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Bacteria, Yeast, Worms, and Flies: Exploiting simple model organisms to investigate human mitochondrial diseases

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Abstract

The extensive conservation of mitochondrial structure, composition, and function across evolution offers a unique opportunity to expand our understanding of human mitochondrial biology and disease. By investigating the biology of much simpler model organisms, it is often possible to answer questions that are unreachable at the clinical level. Here, we review the relative utility of four different model organisms, namely the bacteria *Escherichia coli*, the yeast *Saccharomyces cerevisiae,* the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, in studying the role of mitochondrial proteins relevant to human disease. *E. coli* are single cell, prokaryotic bacteria that have proven to be a useful model system in which to investigate mitochondrial respiratory chain protein structure and function. *S. cerevisiae* is a single-celled eukaryote that can grow equally well by mitochondrial-dependent respiration or by ethanol fermentation, a property that has proven to be a veritable boon for investigating mitochondrial functionality. *C. elegans* is a multi-cellular, microscopic worm that is organized into five major tissues and has proven to be a robust model animal for *in vitro* and *in vivo* studies of primary

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"Here, we review the relative utility of four model organisms, namely the bacteria *Escherichia coli,* the yeast *Saccharomyces cerevisiae,* the microscopic roundworm *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*, emphasizing insights each has afforded in the pursuit of improved understanding of the role and functions of mitochondrial gene mutations and proteins relevant to human disease"

"Utilizing evolutionary homology allows key components held in common across divergent classes of organisms to be recognized and studied"

"In the context of the newly reported structural framework, future mutagenesis and structure/function studies of *E. coli* complex I will likely yield not only fundamental insight into energy coupling mechanisms in complex I but also provide detailed understanding at the molecular level of presumed pathogenic human mutations."

"The peculiarities afforded by yeast as a model organism are accelerating our understanding of mitochondrial function and clarifying the relevance of mitochondrial function to human disease."

"Many therapeutic agents of purported benefit in human RC disease are amenable to mechanistic study in *C. elegans* models of primary respiratory chain dysfunction."

"Over the past ten years mutations in several *Drosophila* orthologs of human mitochondrial disease genes, or genes critical to fundamental mitochondrial functions, have been described."

respiratory chain dysfunction and its potential therapies in humans. Studied for over a century, *D. melanogaster* is a classic metazoan model system offering an abundance of genetic tools and reagents that facilitates investigations of mitochondrial biology using both forward and reverse genetics. The respective strengths and limitations of each species relative to mitochondrial studies are explored. In addition, an overview is provided of major discoveries made in mitochondrial biology in each of these four model systems.

Keywords

Escherichia coli; *Saccharomyces cerevisiae*; *Caenhorabditis elegans*; *Drosophila melanogaster*; mitochondria; model organisms

I. Introduction

Mitochondria are organelles present in all eukaryotic species. Nevertheless, all eukaryotes do not contain prototypic mitochondria, nor are all mitochondria the same within a single organism. The suite of functions performed by mitochondria varies, depending both on environmental conditions and tissue demands. These findings imply that mitochondria are dynamic organelles which, even today, remain under evolutionary pressure. Best scientific estimates trace the origin of mitochondria to a purple eubacteria-like organism that established itself in an endosymbiosis with a primitive nucleated cell approximately 2 billion years ago (Margulis 1996). The transition from this ancestral purple eubacteria into modern mitochondria has been accompanied by major macromolecular rearrangements. Entire metabolic pathways have vanished, most genetic information necessary for mitochondrial structure and function has been transferred to the nucleus, and new protein functions have been acquired within mitochondria largely from their co-evolving eukaryotic host. Indeed, proteins such as the ATP/ADP translocase, the entire family of metabolite carrier proteins, and Mia40 of the intermembrane space protein-assembly machinery were all acquired on the road to becoming modern mitochondria (Chacinska et al. 2009; Gabaldon and Huynen 2004).

