.1	9.5	72%	15	Yes
CH2S	168.0	10.6	76%	15	Yes
CH3S	161.8	9.3	73%	16	Yes
CH4S	164.4	12.4	74%	17	Yes
CH5S	128.8	6.6	58%	16	Yes
NoCrAS	181.1	8.5	82%	15	No
NoAS	182.3	3.4	82%	6	No

Production of Algal-Chimera ATP6 Protein

Western Blot was attempted to determine the amount of algal-chimera ATP6 protein imported into mitochondria. Because the custom-made anti-ATP6 antibody could not distinguish endogenous ATP6 protein from engineered ATP6 protein, we had to rely on the possible difference in size between the endogenous ATP6 protein and the transgenic algal ATP6 protein. The hypothesis was that the MTS cleavage sites for algal-chimera ATP6 and the endogenous ATP6 were different. If that was correct, then there should be two bands on the Western Blot. However, only one band was detected. It is possible that 1) the amount of exogenous algal-chimera ATP6 protein was too low to be detected, especially when the endogenous ATP6 protein was still produced at a normal level and 2) the size difference between algal-chimera ATP6 protein and the endogenous ATP6 protein was too small to be detected at this resolution.

5.3 STRATEGY 2: MITOCHONDRIAL TARGETING SEQUENCES

The hypothesis for strategy 2 was that an addition of exogenous MTS to the recoded *Drosophila* ATP6 protein would lead to increased import efficiency and improvements in functional assays. We have generated 18 transgenic UASB-TG fly strains (5 MTSs x 3 3'UTRs and No-exogenous MTSs x 3 3'UTRs) driven by two different Gal4 systems (daughterless-Gal4 and ubiquitous-Gal4) and incubated at two different conditions (25ºC and 29ºC). Of all the transgenic fly strains, PAS, PAO, and sOAS gave the best overall performance (Figure 27). However, the functional improvements were not as dramatic as those of algal fly strains. While transgenic flies with

different MTS performed significantly better than the control, none of them consistently performed better than the others. The complete results are listed in tabular forms (Table 16-27).

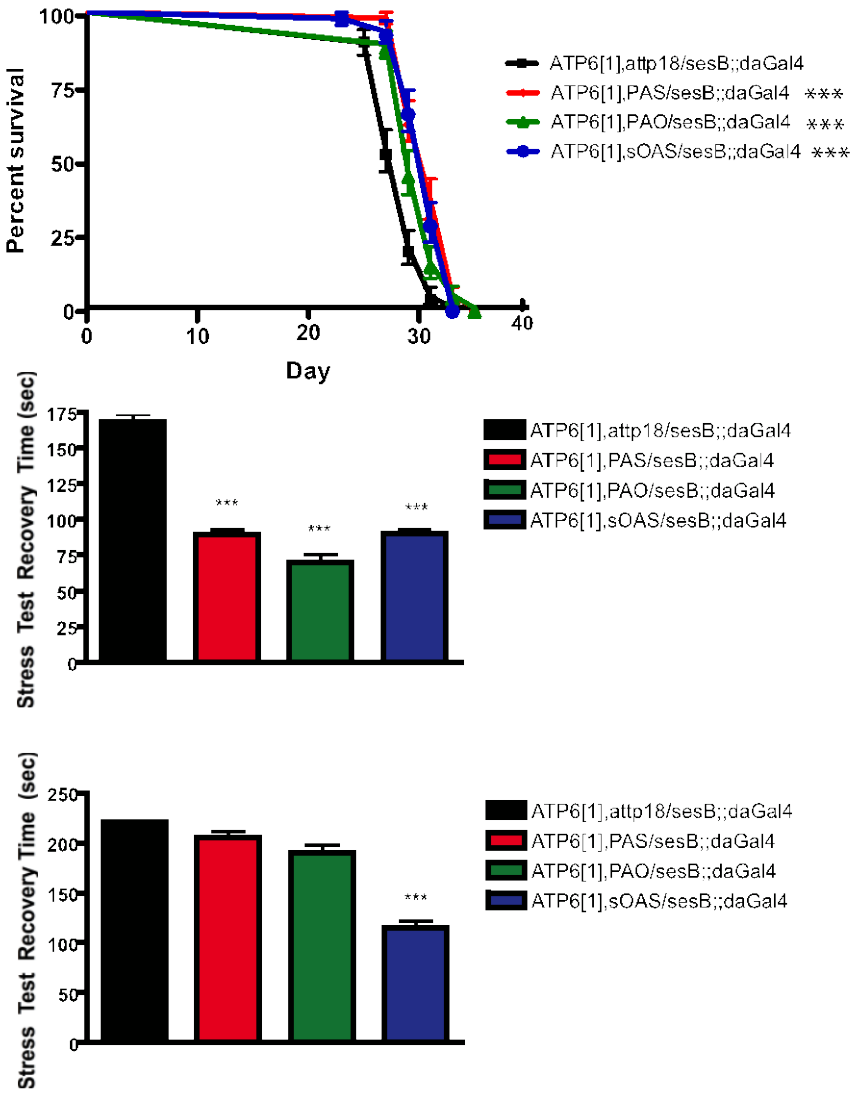

Figure 27. Functional Assays to Determine the Best Transgenic Constructs

(A) Lifespan assays for the best three non-algal fly strains at 25ºC. Transgenic flies with PAS, PAO, and sOAS have longer lifespan than the *ATP6[1];attp18* mutant control. Mechanical stress test recovery time on **(B)** day 16 and **(C)** day 20 at 25ºC. *** = p<0.0001

Table 16. Lifespan of Transgenic Fly Strains Driven by daughterless-Gal4 at 25ºC.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18/sesB[1];;daGal4* (control).

Code	Max lifespan	Median Lifespan	% of Control	N	p-value
attp18	33	29	-	49	
SAO	35	33	114%	45	<.0001
8AV	35	33	114%	42	<.0001
8AO	35	33	114%	43	<.0001
DAO	35	32	110%	48	<.0001
PAV	33	31	107%	43	<.0001
PAS	35	31	107%	49	<.0001
sOAV	35	31	107%	45	<.0001
sOAS	33	31	107%	45	<.0001
sOAO	33	31	107%	48	<.0001
DAV	33	31	107%	45	<.0001
NoAO	33	31	107%	42	<.0001
SAV	31	29	100%	49	0.6137
SAS	35	29	100%	45	.0003
PAO	35	29	100%	46	.0002
DAS	33	29	100%	45	.1557
8AS	31	29	100%	45	.7703
NoAV	33	29	100%	43	.006
NoAS	31	29	100%	45	.0055

Table 17. Lifespan of Transgenic Fly Strains Driven by daughterless-Gal4 at 29ºC.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18/sesB[1];;daGal4* (control).

Code	Max lifespan	Median Lifespan	% of Control	N	p-value
attp18	17	15	-	45	
PAS	19	17	113%	45	<.0001
DAO	17	17	113%	50	<.0001
8AO	19	17	113%	45	<.0001
PAV	17	15	100%	45	0.2383
PAO	19	15	100%	50	0.3618
sOAV	15	15	100%	49	0.0045
sOAS	17	15	100%	45	0.0383
sOAO	17	15	100%	46	0.012
SAV	17	15	100%	45	0.0004
SAS	17	15	100%	45	0.9468
SAO	17	15	100%	45	0.4452
DAV	17	15	100%	45	0.0076
8AV	19	15	100%	45	0.0066
8AS	17	15	100%	55	0.0178
NoAV	17	15	100%	47	0.0492
NoAS	19	15	100%	48	0.1426
NoAO	17	15	100%	43	0.5892
DAS	17	13	87%	45	<.0001

Table 18. Lifespan of Transgenic Fly Strains Driven by ubiquitous-Gal4 at 25ºC.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18;ubiGal4* (control).

Code	Max lifespan	Median Lifespan	% of Control	N	p-value
attp18	39	37	-	47	
sOAO	43	41	111%	48	<.0001
DAO	43	41	111%	45	<.0001
8AO	43	41	111%	44	<.0001
PAV	43	39	105%	47	<.0001
PAS	43	39	105%	46	<.0001
sOAS	43	39	105%	45	<.0001
SAV	43	39	105%	45	<.0001
8AV	41	39	105%	44	<.0001
NoAS	43	39	105%	45	<.0001
NoAO	43	39	105%	44	<.0001
8AS	41	38	103%	30	.0111
PAO	41	37	100%	44	0.0021
sOAV	41	37	100%	47	0.2741
SAS	43	37	100%	44	0.0042
SAO	45	37	100%	45	<.0001
DAV	41	37	100%	45	0.01
DAS	41	37	100%	45	0.5901
NoAV	41	37	100%	44	0.0062

Table 19. Lifespan of Transgenic Fly Strains Driven by ubiquitous-Gal4 at 29ºC.

p-value was measured by Log-rank Test and compared to *ATP6[1];attp18;ubiGal4* (control).

Code	Max lifespan	Median Lifespan	% of Control	N	p-value
attp18	21	19	-	45	
SAS	23	21	110%	49	0.0706
PAS	21	19	100%	45	0.4884
sOAS	21	19	100%	47	<.0001
sOAO	21	19	100%	45	0.0071
SAV	21	19	100%	42	<.0001
SAO	21	19	100%	50	0.0008
DAV	21	19	100%	48	0.0004
DAO	23	19	100%	45	0.0668
8AV	21	19	100%	45	0.0016
8AO	21	19	100%	45	0.2822
NoAS	21	19	100%	45	0.0013
NoAO	21	19	100%	45	0.9366
PAV	19	17	90%	46	<.0001
PAO	19	17	90%	44	<.0001
sOAV	21	17	90%	44	<.0001
DAS	21	17	90%	41	<.0001
8AS	19	17	90%	45	<.0001
NoAV	21	17	90%	46	<.0001

Table 20. Mechanical Stress Test Recovery Time for Transgenic Fly Strains Driven by daughterless-Gal4 at 25ºC on Day 16.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test. Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Day 16	SE	% of Control	N	p-value
attp18	168.2	5.8	-	17	
PAO	69.75	5.7	41%	16	***
SAO	74.19	6.9	44%	16	***
DAV	73.88	10.6	44%	16	***
NoAS	87.07	16.7	52%	15	***
NoAO	89.94	9.0	53%	16	***
PAS	89.13	3.9	53%	16	***
sOAS	90.19	2.8	54%	16	***
sOAO	91.5	8.0	54%	16	***
8AO	92.38	4.6	55%	16	***
DAS	96.75	11.1	58%	16	***
SAV	99.13	3.9	59%	16	***
SAS	98.75	8.5	59%	16	***
8AV	102.1	8.1	61%	15	***
PAV	107.3	8.0	64%	16	**
NoAV	109.6	11.0	65%	15	**
DAO	118.8	12.2	71%	16	**
8AS	140.7	9.7	84%	15	ns
sOAV	149.2	8.1	88%	16	ns