Approximately 1,100 mouse mitochondrial proteins have been identified, which represent on the order of 85% of the total predicted mitochondrial protein content (Pagliarini et al. 2008). It is highly likely that a similar complement of mitochondrial proteins exist in humans (Pagliarini et al. 2008). To date, 184 mitochondria-localized proteins have been implicated in human disorders (Chinault et al. 2009; Schwimmer et al. 2006). Although the vast majority of mitochondrial proteins appear to have been at least tentatively identified, the means by which mutated versions of these proteins lead to mitochondrial diseases that have a broad spectrum of pathogenesis and phenotypic presentations remain largely unexplained. Just as model organisms have helped us identify many of the known mitochondrial proteins, they can also be used to discern their roles in the etiology of mitochondrial diseases to improve our understanding of causative factors for the nearly 60% of mitochondrial disorders which have no definitive etiology (Dimmer et al. 2002). Here, we will review the relative utility of four model organisms, namely the bacteria *Escherichia coli*, the yeast *Saccharomyces cerevisiae,* the roundworm *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*, emphasizing insights each has afforded in the pursuit of improved understanding of the role and functions of mitochondrial proteins relevant to human disease.

II. The Bacteria, *Escherichia Coli*

IIA. Utility of bacteria as a model system in which to study mitochondrial composition and function

Bacteria are single cell, prokaryotic organisms that are typically several microns in length, which is similar in size to mitochondria. They take on a range of shapes including spheres, rods, and spirals (Figure 2A, left image). The cell membrane in gram-negative bacteria, such as *Escherichia coli*, is separated by the periplasm from a peptidoglycan layer and an outer membrane. In contrast, there is no periplasm in gram-positive bacteria and their cell wall is closely juxtaposed to the cytoplasmic membrane. Oxidative phosphorylation exists in many genera of bacteria (Haddock 1980; Zannoni 2004). Recent focus on bacterial electron transport mechanisms stems from their structural simplicity in comparison with eukaryote counterparts, their short generation time on the order of 20–40 minutes, and their relative ease of gene manipulation. Bacteria have proven a useful model system in which to investigate protein structure and function, although these single cell organisms cannot be used to directly study manifestations of human diseases. Knowledge gained in the bacteria model can often be applied to homologous proteins in more complex higher organisms.

IIB. Electron transport chains in bacteria

Electron transport systems are located in the cytoplasmic membrane of numerous species of aerobic, or facultative, bacteria. Specific components involved in electron transfer can be highly diversified between organisms, or even within the same organism depending on growth conditions. In contrast to mitochondria, individual bacteria utilize multiple electron transport chains, often simultaneously. Bacteria have a number of different dehydrogenases, oxidases and reductases that are used for this purpose, as well as a variety of different electron donors and acceptors (Zannoni 2004). Individual enzymes for susbstrate oxidation and reduction are variably expressed according to environmental conditions. For example, when *E. coli* are grown aerobically, many of their electron transport components are distinct from those found in mammalian mitochondria. *E. coli* uses two different NADH dehydrogenases. One is the proton-translocating enzyme, NDH-1, which is very similar to mitochondrial complex I; the other is a single polypeptide enzyme, alternative NADH-quinone oxidoreductase (NDH-2), which does not translocate protons (Yagi et al. 1998). They also have two different quinol oxidases. One is the cytochrome bo_3 complex, which contains a heme-copper center related to the cytochrome aa_3 family that includes mitochondrial complex IV; the other is the cytochrome *bd* complex, which contains heme proteins and a chlorine-Fe-protein (Unden and Bongaerts 1997). Cytochrome *bd* has a much higher affinity for oxygen than cytochrome bo_3 and is induced under lower oxygen pressure. However, *E. coli* have no detectable c-type cytochromes (Unden and Bongaerts 1997). Since bc_1 complex (complex III) is also absent, ubiquinol is oxidized directly in *E. coli*. Thus, *E. coli* clearly has a truncated electron transfer chain relative to that in mitochondria. On the other hand, the electron transport system of the soil bacteria, *Paracoccus denitrificans*, has many features that are similar to those in mitochondria: complex I/NDH-1, succinate dehydrogenase (complex II), bc_1 complex, and cytochorome aa_3 (Baker et al. 1998; Zannoni 2004). *P. denitrificans* does not have a NDH-2 type enzyme, but does have other ba_3 and cbb_3 oxidases (Baker et al. 1998).

IIC. Modeling mitochondrial respiratory complexes in bacteria

Lacking an intricate coding background and mitochondrial assembly pathways, bacterial enzymes typically involve a much simpler subunit composition than those in mitochondria. Nonetheless, bacterial enzymes maintain all fundamental functions established for their mitochondrial “relatives”. The number of protein subunits in mammalian and bacterial respiratory chain complexes, respectively, includes: complex I, 45:14; complex II, 4:4;

complex III, 11:3; complex IV, 13:4. Electron flow from reducing substrates to oxygen is coupled to transmembrane proton movements, just as it is in the mitochondrial respiratory chain.

Complex I, NADH:ubiquinone oxidoreductase, is the main entrance for electrons into the respiratory chains of many bacteria and mitochondria of most eukaryotes. It couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. The bacterial complex I, in general, is made up of 14 different subunits, representing a minimal structural form of complex I (Yagi et al. 1998) (Figure 2A, right image). In *E. coli,* the genes encoding two of the subunits are fused to form one gene, nuoCD. The 14 *E. coli* complex I subunits together have a molecular mass of approximately 530 kDa (Friedrich 1998). Seven are peripheral proteins including the subunits that bind all known redox groups of complex I, namely one FMN and eight or nine iron-sulfur clusters (Friedrich 1998). The remaining seven subunits are hydrophobic membrane proteins, which have recently been shown to fold into 63 α-helices across the cell membrane (Efremov et al. 2010); little is known about their function, but they are most likely involved in quinone reduction and proton translocation (Friedrich 1998). In contrast, mammalian complex I has 45 different protein subunits with a total molecular mass of approximately 1 MDa (Carroll et al. 2006). The majority of these subunits have no known function (Brandt 2006). However, by comparison with their simpler bacterial homologues, it is apparent that the core catalytic structure of mammalian complex I that carries out electron transfer and proton pumping functions involves only 14 subunits, all of which are homologous to the 14 bacterial complex I subunits (Brandt 2006). The homologs of the seven hydrophobic bacterial membrane subunits (NuoA, H, J, K, L, M, and N) are encoded by mitochondrial DNA (mtDNA) in all eukaryotes as ND3, ND1, ND6, ND4L, ND5, ND4, and ND2, respectively. Electron microscopy has established that both mitochondrial and bacterial complex I have a characteristic L-shaped structure that consists of two domains, a peripheral arm and a membrane domain (Brandt 2006; Zannoni 2004). Both complexes have similar electron-transfer and energy-transduction pathways, and are sensitive to the same inhibitors such as piericidin A, capcaisin or acetogenins, suggesting that the bacterial complex I may serve as a useful model system for the study of the human enzyme complex I.

In fact, utilizing *P. denitrificans, Thermus thermophilus, Rhodobacter capsulatus* and *E. coli*, several groups succeeded in identifying 8–9 iron-sulfur clusters, N1a, N1b, N2, N3, N4, N5, N6a, N6b, and N7 in each of the peripheral subcomplex arms of complex I (Chevallet et al. 1997; Dupuis et al. 1998; Flemming et al. 2003; Nakamaru-Ogiso et al. 2008; Nakamaru-Ogiso et al. 2002; Nakamaru-Ogiso et al. 2005; Rasmussen et al. 2001; Velazquez et al. 2005; Yano et al. 1999; Yano et al. 2003; Yano et al. 1994; Yano et al. 1995). Those assignments were recently confirmed by demonstration of the X-ray crystal structure of the hydrophilic peripheral part of *T. thermophilus* complex I that was determined at 3.3 angstrom (Å) resolution (Sazanov and Hinchliffe 2006). In addition, recent information about the structure of the membrane domain subunits became available although at lower resolution (4.5 Å and 3.9 Å) (Efremov et al. 2010). A speculative arrangement (and topology) of the membrane segment of *E. coli* complex I, which was previously proposed based on the projection structure of the membrane domain and detergent-based fractionation study (Baranova et al. 2007; Holt et al. 2003), has now been confirmed by the X-ray crystal structure of bacterial complex I (Efremov et al. 2010). Subunits NuoH (ND1), NuoA (ND3), NuoJ (ND6), and NuoK (ND4L) are present in the vicinity of the peripheral arm, whereas the NuoL (ND5) and NuoM (ND4) subunits are distantly located from the peripheral segment. NuoN (ND2) is located in the middle of the membrane arm. Since similar subcomplex patterns of ND subunits have been obtained with bovine heart complex I after detergent treatment (Sazanov and Walker 2000), this recent insight into structural organization can now be directly applied to mammalian complex I. Thus, it is expected that

investigation into the roles of membrane-bound complex I subunits in proton translocation, ubiquinone/inhibitor binding sites, and the mechanisms linking mutations in these subunits to human mitochondrial diseases will now be quickly advanced.