Table 21. Mechanical Stress Test Recovery Time for Transgenic Fly Strains Driven by daughterless-Gal4 at 25ºC on Day 20.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test. Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Day 20	SE	% of Control	N	p-value
attp18	221	0.0	-	9	
sOAS	114.7	7.3	52%	16	***
DAS	139.2	6.4	63%	16	***
SAO	144.8	7.4	66%	16	***
sOAO	158.3	6.1	72%	16	**
DAO	166.6	11.8	75%	8	**
PAV	167.6	6.2	76%	16	***
NoAS	182.3	3.4	82%	6	***
SAV	188.8	3.4	85%	16	*
PAO	190.3	8.4	86%	16	**
DAV	192	3.8	87%	16	ns
8AO	194	9.4	88%	16	*
SAS	196.6	7.9	89%	16	ns
NoAO	199.9	7.4	90%	15	*
NoAV	203.4	4.6	92%	15	*
PAS	205.7	5.8	93%	16	ns
8AV	216	6.2	98%	16	ns

Table 22. Mechanical Stress Test Recovery Time for Transgenic Fly Strains Driven by daughterless-Gal4 at 29ºC on Day 8.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Day 8	SE	% of Control	N	p-value
attp18	62.56	6.1	-	16	
PAO	6	3.7	10%	16	***
PAS	15.79	3.2	25%	14	**
sOAS	20.63	6.0	33%	16	**
PAV	23.38	8.3	37%	16	*
DAV	31.75	7.5	51%	16	ns
SAO	41.07	7.2	66%	14	ns
NoAS	42	7.2	67%	15	ns
sOAO	46.73	8.1	75%	15	ns
DAO	55.63	7.6	89%	16	ns
8AO	60.31	8.4	96%	16	ns
SAV	60.63	5.7	97%	16	ns
sOAV	61.69	6.5	99	16	ns
SAS	63.88	6.2	102%	16	ns
8AS	68.69	7.8	110%	16	ns
NoAV	70	4.7	112%	16	ns
8AV	71.13	8.3	114%	16	ns
DAS	79.25	5.5	127%	16	ns
NoAO	80.69	6.6	129%	16	*

Table 23. Mechanical Stress Test Recovery Time for Transgenic Fly Strains Driven by daughterless-Gal4 at 29°C on Day 12.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test. Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18/sesB[1];;daGal4* control.

Code	Day 12	SE	% of Control	N	p-value
attp18	262.7	7.5	-	16	
sOAS	130.9	9.5	50%	15	***
8AO	150.8	6.2	57%	16	***
DAO	160.8	3.3	61%	16	***
PAS	161.7	7.7	62%	13	***
PAO	162.9	8.9	62%	14	***
8AV	162.8	5.8	62%	16	***
sOAO	166.6	2.2	63%	15	***
sOAV	171.2	16.8	65%	16	***
NoAV	176.6	17.3	67%	16	***
NoAO	184.7	4.8	70%	16	***
DAS	184.5	3.1	70%	15	**
SAO	186.3	5.2	71%	14	***
DAV	191.3	7.1	73%	14	***
SAV	193.2	6.8	74%	15	***
8AS	209.3	5.7	80%	16	ns
NoAS	214.1	6.2	81%	15	**
PAV	217.2	8.9	83%	15	ns
SAS	222.5	8.3	85%	15	*

Table 24. Mechanical Stress Test Recovery Time for Transgenic Fly Strains Driven by ubiquitous-Gal4 at 25ºC on Day 16.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test. Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18;ubiGal4* control.

Code	Day 16	SE	% of Control	N	p-value
attp18	101.4	12.1	-	16	
PAV	21.06	5.4	21%	16	***
PAS	29.56	8.0	29%	16	***
SAS	33.83	8.5	33%	12	***
SAO	33.75	5.7	33%	16	***
SAV	34.21	6.1	34%	14	***
8AV	35.38	7.2	35%	16	***
NoAS	36.31	7.6	36%	16	***
DAV	39.38	5.6	39%	16	***
sOAO	39.69	6.1	39%	16	***
PAO	46.81	4.9	46%	16	*
DAO	53.75	4.8	53%	16	**
8AO	59.53	7.5	59%	15	*
sOAS	61.53	2.9	61%	15	ns
DAS	65.93	4.4	65%	15	ns
NoAV	70.31	3.4	69%	16	ns
8AS	79.07	10.4	78%	15	ns
sOAV	80.31	16.4	79%	16	ns
NoAO	83.38	4.3	82%	16	ns

Table 25. Mechanical Stress Test Recovery Time for Tansgenic Fly Strains Driven by ubiquitous-Gal4 at 25ºC on Day 20.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test. Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18;ubiGal4* control.

Code	Day 20	SE	% of Control	N	p-value
attp18	146.2	4.0	-	16	
PAV	65.06	12.1	45%	16	***
SAO	70.31	4.5	48%	16	***
8AV	79.38	14.0	54%	16	***
SAS	80.5	5.5	55%	12	***
SAV	84.64	8.5	58%	14	***
DAS	88.87	4.7	61%	15	***
PAS	90.56	5.5	62%	16	***
PAO	92.63	6.7	63%	16	***
sOAO	94.25	6.6	64%	16	***
DAV	95.06	6.1	65%	16	***
8AO	104.4	9.0	71%	16	**
sOAS	114.4	7.7	78%	16	**
sOAV	119.1	3.7	81%	16	**
DAO	120.8	11.2	83%	16	*
NoAS	151	15.4	103%	15	ns
NoAO	170.3	4.5	116%	16	ns
8AS	212.7	14.7	145%	7	ns
NoAV	225.7	8.3	153%	16	***

Table 26. Mechanical Stress Test Recovery Time for Transgenic Fly Strains Driven by ubiquitous-Gal4 at 29ºC on Day 8.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test. Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18;ubiGal4* control.

Code	Day 8	SE	% of Control	N	p-value
attp18	15.25	2.9	-	16	
PAS	6.75	0.7	44%	14	ns
PAV	11.75	3.2	77%	16	ns
DAV	13.31	3.0	87%	16	ns
SAO	13.56	3.7	89%	16	ns
sOAO	15.2	2.6	100%	15	ns
NoAS	15.69	3.3	103%	16	ns
PAO	19.44	4.7	127%	16	ns
8AO	19.81	3.8	130%	16	ns
8AV	21.81	4.5	143%	16	ns
sOAV	22.31	4.7	146%	16	ns
sOAS	23.75	5.1	156%	16	ns
DAO	27.25	5.8	179%	16	ns
SAS	31.19	6.4	205%	16	ns
DAS	36.63	8.8	240%	16	ns
SAV	39.21	4.2	257%	14	**
NoAO	40.08	5.2	263%	16	**
NoAV	42.25	3.8	277%	16	***
8AS	57.25	6.3	375%	16	***

Table 27. Mechanical Stress Test Recovery Time for Transgenic Fly Strains Driven by ubiquitous-Gal4 at 29ºC on Day 12.

Transgenic fly strains were grouped by MTS for One-way ANOVA Kruskal-Wallis Test. Dunn's Multiple Comparison Test were used to determine the specific p-value of each transgene against *ATP6[1];attp18;ubiGal4* control.

Code	Day 12	SE	% of Control	N	p-value
attp18	167.7	17.7	-	10	
SAV	57.15	4.0	34%	13	***
PAS	63.63	4.9	38%	16	***
SAO	71.13	9.0	42%	16	**
sOAS	74.06	3.9	44%	16	***
SAS	74.73	5.3	45%	15	*
sOAV	83.5	7.7	50%	16	**
PAO	89.31	7.1	53%	16	**
sOAO	89	8.5	53%	15	*
8AO	92.69	10.1	55%	16	***
PAV	99.69	5.1	59%	16	ns
8AV	103	7.3	61%	16	**
DAV	111	11.9	66%	16	**
NoAS	121.3	8.9	72%	15	*
NoAO	122.9	5.1	74%	14	**
DAS	124.9	8.6	74%	15	*
NoAV	127.2	5.9	76%	15	**
8AS	136.3	10.2	81%	16	ns

5.4 STRATEGY 3: *SOD2* 3'UTR OR *OXA1* 3'UTR

Because *SOD2* 3'UTR and *OXA1* 3'UTR were tested along with different MTS, the results were shown in tabular form above. The hypothesis for strategy 3 was that by attaching *SOD2* 3'UTR or *OXA1* 3'UTR to the C-terminus of the recoded *ATP6* gene the mRNA would be brought closer to the mitochondrial surface, facilitating protein import and improving functional assay performances. Although many transgenic fly strains with *SOD2* 3'UTR and *OXA1* 3'UTR performed better than the control (*SV40* 3'UTR), neither performed consistently better than the other.

5.5 STRATEGY 4: SUBOPTIMALLY-ENCODED CODONS

Suboptimally-encoded transcripts are translated more slowly and, thus, may allow for increased co-tranlsational import and improved efficiency of import with less toxicity resulting from cytosolic aggregation. The hypothesis for strategy 4 was that better synchronization of translation and import would lead to improved functional assay outcomes. Five pairs of transgenic fly strains with regular recoded codons and suboptimally-encoded codons were tested (Figure 28). (A) The difference in lifespan between SAO and SsoAO is not significant ($p=0.082$). (B) Transgenic fly strain with regular recoded codons SAS has a significantly longer lifespan than that with suboptimally-encoded codons SsoAS ($p<0.0001$). (C) Fly strain with

suboptimally encoded codons DsoAS has a significantly longer lifespan than that with regular recoded codons DAS ($p<0.0032$). (D) The difference in lifespan between DAO and DsoAO is not significant ($p=0.95$). (E) Transgenic fly strain with regular recoded codons NoAS has a significantly longer lifespan than that with suboptimally-encoded codons (NosoAS) ($p<0.0013$). There was no consistent trend to show that transgenic fly strains with suboptimally-encoded codons performed better than those with regular recoded codons.

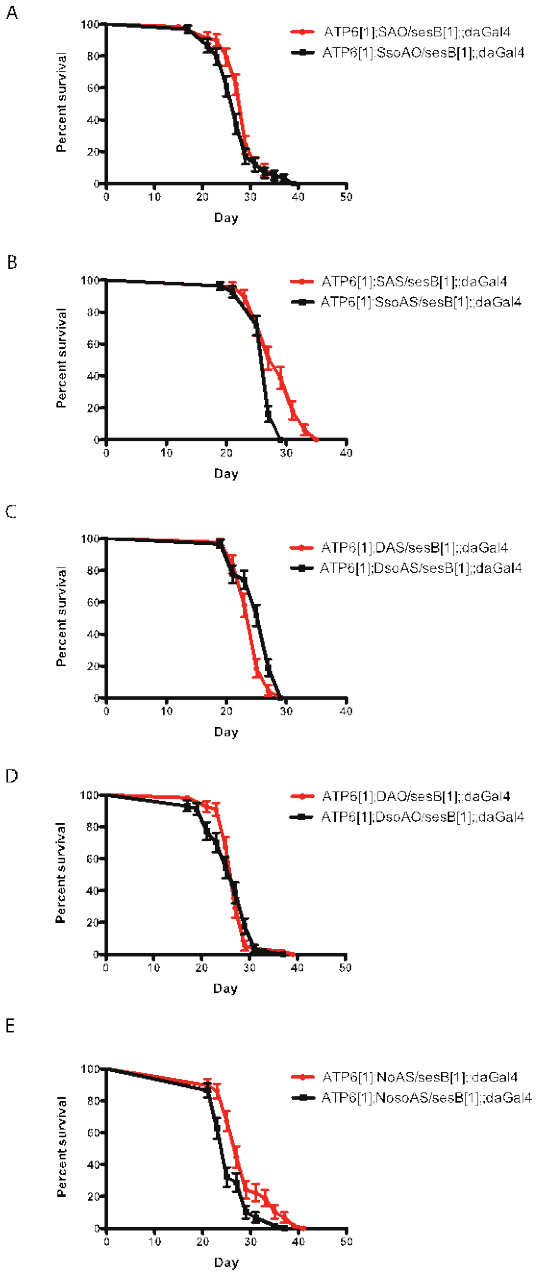

Figure 28. Lifespan of Transgenic Fly Strains with Suboptimally-Encoded ATP6.