Complex III, also called ubiquinol:cytochrome c oxidoreductase or the bc_1 complex, is a key component for both bacterial respiration and photosynthesis. The simplest form of the cytochrome bc_1 is found in prokaryotes and is comprised of three redox-active subunits, which bear two b-type hemes, one c-type heme, and one [2Fe-2S] cluster (Rieske protein) as prosthetic groups (Zannoni 2004). Photosynthetic bacteria like *Rhodobacter* species have provided powerful models for studying the function and structure of this enzyme. In recent years, extensive use of spontaneous and site-directed mutants, investigation of new inhibitors, and engineering of novel *bc*1 complexes have provided us with a wealth of information on the functional mechanisms, subunit interactions, and assembly of this important enzyme. Recent resolution of the structure of various mitochondrial bc_1 complexes in different crystallographic forms (Berry et al. 2000) has consolidated previous findings, and raised new issues, such as the unique mobility of the Rieske protein subunit during Qo site catalysis (Lee et al. 2006; Lee et al. 2008). Multidisciplinary approaches combining physiologic, molecular genetic, biochemical, and biophysical techniques have been extremely successful in the bacterial bc_1 complex to serve as a guiding light for all organisms and their organelles.

Complex IV, cytochrome c oxidase, is a large transmembrane protein complex found in both bacteria and mitochondria. It is the last enzyme in the electron transport chain of mitochondria. It receives an electron from each of four cytochrome c molecules, transfers them to one oxygen molecule, and then converts molecular oxygen to two molecules of water. While the canonical mitochondrial complexes have been investigated for almost five decades, the corresponding bacterial enzymes have been established only recently as attractive model systems in which to address basic reactions in electron transfer and energy transduction. Two different preparations have previously been studied for the aa_3-type cytochrome c oxidase isolated from *P. denitrificans* (Richter and Ludwig 2009): it was originally isolated as a two-subunit enzyme in the presence of Triton X-100 (Richter and Ludwig 2009) and later isolated as a four-subunit complex using dodecyl maltoside for solubilization (Richter and Ludwig 2009). These studies demonstrated that cytochrome c oxidase subunits I–III (but not subunit IV) exhibit high sequence identities with their corresponding mtDNA-encoded subunits of the eukaryotic enzyme. Not unexpectedly, both bacterial preparations were indistinguishable from the 13-subunit enzyme isolated from mammalian mitochondria in terms of their energy transduction properties and basic 3-D structure as deduced from X-ray crystal analyses. Furthermore, subunits III and IV do not contribute to the redox-related signals observed by fourier transform infrared (FTIR) spectroscopy (Hellwig et al. 1998), nor do they influence the electron transfer reaction to any appreciable extent. Recent research questions ranging from primary steps in cofactor insertion to supramolecular architecture of electron transfer complexes, can also be favorably addressed in prokaryotic systems to improve understanding of prototypic structures and mechanisms common to all family members.

IID. Investigating complex I dysfunction by modeling point mutations in mtDNA-encoded ND subunits

Mitochondrial DNA contains 37 genes, thirteen of which encode polypeptides that are all essential components of the respiratory chain. Indeed, these 13 proteins are highly conserved subunits in respiratory enzyme complexes (complex I, III, IV, and or ATP synthase) from bacteria to mitochondria. Seven of these proteins constitute the core hydrophobic membrane subunits of complex I. A number of point mutations reported in these subunits have been implicated in human mitochondrial diseases including Leber Hereditary Optic Neuropathy

(LHON) and Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) (DiMauro and Schon 2003). Complex I dysfunction has also been implicated in a variety of human diseases including heart failure, diabetes mellitus, and several neurodegenerative diseases such as Parkinson's disease (DiMauro and Schon 2003; Singer et al. 1993). Although complex I dysfunction is postulated to promote apoptosis through enhanced reactive oxygen species (ROS) production from the damaged enzyme (Sheeran and Pepe 2006), the pathophysiology of complex I-related diseases is complex and multifactorial. However, at least identifying the detrimental mtDNA mutations offers a rational approach to investigate pathogenesis. Analysis of the bacterial enzymes has a clear advantage when the target protein is mtDNA-encoded in eukaryotes. Studying the effects and implications of engineered mutations in the ND subunits of the complex I enzyme relative to its structure, respiratory chain function, and/or reactive oxygen species production is a prerequisite to understand how individual disease mutations may contribute to disease states.

In that respect, *E. coli* complex I is a powerful model system in which to exploit the convenient tool of gene manipulation (especially site-specific mutagenesis), which is virtually impossible in mammalian complex I for mtDNA-encoded subunits such as ND5. Even commonly used RNA interference technique cannot be applied to knockdown mtDNA-encoded subunits. Transmitochondrial cybrid systems are useful but offer very limited capacity to carry out mechanistic studies at the enzyme level. Fortunately, human disease mutations in mitochondrial complex I involve amino acid residues that are mostly conserved in *E. coli* complex I. In the case of ND5, they are perfectly conserved (Figure 3, red font). Some are also conserved with other multi-subunit secondary Na^+/H^+ antiporters, which show high sequence similarity to ND5 and to other transporter module subunits, such as ND2 and ND4 of complex I. F124L causes Leigh Disease, E145G causes MELAS, A171V, G239S and G465E cause LHON, M237L causes MELAS/Leigh /LHON overlap syndrome, and D393N/G causes MELAS/Leigh Disease. Very recently, mutational analyses of the corresponding mutations at E144 and D400 of the *E. coli* NuoL (ND5) subunit suggested that these residues are critical for the normal complex I functions of electron transfer and/or proton pumping (Nakamaru-Ogiso *et al.* submitted). The A52T mutation in ND1 that causes LHON was introduced into its homologous subunit, NQO8 of *P. denitrificans* complex I, revealing that the mutated residue plays an important role in ubiquinone reduction by complex I (Zickermann et al. 1998). Other ND1 pathogenic mutations, such as the E24K LHON/MELAS mutation and the R25Q, G131S, and E214K MELAS mutations, were also found to play an important role for complex I activities (Sinha et al. 2009). The equivalent *E. coli* mutant of the common M64V LHON mutation in ND6 demonstrated a mild effect on *E. coli* complex I activity (Kao et al. 2005). These data demonstrate the feasibility and utility of modeling amino acid substitutions caused by mtDNA mutations in homologous positions in the prokaryotic enzyme.

However, there are clearly some limitations to the utilization of the bacterial system. Although the residue associated with the E143K LHON mutation in ND1 is almost completely conserved, the corresponding *E. coli* mutant (E157K) actually augmented peripheral complex I activity to 160% of control (Sinha et al. 2009). Slight mitochondrial proliferation with abnormal mitochondria has previously been reported in affected human patients (Valentino et al. 2004). Introducing human pathogenic mutations at non-conserved residues in ND1 has recently been attempted in its homologus subunit, NuoH, of *E. coli* complex I (Maliniemi et al. 2009). The overall effects of mutations were milder in this system such that results did not support the pathogenicity of the sole ND1-L285P/NuoH-V297P mutation or the suppressor effect of the ND1-Y277C/NuoH-L289C mutation on the former one, which has been previously suggested from the study of clinical/biochemical phenotypes in affected family members carrying these mutations (Howell et al. 1991). As

another example, Hofhaus *et al.* (Howell et al. 1991) and Park *et al.* (Park et al. 2007) reported that NADH dehydrogenase-dependent respiration measured in digitonin-permeabilized cybrid cells was specifically decreased by 40% in cells harboring a homoplasmic ND4 R340H mutation that is associated with LHON. The corresponding *E. coli* R369H mutant in NuoM showed only slightly decreased complex I activity (70% of control) (Torres-Bacete et al. 2007), while the corresponding *R. capsulatus* R368H mutant in NuoM showed a clear impairment in oxidative phosphorylation capacity (Lunardi et al. 1998).

In conclusion, the bacterial system can be beneficial for comprehensive studies investigating effects of mtDNA mutations known to cause human complex I deficient mitochondrial diseases. However, much more detailed information is first needed on the basic structure and function of complex I. Without basic knowledge, it will be extremely difficult to assess whether pathogenic human mutations are in functionally-important regions (such as inhibitor/ubiquinone binding sites and/or proton-pumping sites) and what effects on complex I function might be anticipated. Knowledge of mitochondrial complex I is still too limited to completely understand human mitochondrial diseases involving complex I dysfunction. However, for the first time, the X-ray crystal structures of the entire complex I from *T. thermophilus* as well as the membrane domain of *E. coli* complex I have been determined (Efremov et al. 2010). This structural framework provided surprising new information that NuoL contains an unusually long, conserved, amphipathic helix that extends ~110 Å to a position near the catalytic site from the end of the membrane arm (Efremov et al. 2010), suggesting that it constitutes a mechanical link capable of transmitting conformational changes. This structural information suggests an unprecedented, unique, and unexpected role for ND5 (NuoL) in the redox-driven proton pumping mechanism of complex I. In the context of the newly reported structural framework, future mutagenesis and structure/function studies of *E. coli* complex I will likely yield not only fundamental insight into energy coupling mechanisms in complex I but also provide detailed understanding at the molecular level of presumed pathogenic human mutations.