Transgenic fly strains with regular recoded codons are shown in red and those with suboptimally-encoded codons are shown in black. All flies were incubated at 25ºC. **(A)** SAO vs. SsoAO. **(B)** SAS vs. SsoAS. **(C)** DAS vs. DsoAS. **(D)** DAO vs. DsoAO and **(E)** NoAS vs. NosoAS.

5.6 STRATEGY 5: MITOCHONDRIAL BIOGENESIS FACTORS

The best performing transgenic strains in strategy 1 and 2 (NoCrAO, PAS, PAO and sOAS) were chosen to further test whether the overexpression of mitochondrial biogenesis factors (PGC1-α, AMPK, and DSP1) would improve functional outcomes. The hypothesis for this strategy was that overexpressing AMPK or PGC1-α would increase the total number of mitochondria through activation of nuclear respiratory factor -1, -2 and estrogen-related receptor- α, which induce genes that encode mitochondrial proteins [327]. This might help overcome the energy deficits caused by the *ATP6* mutation and improve the functional status of *ATP6[1]* mutant flies.

Overexpression of PGC1-α at 25ºC (Figure 29) led to decreased lifespan in NoCrAO fly strain (A). The median lifespan did not show improvement for (B) PAS and (D) sOAS when PGC1-α was overexpressed. The median lifespan did increase for (C) PAO with PGC1-α overexpression when compared to the PAO strain by 7% but it was not statistically significant. At 29ºC, overexpressing PGC1-α did not improve median survival for all four transgenic strains tested (Figure 30). For mechanical stress tests, transgenic fly strains with PGC1-α overexpression consistently performed worse than those without PGC1-α overexpression (Figure 31 and 32).

Overexpression of AMPK at 25ºC (Figure 33) led to decreased lifespan in NoCrAO fly strain (A). The median lifespans for PAS, PAO, and sOAS showed statistically significant increase when AMPK was overexpressed (B-D). Overexpression of AMPK at 29°C (Figure 34) led to increased median lifespan in PAO and sOAS but not in NoCrAO and PAS. For mechanical stress tests, transgenic fly strains with AMPK overexpression performed worse than those

without AMPK overexpression (Figure 35 and 36) except in the cases of PAS and PAO at 29°C (Figure 36 B and C).

Attempts to generated transgenic flies with DSP1 overexpression were not successful because virgin *ATP6[1];FM0/UAS-TG;;TM6/daGal4* exhibited reduced viability and sufficient animals could not be obtained.

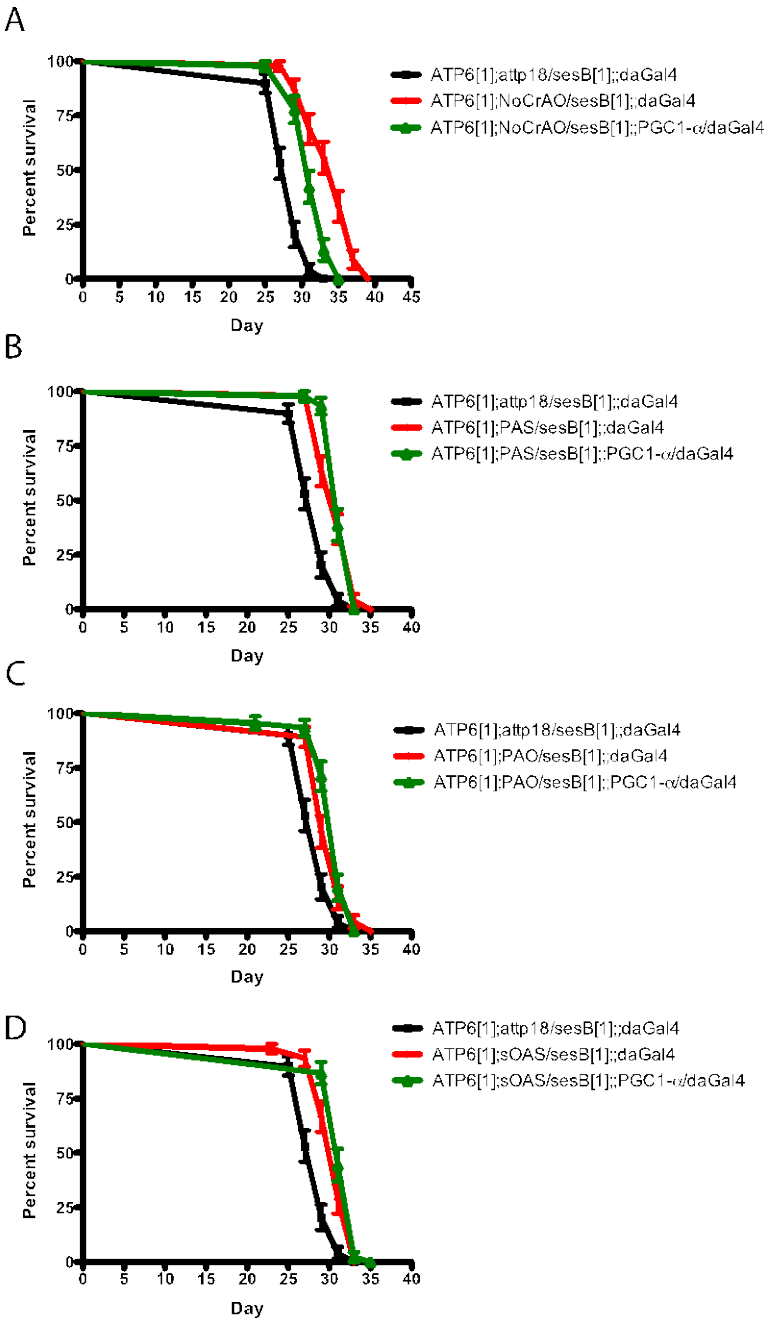

Figure 29. Lifespan of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of PGC1-α at 25°C.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

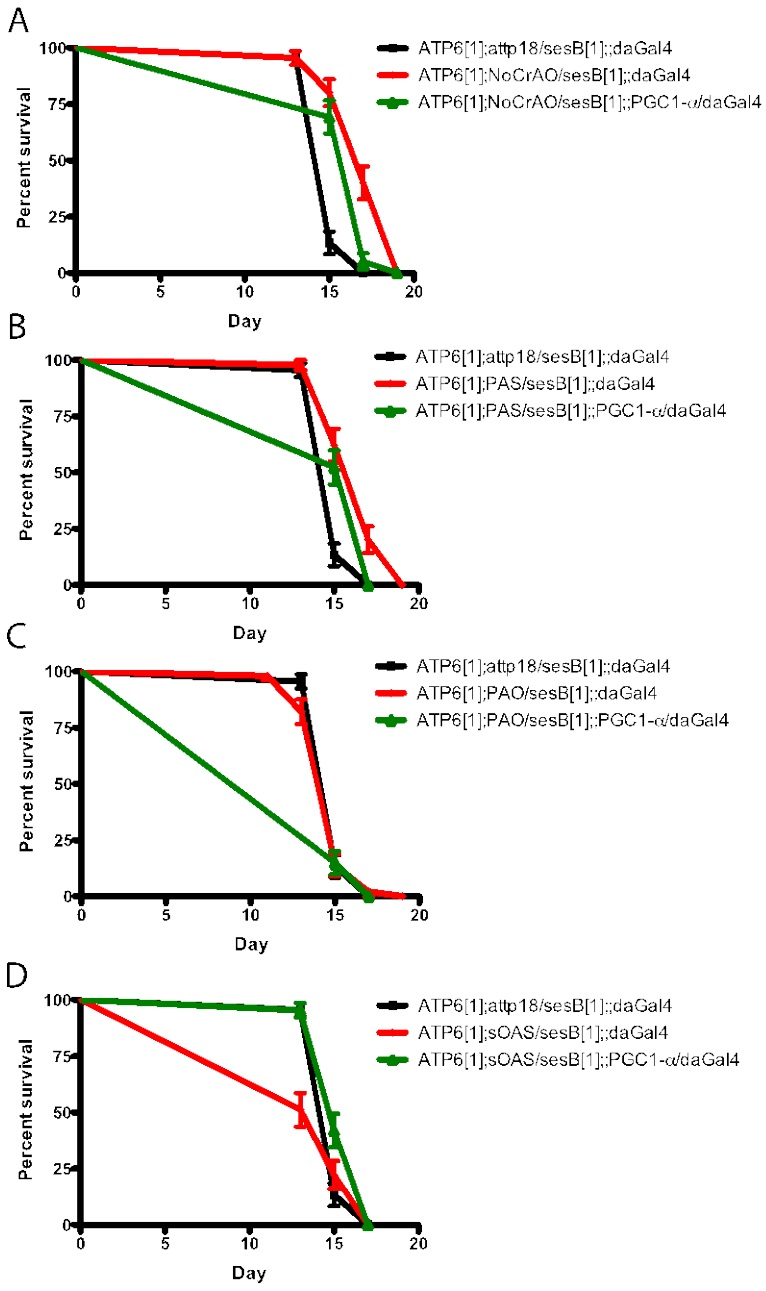

Figure 30. Lifespan of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of PGC1-α at 29ºC.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

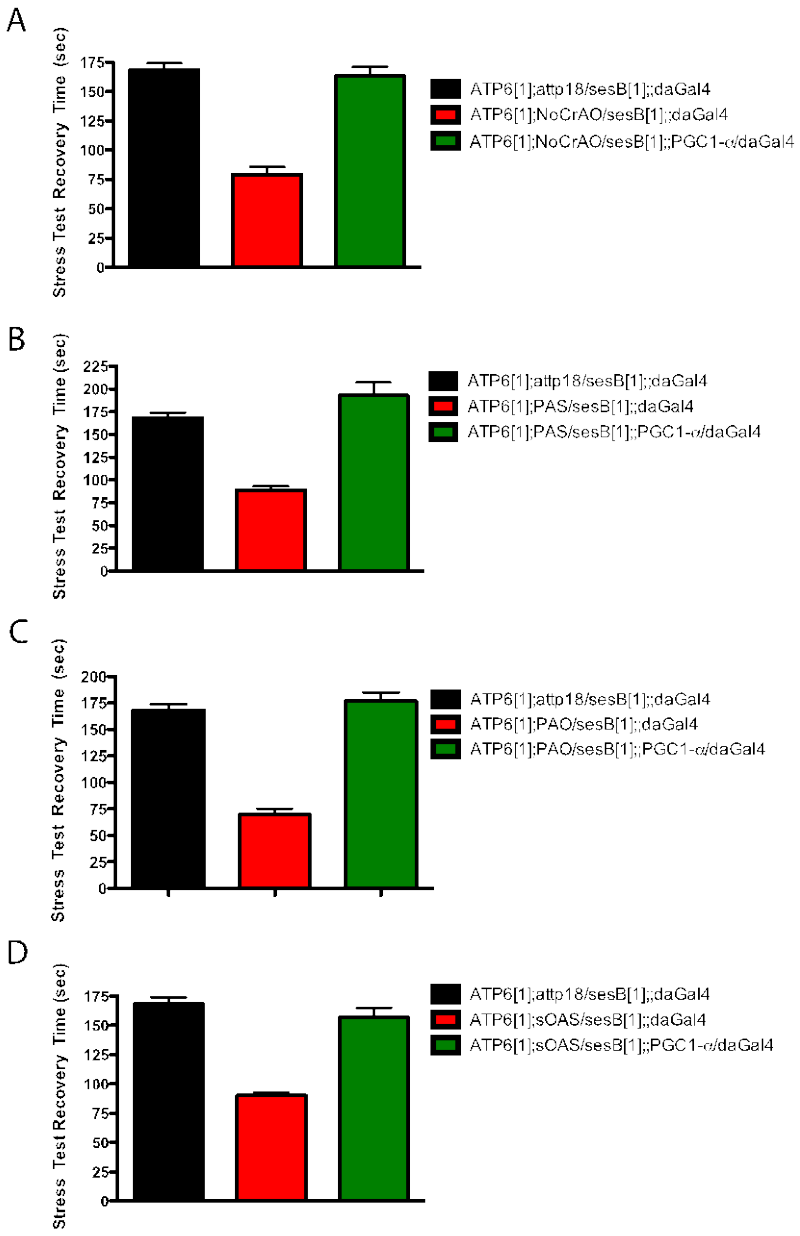

Figure 31. Mechanical stress test recovery time of transgenic strains with nucleus-encoded ATP6 and overexpression of PGC1-α at 25ºC on Day 16.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

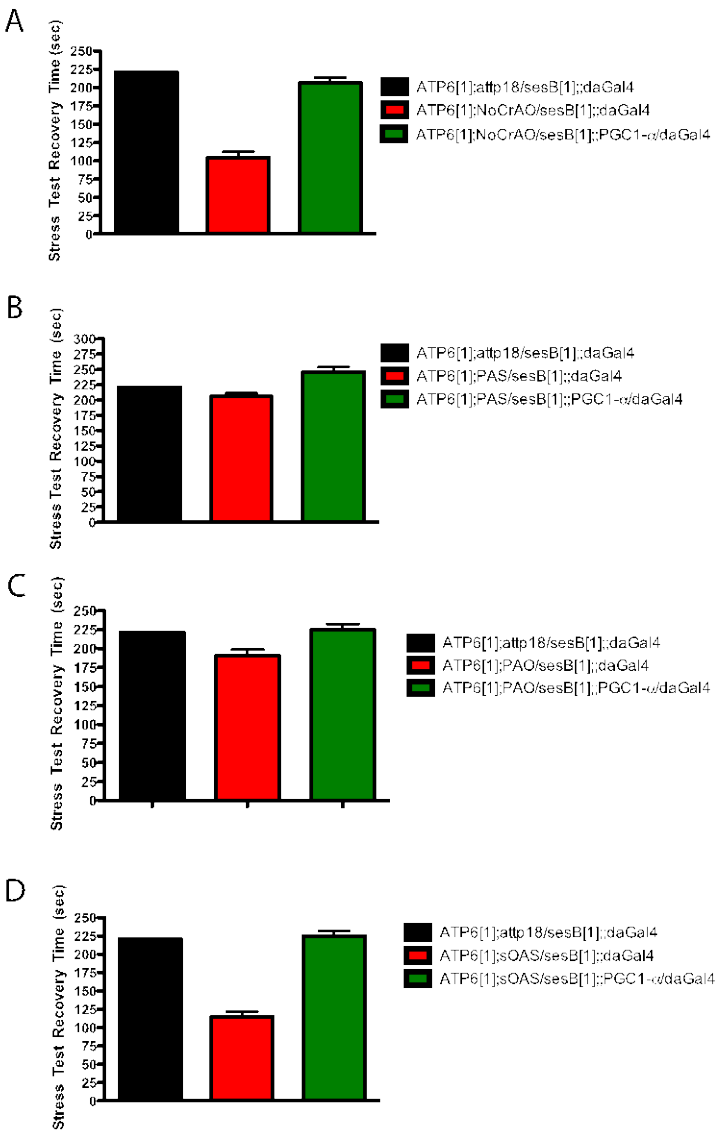

Figure 32. Mechanical Stress Test Recovery Time for Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of PGC1-α at 25ºC on Day 20.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

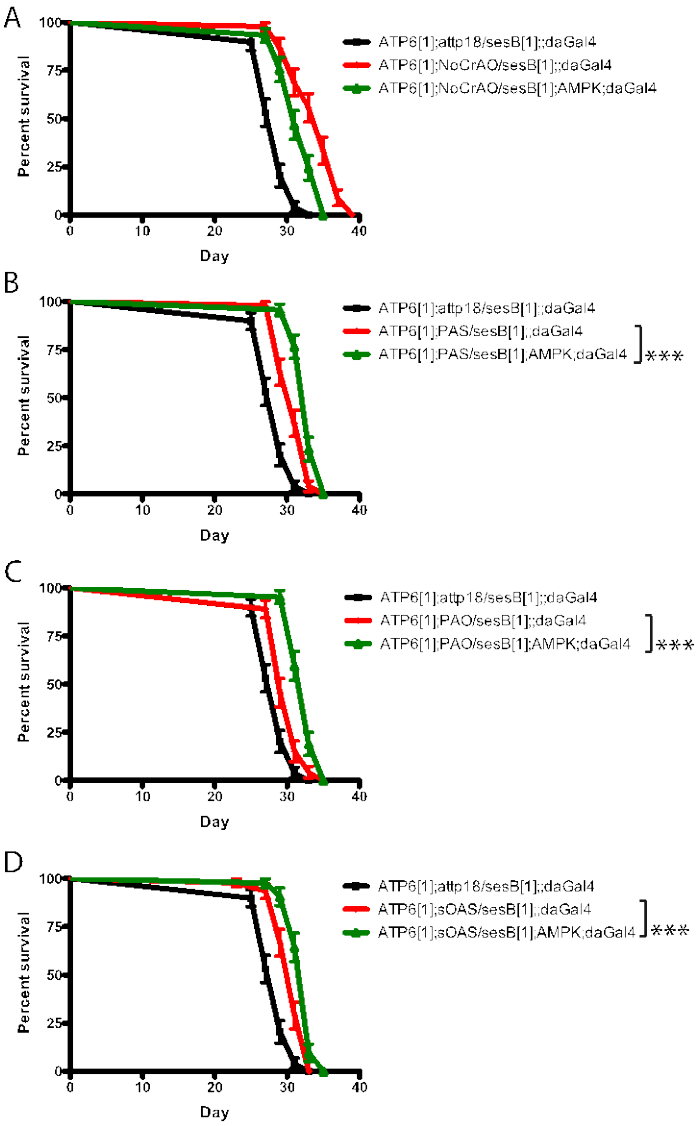

Figure 33. Lifespan of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of AMPK at 25ºC.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

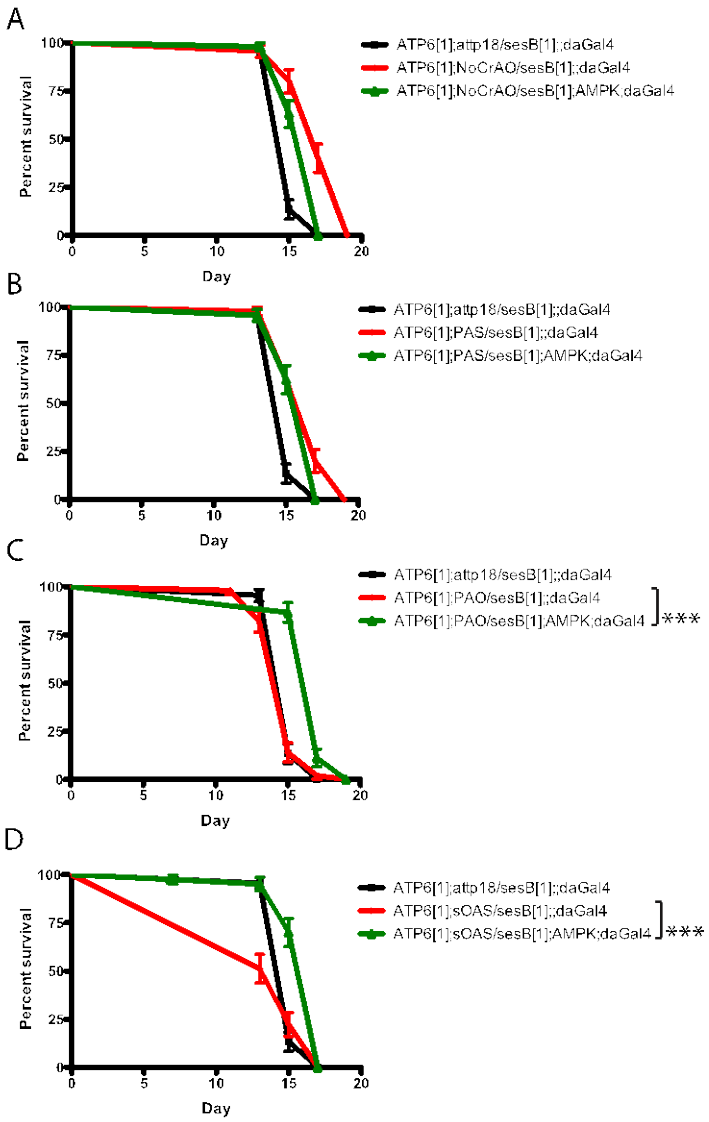

Figure 34. Lifespan of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of AMPK at 29ºC.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