III. The Yeast *Saccharomyces cerevisiae*

The term ‘yeast’ is often used synonomously with *Saccharomyces cerevisiae* – the powerful genetic model that also doubles as the well-known Baker’s and Brewer’s Yeast. *S. cerevisiae* is, however, only one of 1,500 yeast species that comprise the sub-kingdom Fungi. These organisms are some of the simplest eukaryotes known. Most yeast reproduce asexually by budding, although a few, such as S*chizosaccaromyces pombe,* do so by binary fission. Typical yeast, such as *S. cerevisiae*, measure only 5 to 10 microns in diameter (Figure 2B).

IIIA. Comparison of yeast and mammalian mitochondrial biology

As a single-celled organism, *S. cerevisiae* has no *physiologic* relevance to humans. However, it is a genetically-tractable eukaryote replete with many mitochondrial proteins that are orthologous to human proteins. Since the last common ancestor of fungi and animals dates back to approximately 450 million years ago (Margulis 1996), an estimated 75% of the evolutionary history of mitochondria in these two sub-kingdoms has been shared. Recent estimates suggest that up to 700 proteins in *S. cerevisiae* are required for mitochondrial function (Pagliarini et al. 2008; Perocchi et al. 2008; Reinders et al. 2006; Steinmetz et al. 2002). Of the 601 known yeast mitochondria proteins, 222 have clear human orthologs (see Table I for database links). Indeed, most of the recent approaches used to identify mitochondrial proteins in mammals (Pagliarini et al. 2008; Smith et al. 2007) were piloted in yeast. These include large-scale gene expression analyses (Epstein et al. 2001), mass spectrometry-based proteomics (Reinders and Sickmann 2007), sub-cellular localization

analysis using fluorescent reporters (Westermann and Neupert 2000), deletion phenotyping (Pan et al. 2004), and many computational methods designed to identify mitochondrial proteins by virtue of signature trafficking sequences (Nakai and Horton 1999). *S. cerevisiae* has also proven to be a powerful tool for studying diverse cellular processes such as mitochondrial biogenesis (Bolotin-Fukuhara and Grivell 1992; Chacinska et al. 2009), mitochondrial DNA (mtDNA) packaging and inheritance (Contamine and Picard 2000), retrograde regulation (Jazwinski 2005), mitochondrial metabolism (Christensen and MacKenzie 2006), and electron transport chain assembly and activity (Barrientos 2003). In both humans and yeast, the functions of mitochondria are now recognized to extend far beyond the synthesis of energy in the form of ATP. Rather, mitochondrial proteins participate in diverse processes ranging from ion flux regulation (Cardoso et al. 2010), ubiquinone production (Tran and Clarke 2007b), pyrimidine biosynthesis and single-carbon metabolism (Christensen and MacKenzie 2006) to amino acid catabolism (Metzler 1977), iron sulfur cluster assembly (Sheftel et al. 2010), and protein quality control (Koppen and Langer 2007; Rep and Grivell 1996).

Several properties of *S. cerevisiae* made it the early model of choice for studies of mitochondria. Foremost, *S. cerevisiae* is a facultative anaerobe that preferentially ferments glucose to ethanol under aerobic conditions. When glucose becomes limiting, ethanol is oxidized to produce reducing equivalents that fuel mitochondrial respiration. Thus, relatively few mitochondrial proteins are essential for viability in S. *cerevisiae*. Those mitochondrial proteins that do remain essential are restricted to protein import and maturation, iron sulfur cluster assembly, flavin mononucleotide synthesis, and uracil biosynthesis (Chacinska et al. 2009; Dimmer et al. 2002). Coupled with the ability to survive in either haploid or diploid states, their capacity to toggle between two metabolic states means many genetic disruptions that adversely affect mitochondrial functions in other species can be easily maintained and studied in *S. cerevisiae*. An additional advantage afforded by *S. cerevisiae* is the ease with which genes can be knocked out or heterologous proteins knocked-in and expressed. Such approaches have been used successfully in the quest to identify novel gene candidates responsible for human mitochondrial disorders. Two illustrative examples are provided by the nuclear-encoded *COX10* and *BCS1L* genes, where gene polymorphisms were suspected of causing a tubulopathy and leukodystrophy disorder and GRACILE syndrome, respectively. In both cases, yeast ‘complementation assays’ confirmed the role of these genes and polymorphisms in each disorder (Hinson et al. 2007).