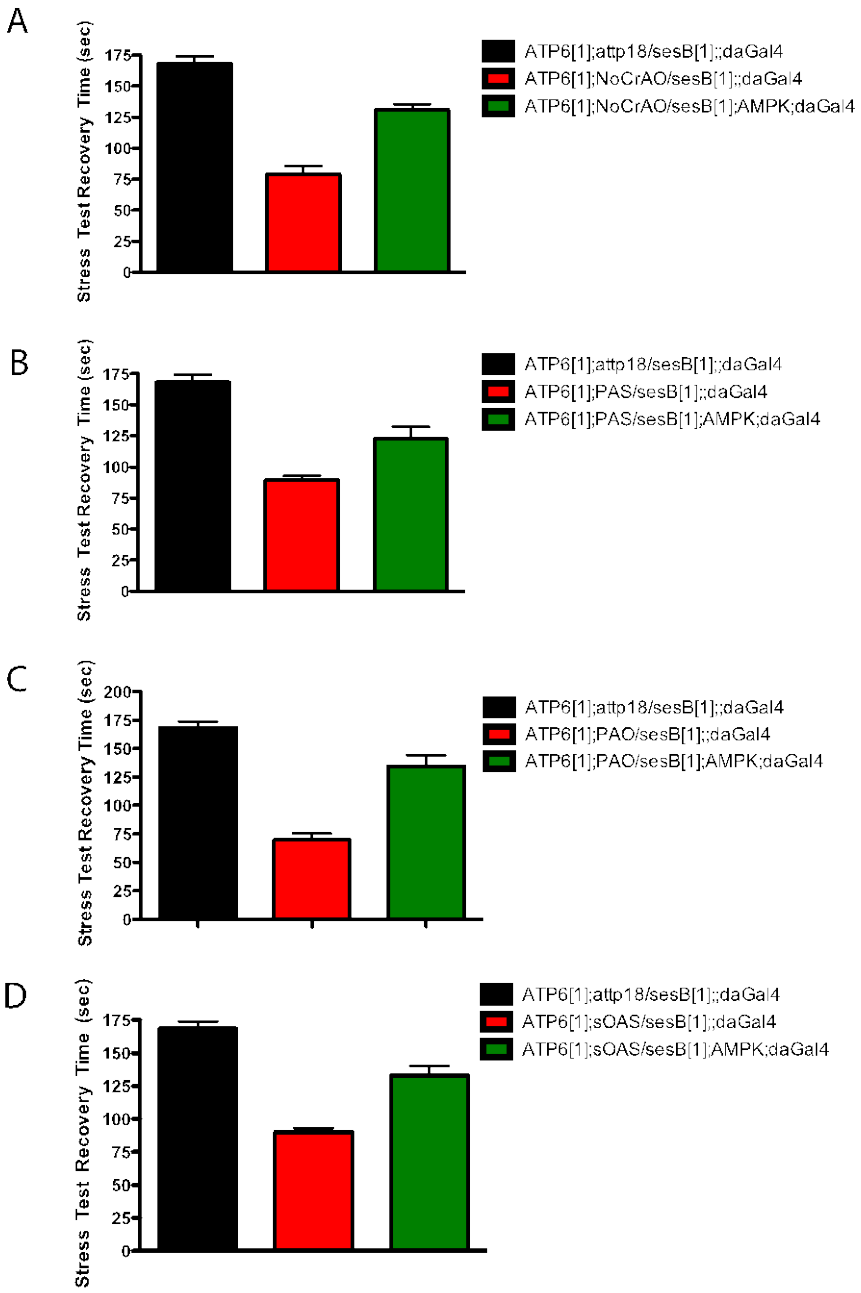

Figure 35. Mechanical Stress Test Recovery Time of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of AMPK at 25ºC on Day 16.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

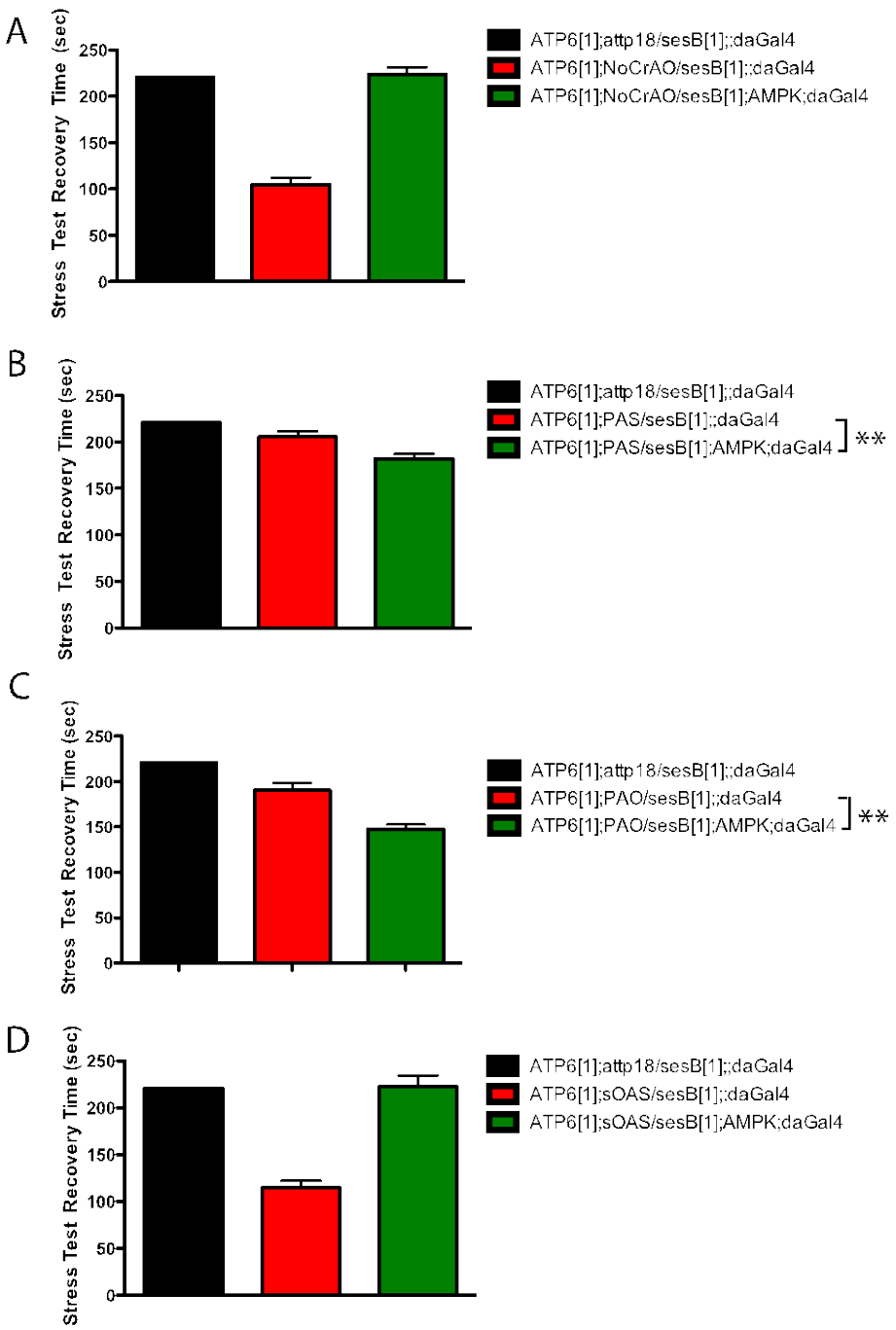

Figure 36. Mechanical Stress Test Recovery Time of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of AMPK at 25ºC on Day 20.

Transgenic fly strains with **(A)** NoCrAO, **(B)** PAS, **(C)** PAO, and **(D)** sOAS.

5.7 STRATEGY 6: MITOCHONDRIAL AUTOPHAGY FACTOR

The hypothesis for this strategy was that overexpressing ATG1 might decrease the number of defective mitochondria and promote mitochondria turnover to compensate for the degradation, improving the efficacy of a rescue. ATG1 overexpression is known to increase autophagy and is protective for PINK1 pathogenesis [328]. We examined overexpression of ATG1; however, no larva were observed on the medium, suggesting that broadly-expressed ATG1 in *Drosophila* is developmentally lethal. We did not pursue this avenue of investigation further.

5.8 STRATEGY 7: MITOCHONDRIAL PORTEIN FOLDING FACTOR

mtHSP70 is a critical mitochondrial chaperone involved in stabilizing hydrophobic proteins and assisting their import into the inner mitochondrial membrane. The hypothesis for this strategy was that overexpressing mtHSP70 might stabilize and assist nucleus-encoded ATP6 protein import. Double transgenic *ATP6[1]* flies with both UAS-TG and UAS-mtHSP70 were generated, driven by daGAL4, and incubated at 25ºC and at 29ºC to test for rescue. No significant differences in functional assays (lifespan and stress test recovery time) were observed (Table 28-30).

Table 28. Lifespan of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of mtHSP70 at 25ºC.

Genotype	Max Lifespan	Median Lifespan	N
ATP6[1];sesB[1]/+;;attp2/daGal4	37	35	49
ATP6[1];attp18/sesB[1];;daGal4	35	31	47
ATP6[1];sesB[1]/+;;mtHSP70/daGal4	33	29	48
ATP6[1];NoCrAV/sesB[1];;daGal4	39	37	46
ATP6[1];NoCrAV/sesB[1];;mtHSP70/daGal4	35	31	44
ATP6[1];NoCrAS/sesB[1];;daGal4	37	33	48
ATP6[1];NoCrAS/sesB[1];;mtHSP70/daGal4	33	31	45
ATP6[1];NoCrAO/sesB[1];;daGal4	39	35	44
ATP6[1];NoCrAO/sesB[1];;mtHSP70/daGal4	35	31	46
ATP6[1];PAV/sesB[1];;daGal4	33	31	43
ATP6[1];PAV/sesB[1];;mtHSP70/daGal4	35	31	44
ATP6[1];PAS/sesB[1];;daGal4	35	31	49
ATP6[1];PAS/sesB[1];;mtHSP70/daGal4	33	31	45
ATP6[1];PAO/sesB[1];;daGal4	35	29	46
ATP6[1];PAO/sesB[1];;mtHSP70/daGal4	33	29	51
ATP6[1];sOAS/sesB[1];;daGal4	33	31	45
ATP6[1];sOAS/sesB[1];;mtHSP70/daGal4	33	31	44
ATP6[1];sOAO/sesB[1];;daGal4	33	31	48
ATP6[1];sOAO/sesB[1];;mtHSP70/daGal4	33	31	45
ATP6[1];SAS/sesB[1];;daGal4	35	29	45
ATP6[1];SAS/sesB[1];;mtHSP70/daGal4	33	31	43
ATP6[1];SAO/sesB[1];;daGal4	35	33	45
ATP6[1];SAO/sesB[1];;mtHSP70/daGal4	33	29	47
ATP6[1];8AS/sesB[1];;daGal4	31	29	45
ATP6[1];8AS/sesB[1];;mtHSP70/daGal4	33	31	42
ATP6[1];8AO/sesB[1];;daGal4	35	33	43
ATP6[1];8AO/sesB[1];;mtHSP70/daGal4	33	31	42
ATP6[1];NoAS/sesB[1];;daGal4	31	29	45
ATP6[1];NoAS/sesB[1];;mtHSP70/daGal4	33	31	43
ATP6[1];NoAO/sesB[1];;daGal4	33	31	42
ATP6[1];NoAO/sesB[1];;mtHSP70/daGal4	33	29	48

Table 29. Lifespan of Transgenic Fly Strains with Nucleus-Encoded ATP6 and Overexpression of mtHSP70 at 29ºC.

Genotype	Max Lifespan	Median Lifespan	N
ATP6[1];sesB[1]/+;;attp2/daGal4	17	15	48
ATP6[1];attp18/sesB[1];;daGal4	17	15	45
ATP6[1];sesB[1]/+;;mtHSP70/daGal4	15	15	23
ATP6[1];NoCrAV/sesB[1];;daGal4	17	17	42
ATP6[1];NoCrAV/sesB[1];;mtHSP70/daGal4	17	15	51
ATP6[1];NoCrAS/sesB[1];;daGal4	19	15	46
ATP6[1];NoCrAS/sesB[1];;mtHSP70/daGal4	17	15	44
ATP6[1];NoCrAO/sesB[1];;daGal4	21	17	45
ATP6[1];NoCrAO/sesB[1];;mtHSP70/daGal4	17	15	47
ATP6[1];PAV/sesB[1];;daGal4	17	15	45
ATP6[1];PAV/sesB[1];;mtHSP70/daGal4	15	15	43
ATP6[1];PAS/sesB[1];;daGal4	19	17	45
ATP6[1];PAS/sesB[1];;mtHSP70/daGal4	15	13	44
ATP6[1];PAO/sesB[1];;daGal4	19	15	45
ATP6[1];PAO/sesB[1];;mtHSP70/daGal4	15	13	45
ATP6[1];sOAS/sesB[1];;daGal4	17	15	45
ATP6[1];sOAS/sesB[1];;mtHSP70/daGal4	15	15	51
ATP6[1];sOAO/sesB[1];;daGal4	17	15	46
ATP6[1];sOAO/sesB[1];;mtHSP70/daGal4	15	13	56
ATP6[1];SAS/sesB[1];;daGal4	17	15	45
ATP6[1];SAS/sesB[1];;mtHSP70/daGal4	15	15	49
ATP6[1];SAO/sesB[1];;daGal4	17	15	45
ATP6[1];SAO/sesB[1];;mtHSP70/daGal4	15	13	48
ATP6[1];8AS/sesB[1];;daGal4	17	15	55
ATP6[1];8AS/sesB[1];;mtHSP70/daGal4	17	15	44
ATP6[1];8AO/sesB[1];;daGal4	19	17	45
ATP6[1];8AO/sesB[1];;mtHSP70/daGal4	15	15	48
ATP6[1];NoAS/sesB[1];;daGal4	19	15	48
ATP6[1];NoAS/sesB[1];;mtHSP70/daGal4	15	15	50
ATP6[1];NoAO/sesB[1];;daGal4	17	15	43
ATP6[1];NoAO/sesB[1];;mtHSP70/daGal4	15	13	45

Table 30. Mechanical Stress Test Recovery Time of Transgenic Strains with Nucleus-Encoded ATP6 and Overexpression of mtHSP70 on Day 16 at 25ºC.

Genotype	Mean	SE	N
ATP6[1];sesB[1]/+;;attp2/daGal4	101.7	4.7	15
ATP6[1];attp18/sesB[1];;daGal4	112.2	10.8	14
ATP6[1];sesB[1]/+;;mtHSP70/daGal4	123.1	6.9	13
ATP6[1];NoCrAV/sesB[1];;daGal4	95.9	2.1	17
ATP6[1];NoCrAV/sesB[1];;mtHSP70/daGal4	85.7	8.0	16
ATP6[1];NoCrAS/sesB[1];;daGal4	104.1	3.8	17
ATP6[1];NoCrAS/sesB[1];;mtHSP70/daGal4	77.9	5.8	17
ATP6[1];NoCrAO/sesB[1];;daGal4	69.1	5.3	18
ATP6[1];NoCrAO/sesB[1];;mtHSP70/daGal4	76.1	4.0	15
ATP6[1];PAV/sesB[1];;daGal4	107.2	8.0	16
ATP6[1];PAV/sesB[1];;mtHSP70/daGal4	70.4	7.8	16
ATP6[1];PAS/sesB[1];;daGal4	89.1	3.9	16
ATP6[1];PAS/sesB[1];;mtHSP70/daGal4	85.4	9.7	14
ATP6[1];PAO/sesB[1];;daGal4	69.8	5.7	16
ATP6[1];PAO/sesB[1];;mtHSP70/daGal4	101.9	9.3	16
ATP6[1];sOAS/sesB[1];;daGal4	90.2	2.8	16
ATP6[1];sOAS/sesB[1];;mtHSP70/daGal4	79.1	3.1	14
ATP6[1];sOAO/sesB[1];;daGal4	91.5	8.0	16
ATP6[1];sOAO/sesB[1];;mtHSP70/daGal4	119.9	15.3	14
ATP6[1];SAS/sesB[1];;daGal4	98.8	8.5	16
ATP6[1];SAS/sesB[1];;mtHSP70/daGal4	73.7	6.8	15
ATP6[1];SAO/sesB[1];;daGal4	77.9	5.0	16
ATP6[1];SAO/sesB[1];;mtHSP70/daGal4	69.5	5.0	16
ATP6[1];8AS/sesB[1];;daGal4	140.4	9.1	16
ATP6[1];8AS/sesB[1];;mtHSP70/daGal4	96.5	5.0	15
ATP6[1];8AO/sesB[1];;daGal4	92.4	4.6	16
ATP6[1];8AO/sesB[1];;mtHSP70/daGal4	99.9	3.1	15
ATP6[1];NoAS/sesB[1];;daGal4	87.1	16.7	15
ATP6[1];NoAS/sesB[1];;mtHSP70/daGal4	88.3	2.9	15
ATP6[1];NoAO/sesB[1];;daGal4	89.9	9.0	16
ATP6[1];NoAO/sesB[1];;mtHSP70/daGal4	105.7	7.6	15

5.9 STRATEGY 8: MITOCHONDRIAL PROTEIN INSERTION FACTOR

OXA1 is known to be a critical protein for inner mitochondrial membrane insertion. The hypothesis was that overexpressing OXA1 would improve the efficiency of insertion and improve efficacy of allotopic expression *in vivo*. Double transgenic *ATP6[1]* strains with both UAS-TG and UAS-OXA1 were generated, driven by daGAL4, and incubated at 25°C for testing (Figure 37). Transgenic strains with overexpression of OXA1 showed malformed wings and significantly shorter lifespan, possibly due to toxicity (Figure 37).

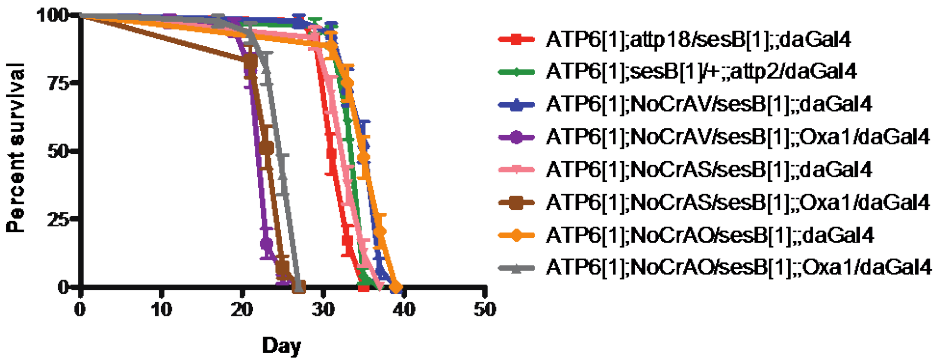

Figure 37. Lifespan of Transgenic Strains With Nucleus-Encoded ATP6 and Overexpression of OXA1 at 25ºC.

Transgenic fly strains with different algal *ATP6* transgenes, with and without the overexpression of OXA1.

5.10 STRATEGY 9: TRANSLATIONAL INHIBITORS (TLIs)

It is known the *ATP6[1]* mutants express a stable mutant protein bearing the glycine to glutamate substitution. As ATP6 is a critical component of a complex and elaborately assembled multi-subunit ATP synthase, it is likely that allotopically expressed wild type protein will be in competition for inclusion in the mature synthase complex. Flies expressing *TLI-MRP::ATP6* or *TLI-RNP::ATP6* have been shown to reduce steady state expression of the endogenous ATP6 protein. We examined whether *TLI-MRP::ATP6* or *TLI-RNP::ATP6 expression* along with the *NoCrAO* transgene showed significantly increased lifespan and decreased mechanical stress test recovery time (Figure 38). Notice that *NoCrAO* fly strain with a *VK27* background did not show a statistically significant improvements in lifespan and mechanical stress test recovery time

comparing to *ATP6[1]* mutants in a *VK27* background. This was in contrast to *NoCrAO* transgenic fly strain in *attp18* background, which showed significantly improvement in functional assays comparing to *ATP6[1]* mutants in an *attp18* background.

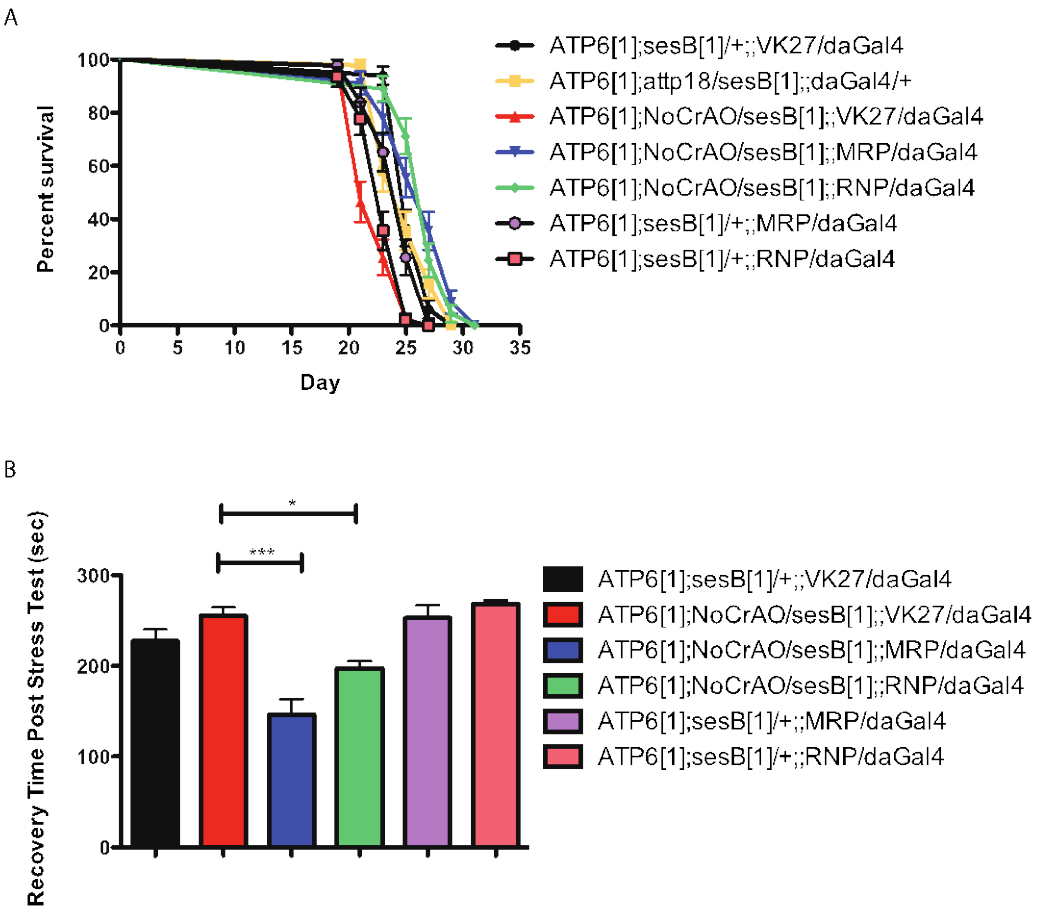

Figure 38. Functional Assays for Transgenic Flies Expressing *NoCrAO* transgene and TLI.

(A) Lifespan of transgenic flies expressing algal *ATP6* transgene (*NoCrAO*) with MRP or RNP showed significantly improvement comparing to *NoCrAO* alone or MRP or RNP alone. **(B)** Transgenic flies with *NoCrAO* with TLI expression showed significantly decreased mechanical stress test recovery time.