The mitochondrial proteome has been shown to markedly differ between mouse tissues (Pagliarini et al. 2008). Analysis of 14 different tissues revealed that, on average, each tissue expressed only 700 of the known 1,098 mouse mitochondrial proteins (considering a 10% false discovery rate). Mitochondrial protein composition between tissue pairs were found to overlap by an average of 75%. However, a core set of approximately 300 proteins was present in every tissue, primarily representing proteins involved in oxidative phosphorylation (OXPHOS), the citric acid cycle, and folate metabolism. Analysis across 500 fully-sequenced species suggested much of this core mitochondrial proteome has been retained throughout evolution. Consistent with this idea, when *S. cerevisiae* is cultured on different non-fermentable carbon sources (e.g., lactate, ethanol and glycerol) a core set of mitochondrial proteins also can be discerned. On top of this core, there is a food type-specific set of mitochondrial proteins that is expressed under each condition (Steinmetz et al. 2002). Furthermore, 58 of 601 known yeast mitochondrial proteins are orthologous to genes causative of human disorders, most of which correspond to core components of intermediary metabolism and energy transduction.

IIIB. Modeling Human Mitochondrial Diseases in Yeast – Past, Present & Future

In humans, diseases associated with mitochondrial dysfunction can be broadly grouped into those arising from genes of nuclear origin and those arising from mutations in mitochondrial DNA (mtDNA). Diseases of nuclear origin can be further classified into diseases due to (i) defective mitochondrial biogenesis, (ii) defective mitochondrial protein import and export, (iii) defective assembly of the oxidative phosphorylation (OXPHOS) system, (iv) defective activity of the OXPHOS system, (v) defective enzymes of intermediary metabolism, (iv) defective mtDNA maintenance, and (vi) defective quality control proteins. *S. cerevisiae* has been used to model several human diseases spanning each of these classes and, in many cases, played an important role in facilitating identification of the genetic mutation or the causative mechanism underlying the human disease. A list of several such examples is provided in Table II. Several excellent reviews also provide detailed historical accounts of the role yeast have played in understanding human mitochondrial disorders (Barrientos 2003; Bassett et al. 1996; Fontanesi et al. 2009; Foury and Kucej 2002; Schwimmer et al. 2006).

The explosive growth of molecular biology in the 1990s, and of systems biology more recently, has led to the introduction of novel models and techniques for the study of human mitochondrial disorders. This has, in some respects relegated yeast to ‘just another tool in a box of many’. Nonetheless, yeast continues to yield major advances in the study of mitochondria. In the following section, we highlight several areas where the peculiarities afforded by yeast as a model organism are accelerating our understanding of mitochondrial function and clarifying the relevance of mitochondrial function to human disease. We end this section on a speculative note, looking at least one yeast research endeavor that might hold promise for understanding previously unappreciated forms of mitochondrial diseases.

IIIC. Yeast models of mitochondrial disease

i. Complex I deficiency – when nothing is something—In human mitochondria, 88 proteins collectively form the 5 complexes of the energy-generating electron transport chain (ETC). Although this number varies in yeast by species, *S. cerevisiae* contains 90 proteins that are currently annotated as respiratory chain-associated (MITOP, see Table 1), including a small number of paralogues and respiratory chain assembly factors. Two basic types of ETC proteins can be distinguished: (i) ‘structural core proteins’, which play an integral role in electron transfer or positive charge-translocation and derive from the original endosymbiont; and (ii) ‘supernumerary proteins’, representing all remaining proteins of largely unknown function that evolved as ‘add-ons’ to the ancient structural-core ETC. The eubacterial genome of present day mitochondria reflect their evolutionary past: in humans, the ancestral genome has been reduced to 13 core ETC protein-coding genes (ND1 to ND6 and ND4L of complex I, CYB of complex III, COI to COIII of complex IV, and ATP6 and ATP8 of complex V). In *S. cerevisiae,* the mitochondrial genome encodes just eight proteins (*CYTB, COX1, COX2, COX3, ATP6, ATP8, ATP9,* and a ribosomal protein, *VAR1*) (Bouchier et al. 2009; Tsang and Lemire 2003). These tiny genomes also demonstrate that even the smallest of ETC complexes cannot assemble spontaneously. Rather, it becomes clear that multiple factors are needed to control the assembly of each complex when one considers that (i) approximately half of all ETC proteins are integral membrane proteins, (ii) subunits decorate structural-cores on both cytosolic- and matrix-side of the inner membrane, (iii) most subunits have to navigate two membranes before undergoing additional peptide processing, and (iv) prosthetic groups are loaded into structural-core proteins after they reach their final destination. Yeast has played a pivotal role in elucidating the complicated process required to temporally and spatially control the assembly of each ETC complex.