6.0 DISCUSSION

The task of examining the efficacy of ATP6 allotopic expression in an animal model has never been undertaken before. We examined 9 strategies and found that utilizing algal ATP6 subunit delivered the best functional results while other strategies provided limited improvements.

Drosophila is an excellent animal model for screening because 1) it is relatively easy to generate transgenic animals and proper genetic controls; 2) they have short life cycles (~14 days) and relatively short lifespans; and 3) they can be generated in large numbers. In this project, we utilized more than 30 transgenic strains driven by 2 Gal4 systems at two different temperatures. It would have been much more difficult to do that in a mammalian model. The phenotypes of the *ATP6[1]* mutant models mirror closely those of human patients, which allow us to infer that if we could achieve phenotypic rescue in *Drosophila* it would not be unreasonable to expect similar functional improvements in humans. As stated earlier, the *ATP6[1]* mutant flies have 98% heteroplasmy, which is higher than any viable organism documented. This provides a very rigorous model to test the efficacy of allotopic expression but also makes it much more challenging to show a positive phenotypic rescue. For example, if we had a fly model with a 90% heteroplasmy and we were able to import 5% engineered ATP6 protein into the mitochondria, it might be sufficient to alleviate the severity of the phenotype (in humans, 70-90% *ATP6* heteroplasmy leads to NARP while 90%+ *ATP6* heteroplasmy leads to MILS, a much

more severe disease). However, being able to import 5% engineered ATP6 protein into the mitochondria might not be sufficient in our flies because they had a 98% heteroplasmy. Flies with a 93% heteroplasmy might show similar phenotypes as those with a 98% heteroplasmy. Therefore, a much higher percentage of engineered ATP6 import is required to show any rescue effect.

Because it was a large-scale screening effort, we focused on using daughterless-GAL4 and ubiquitous-GAL4, both broadly-expressed promoters, to drive our transgenic constructs. Although daughterless-GAL4 and ubiquitous-GAL4 are commonly used, their expression profiles are not entirely well-described. Most importantly, their expression levels tend to fluctuate during development, adulthood, and aging, so the transcription levels may vary, affecting translation and the amount of engineered ATP6 proteins actually generated. Although daughterless-GAL4 and ubiquitous-GAL4 are both broadly-expressed, the flies driven by ubiquitous-GAL4 tend to live longer than those driven by daughterless-GAL4. The reason is unknown. It is also unclear why transgenic animals driven by ubiquitous-GAL4 tend to experience recurrent seizures after initial recovery post mechanical stress test. However, it does point out the importance of examining different GALl4 systems and determining one that is most suitable for the experiments on hand. The importance of tissue-specific GAL4 system will become obvious when we discuss overexpressing factors that are involved in various mitochondrial functions.

The background of the animals is also of significance. As pointed out in figure 38, flies expressing algal *ATP6* in a *VK27* background did not show any rescue effect comparing to

ATP6[1] mutants in a *VK27* background. However, significant improvements in functional assays were achieved in flies expressing algal *ATP6* in an *attp18* background comparing to *ATP6[1]* mutants in an *attp18* background. Also, the median lifespan for female offspring that had a paternal parent from an *attp18* background was longer than the median lifespan for female offspring that had a paternal parent from a Canton S background (29 days vs. 23 days), even though the maternal parents from both matings were identical (*ATP6[1];sesB;;daGal4*). This highlighted the importance of utilizing suitable controls and testing multiple fly lines.

We incubated the animals at two different temperatures at 25°C and 29°C. Increasing the temperature will increase the metabolism and speed up the life cycle of *Drosophila*. However, a compressed life cycle may mask a moderate rescue, and the stress response induced by increased temperature may alter protein expression and functions. When we evaluated the transgenic animals, we did realize that the transgenic fly strains that performed best at 25°C might not be the same ones that performed best at 29°C.

Stress tests were performed on day 16 and day 20 for flies that were incubated at 25°C and on day 8 and day 12 for flies that were incubated at 29°C. Because *ATP6[1]* mutant phenotypes are progressive, with mechanical stress test and strobe light test recovery times comparable to those of WT flies when they are young, it is essential to determine an optimal time during their adulthood to perform stress test assays. Day 16 and day 20 were reasonable choices as many of the transgenic fly strains showed significantly improved recovery time comparing to the control. The optimal time also depends on the GAL4 that is driving the transgene. For example, most stress tests conducted on day 8 for the flies driven by ubiquitous-GAL4 did not

show significant differences comparing to the control, suggesting that the flies, on day 8, had not entered their symptomatic phase yet. Moreover, the stress test recovery time for 8-day-old flies incubated at 29°C did not necessarily correspond to that for 16-day-old flies incubated at 25°C. There was a 4-day gap between the two successive stress tests at both temperatures. However, because the flies had a compressed life cycle at 29°C, the physiology of the flies on day 12 at 29°C most likely would be very different from that of the flies on day 20 at 25°C.

Another challenge associated with performing behavioral assays in animals is the variability. Changes in seasons, slight variations in humidity and temperature in the incubators, conditions of the medium, the time of the day when the tests are conducted can all introduce variables into the functional outcomes. Care has been taken as much as possible to maintain a constant environment for the animals and minimize these variables, but it is still possible that the fluctuations in the environment and experimental treatment may affect functional test performances.

Despite the challenges, we also obtained some exciting results. Of all the strategies tested, it was somewhat surprising that algal ATP6 subunit delivered the best functional outcome. Because the rest of the ATP synthase originated from *Drosophila*, an algal ATP6 subunit might not fit properly into the rest of the complex. Indeed, although one of the transgenic strains showed a 21% increase in lifespan comparing to that of the *ATP6[1];attp18* control, it was still significantly shorter than the lifespan of WT animals (Figure 39). Further improvements in functional tests were observed in algal-chimera transgenic animals (CH5S delivered a 28% increase in lifespan). A reasonable explanation was that all the algal-chimera utilized the same

algal MTS and the algal MTS provided a similar advantage for all the engineered ATP6 proteins (full-length algal ATP6 or algal-chimera ATP6) to be imported into mitochondria. Once inside the mitochondria, however, the algal-chimera ATP6 protein may assemble in and/ or function within the mature complex better than the full-length algal ATP6 protein. We were limited in our ability to map such critical domains due to the number of chimeras examined. We sought to determine the relative amount of full-length algal and algal-chimera ATP6 proteins imported into the mitochondria. Unfortunately, there was no commercially available ATP6 antibody against *Chlamydomonas reinhardtii*, and our custom-made anti-ATP6 antibody could not distinguish endogenous and engineered ATP6 protein. We tried to circumvent this by exploiting a possible difference in size between the MTS-processed algal-chimera ATP6 protein and the endogenous *Drosophila* ATP6 protein, as the recognition epitope of the custom-made anti-ATP6 antibody was also present in the algal-chimera ATP6 proteins. We expected that would lead to two bands of different sizes on a Western Blot. However, the cleavage site of the algal MTS was predicted to be 107aa [279] and the size of the MTS-processed algal-chimera ATP6 would be 25.6kDa, which was very similar in size to the endogenous *Drosophila* ATP6 protein 26.4kDa. We were able to observe only one band in Western Blot. This could be due to several reasons. The sizes of the MTS-processed algal ATP6 and the endogenous ATP6 were similar and thus we were not able to differentiate them at this resolution. Another possibility was that the amount of algal ATP6 protein that was delivered into the mitochondria was so low that it could not be observed on a standard Western Blot. Attaching a marker such as myc-tag would have been helpful; however, we could not determine a site where the myc-tag could be attached on the algal protein without compromising its function. However, based on the functional readouts of algal ATP6 transgenic strains versus engineered fly *ATP6* transgenic strains, it is reasonable to suggest that

the length and the hydrophilicity of the algal MTS were the most important factors in providing the rescues we observed in these experiments.

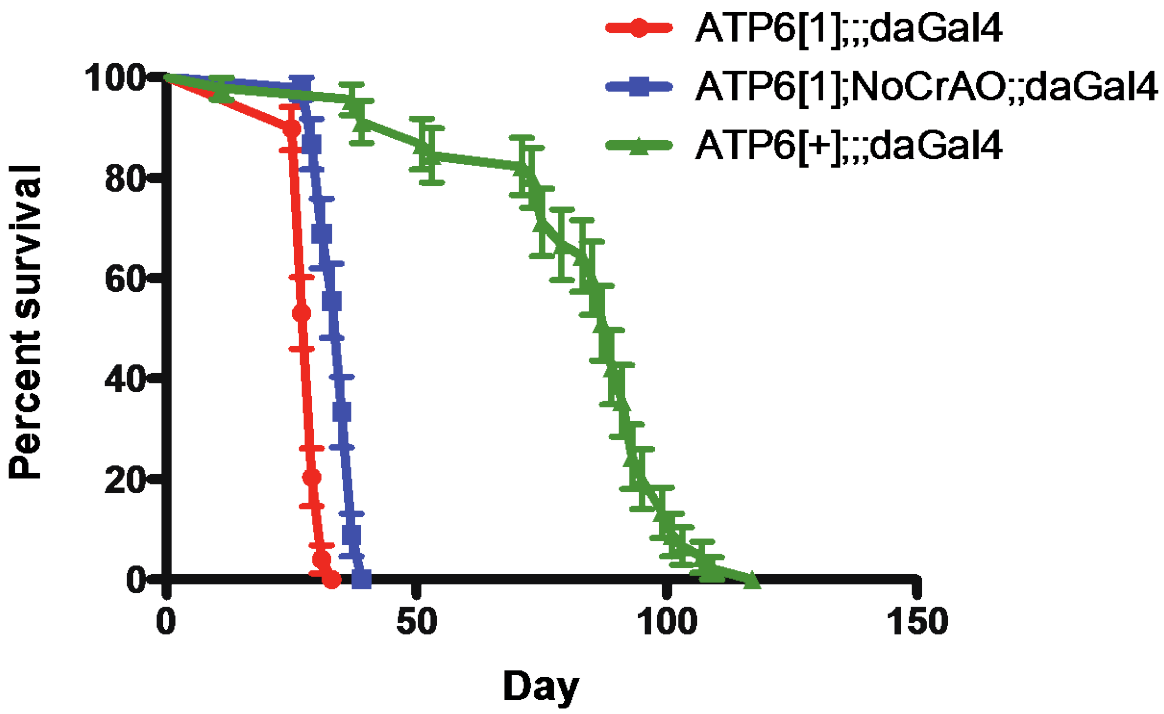

Figure 39. Improvement in Lifespan in *ATP6[1]* Mutant Flies with Algal ATP6 Expression.

Expression of algal ATP6 in *ATP6[1]* mutant flies provided statistically significant improvement in lifespan. Average lifespan for *ATP6[1]* mutant flies is 29 days. *ATP6[1];NoCrAO;;daGal4* is 35 days, and *ATP6[+];;;daGal4* is 89 days.

We have also attempted to perform ATP synthase activity assay. This assay provides the most definitive proof that the allotopically-expressed ATP6 is incorporated properly and functional. However, multiple protocols have been tested and we have not been able to obtain reproducible results. This result is hardly atypical as ATP synthase assay is very sensitive to protein and detergent concentration [270], and some *in vitro* studies of ATP6 allotopic expression have also failed to show improved ATP synthase activity outcome [257, 270].

When testing different MTSs and 3'UTRs, our hypothesis was that a single construct would be the optimal combination and provide significantly better functional readouts than the rest of the constructs. We would then utilize that one particular construct to test other strategies. However, most transgenic constructs with different MTSs and 3'UTRs delivered moderate improvements and none of them stood out. It was probably because none of the MTSs were as hydrophilic and as long as the algal MTS, and thus their potentials were limited. Moreover, the very hydrophobic fly ATP6 protein might be too difficult to be imported into the mitochondria even with exogenous MTS and 3'UTR in sufficient quantities. However, we did see improvements in both lifespan and mechanical stress test recovery time, so they were able to deliver some improvement although not at levels that we would have liked. One obvious question was how efficiently was engineered ATP6 protein imported into the mitochondria? Unfortunately, our custom *Drosophila* anti-ATP6 antibody could not distinguish the engineered ATP6 protein from the endogenous mutant ATP6 protein. It would have been useful to determine the efficacy of each strategy by first measuring the total engineered ATP6 proteins that were translated and then performing mitochondrial purification and measuring the amount of engineered ATP6 proteins that were imported into the mitochondria.

Overall, where we did get siginifcant rescues nothing came close to giving us a complete rescue (Figure 39). It is likely that only a small amount of *Drosophila* ATP6 was imported as allotopic expression of human ATP6 in cybrids yields a 18.5% import efficiency [255]. However, it is also possible that ATP6 protein level is expressed at a very high level that it becomes toxic to the animals as daughterless-Gal4 is a ubiquitous promoter that is expressed in all tissues. We have a *Drosophila* anti-ATP6 antibody, however, we cannot distinguish mutant, wildtype or engineered forms of the protein at this time. Non-protein-coding RNAs can affect transcription [329]. Primers can be designed to determine the transcription efficiency as measured by quantitative RT-PCR to rule out inefficient transcription. We could possibly distinguish between low expression and overexpression toxicity with Western blotting using anti-ATP6. Many possibilities exist as to what happens downstream after the nucleus-encoded *ATP6* gene is inserted. Questions such as transcription efficiency, translation efficiency, how the ATP6 proteins interact with other proteins, such as heat shock proteins, in the cytosol, whether the engineered, hydrophobic ATP6 proteins would aggregate and be targeted for degradation, whether the entire ATP6 polypeptide was imported into the mitochondria instead of tethering at the import pore, and whether it folds into proper conformation and inserts correctly into the ATP synthase complex remain to be answered.

Transgenic fly strains with suboptimally-encoded codons did not consistently perform better than the fly strains with regular recoded codons in functional assays. In theory, synchronizing the speed of translation and the speed of import should allow, presumably, more engineered ATP6 protein to be imported into the mitochondria. However, there were so many

factors at play such as hydrophobicity and competition from the endogenous ATP6 proteins that this might not even be a crucial factor.

Another potential issue was that an imprecise number of ATP6 subunit was imported. As ATP6 synthase complex has multiple subunits, importing excessive number of ATP6 may interfere with proper incorporation and toxicity while lower-than-required ATP6 importation may not deliver optimal rescue result. Being able to quantify the amount of proteins that are transported into the mitochondria will be an important first step before we can control the amount of proteins that are imported.

We sought to overexpress several factors that played important roles in mitochondrial functions. We examined PGC1-α, AMPK, DSP1 for their roles in mitochondrial biogenesis, mtHSP70 for its role in mitochondrial protein folding and unfolding, ATG1 for its role in autophagy, and OXA1 for its role in the conservative pathway for mitochondrial import. For PGC1-α, overexpressing PGC1-α led to decreased lifespan, which was consistent with a published report [324]. However, overexpressing PGC1-α in the digestive tract increased lifespan in wild type *Drosophila*. A follow-up on this experiment would be to utilize intestinal-specific driver to increase PGC1-α expression in the *ATP6[1]* mutant flies. A caveat is that the mitochondrial mutation in the *ATP6[1]* mutant flies is present in 98% of the mitochondria that presents in all tissues; therefore, whether driving expression only in the intestinal tract is sufficient to increase lifespan is unclear. However, limiting expression with tissue specificity may circumvent potential toxicity. For AMPK, the literature of its effects on *Drosophila* is mixed. A report showed that overexpressing AMPK in adult fat body or adult muscles improved

lifespan [330], but flies that were fed with metformin, which activated AMPK, did not have increased longevity [331]. All our transgenic flies were driven by broadly-expressed daughterless-GAL4, and PAS, PAO and sOAS transgenic flies showed significant lifespan improvement at 25°C. Utilizing tissue-specific Gal4 may help further improve the efficacy of AMPK overexpression in the *ATP6[1]* mutants. Overexpressing DSP1 was unsuccessful due to the difficulty in obtaining the desired genotype. Overexpressing ATG1 is developmentally lethal when driven by daughterless-GAL4. Therefore, a follow-up experiment will be to limit its expression to muscle-specific GAL4 or neuron-specific GAL4. For mtHSP70, there was no significant improvement in lifespan or stress test recovery time in flies overexpressing mtHSP70. It could be due to tissue specificity or other factors upstream (e.g. hydrophobicity) that lowered the efficacy of mtHSP70 function. Flies overexpressing OXA1 showed obvious toxicity with significantly shortened lifespan and malformed wings. It is unclear why overexpressing OXA1 would lead to malformation. It is possible that overexpressing OXA1 might have led to increased insertion of other proteins that utilized the conservative pathway and overwhelmed the import system. Moreover, because ATP6 does not require import under natural circumstances, we made the best guess that it would utilize the conservative pathway. It is possible that engineered ATP6 utilized another pathway, but it is unlikely due to the protein characteristics that utilize each pathway. In conclusion, tissue-specificity seems to be a crucial determinant in overexpressing proteins that play roles in mitochondrial functions.

Translational Inhibitor (TLI) was developed in our lab to address another major obstacle with ATP6 protein import – competition between endogenous ATP6 and engineered ATP6 to get incorporated into the ATP synthase complex. It is an engineered RNA that is complementary to

the target mRNA. Once it binds to the target mRNA, it prevents the docking of the mitochondrial ribosomes and thus reduces translation of the target protein. In this project, we utilized two different non-coding leader sequences (NCLs) – MRP and RNP - to determine which one delivered a better outcome. We did notice that the lifespan and the mechanical stress test recovery time improved when flies co-expressed algal transgenes and translational inhibitors comparing to flies expressing algal transgenes alone. However, as stated earlier, the flies expressing algal transgenes in a VK27 background did not perform better than the VK27 control. This is different from the results we observed when the flies expressing algal transgenes were in attp18 background. This shows the importance of background and how it can affect phenotypic outcomes significantly.

The most promising avenue of future research is to study MTS as a major determinant of import efficiency. The main difference between the algal ATP6 and the recoded *Drosophila* ATP6 was the size and hydrophilicity of the MTS and N-terminus of the nascent polypeptide. In yeast, using a tandem duplication of the *Neurospora crassa* ATPase9 MTS was sufficient to overcome the obstacle of importing ATP8, when a single ATPase9 MTS was not able to do so [332]. We could utilize a similar method (e.g. duplicate P1 MTS). However, the main obstacle - the hydrophobicity of the ATP6 protein – remained.

Overall, algae *Chlamydomonas reinhardtii* ATP6 protein provides the best rescue efficacy to a *Drosophila* strain with an *ATP6[1]* mutation utilizing allotopic protein expression. Based on our results, we conclude that MTS is a major determinant of import efficiency. Other factors that have been shown to be efficacious in *in vitro* conditions provide modest

improvements in an *in vivo* animal model. Competition from the endogenous ATP6 protein can be effectively addressed by TLI. However, reducing ATP6 hydrophobicity will be crucial in facilitating ATP6 protein import into mitochondria and improving functional outcomes. One way to circumvent ATP6 protein hydrophobicity altogether has already been investigated: allotopic *ATP6* RNA import. Recoded *ATP6* gene is expressed in the nucleus and *ATP6* mRNA is transcribed. However, the mRNA will be transported into the mitochondria and the mitochondrial translational machinery will translate the protein, and thus circumventing the challenge of importing a hydrophobic protein through the TIM-TOM complex. There are four components to this engineered *ATP6* sequence, similar to the TLI construct shown in Figure 18B, except that the antisense RNA is replaced by the recoded *ATP6* mRNA sequence. The MTS used in this dissertation will be replaced by non-coding leader sequences such as 5s, MRP, and RNP. 5s rRNA is readily found in the mitochondria [333, 334], and MRP and RNP are readily imported into mammalian mitochondria [335]. These structural elements will target the engineered *ATP6* mRNA into the mitochondria. This strategy has already been met with success. In a project headed by graduate student Atif Towheed in the Palladino lab, it has been shown that importing engineered *ATP6* RNA and TLI leads to significantly improved lifespan and locomotor function (Towheed et al., manuscript in preparation).

The ultimate goal of *ATP6* allotopic expression research is to translate the functional improvements in an animal model to human patients. To this end, developing a targeted delivery system is necessary. For treatment of Leber's hereditary optic neuropathy (LHON), delivery of *ND4*, also a mitochondrially-encoded gene, by Adeno-Associated Virus (AAV) has already been tested for efficacy and safety in the mouse system [336-338] and *ex vivo* primate eyes [339]. A

human clinical trial is underway [340, 341]. AAV can accommodate genes up to 5kb in size, and thus it can deliver most mitochondrial genes [338]. Although the mode of delivery of *ND4* may apply to *ATP6*, there are several fundamental differences between the two mutations. Patients suffering visual loss from LHON usually have 100% mutant mtDNA homoplamy. This suggests that even a very small amount of transgene delivery to LHON patients might be sufficient to prevent visual loss. Moreover, *ND4* delivery is narrowly targeted to the eye by injection, while *ATP6* will potentially be delivered to multiple organs, raising the challenge of delivering the optimal dosage of transgene (and the translated protein) that is efficacious but non-toxic to the cells.

Besides virus-mediated delivery system, embryonic extrapolation represents another potential venue in treating patients with *ATP6* mutations at a very early stage. Extrapolation involves delivering short high-voltage pulses to temporarily permeabilize the cell membrane, which allows a variety of molecules, including DNA, to be imported into a cell. It is possible that after *in vitro* fertilization process that the zygote or the blastomeres be subjected to electroporation, allowing the recoded *ATP6* transgene be delivered into the cell. The challenges associated with this strategy include the stability of *ATP6* transgene in the cell and integration issue with the existing nuclear genome; however, it has been successfully demonstrated in a ND4 rat model [342].

Because pharmacotherapy has been studied extensively but none of the drug cocktails is particularly promising, it is reasonable to explore gene therapy for potential cures. Overall, ATP6 protein allotopic expression *in vitro* has been explored for more than a decade with conflicting

reports on its efficacy and feasibility, and ATP6 protein allotopic expression *in vivo* has only been explored by two labs so far (Palladino lab and Pinkert lab from Auburn University). Many obstacles such as hydrophobicity, localization, endogenous mutant ATP6 competition, proper incorporation still exist and require further investigation. However, it is highly like that with *ATP6* RNA import and other technical developments these challenges will be overcome. Subsequent optimization of a delivery system *in vitro* and *in vivo* will ensue, and based on the example of *ND4* it is believed that allotopic expression of *ATP6* is a promising genetic approach to treating mitochondrial diseases in humans.

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