NADH:ubiquinone oxidoreductase (complex I) deficiency is associated with a wide range of clinical phenotypes including Leigh Syndrome, Leber Hereditary Optic Neuropathy, and mitochondrial encephalopathy, lactic acidosis, and stroke (MELAS). With the advent of large scale sequencing tools it came as a surprise to find that mutations in complex I structural genes were absent in approximately 60% of cases where NADH:ubiquinone oxidoreductase deficiency could be confirmed biochemically (Smeitink et al. 2001). Such findings suggested auxiliary proteins were required for complex I assembly.

Many yeast species contain alternative NADH dehydrogenases that operate alongside complex I. These enzymes provide additional pathways for the transfer of reducing equivalents from NADH to the ETC without the concomitant pumping of protons across the inner mitochondrial membrane (Kerscher 2000). In some yeast species, complex I function has been lost entirely. Such is the case for *S. cerevisiae,* where three alternative NADH dehydrogenases operate to deliver reducing equivalents from the cytosol (NDE1, NDE2) or matrix (NDI1) to the mitochondrial ETC (Fang and Beattie 2003). Reasoning that loss of complex I should remove any evolutionary pressure to retain its assembly factors, Shoubridge and colleagues (Ogilvie et al. 2005) used an *in silico* approach to subtractively identify genes that had been differentially lost in *S. cerevisiae* but retained in *Y. lipolytica* and *D. hansenii*, which have both retained complex I functionality. This approach led to the identification of B17.2L (encoded by *NDUFAF2* in humans), which is a protein necessary for the maturation of an 830 kDA subassembly of complex I. The authors not only showed that a patient presenting with a progressive encephalopathy had a null mutation in *NDUFAF2* but that complex I dysfunction in fibroblasts from this patient was completely reversible by retroviral-mediated expression of B17.2L.

In a similar but broader fashion, Mootha and colleagues (Pagliarini et al. 2008) applied phylogenetic profiling across 42 fully-sequenced eukaryotic species to show that a set of 15 core complex I proteins had been independently lost four times across the course of evolution (two yeast and two protozoan clades). These authors also identified a set of 19 proteins that shared the same phylogenetic profile as the 15 core complex I proteins, making them candidates for complex I assembly factors. Biochemical analyses confirmed one of these proteins, *C8Orf38,* was indeed required for complex I activity and that mutations in this gene caused a novel form of Leigh Syndrome. Later studies confirmed that a second predicted protein, *C20orf7,* was also a *bona fide* complex I assembly factor (Gerards et al. 2009; Sugiana et al. 2008). Intriguingly, several of the remaining 17 predicted assembly factors encode metabolic enzymes of known function. It is unclear if these proteins evolved as an attachment to the structural-core to enhance substrate tunneling or if they serve as true folding factors.

ii. Fumarase – An old enzyme with a new function—Studies using the yeast *S. cerevisiae* have significantly advanced recent understanding of the tumor susceptibility syndrome, Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC), characterized by benign cutaneous and uterine leiomyomas, uterine leiomyosarcomas, and renal cell carcinomas (Launonen et al. 2001). HLRCC is almost always associated with bi-allelic inactivation of the citric acid cycle enzyme fumarase, which reversibly hydrates fumarate to malate. While the majority of fumarase is delivered to the mitochondrial matrix, a small fraction remains cytosolic (Karniely et al. 2006). This phenomenon arises because some fumarase molecules mature in the cytosol before being imported into mitochondria. Early maturation is not a problem for most mitochondria-targeted proteins since targeting pre-sequences direct uptake of mature proteins into the mitochondria. The mature fold for fumarate effectively masks its mitochondria import pre-sequence, however, leaving the protein stranded in the cytosol. The interesting observation that cytosolic fumarase is conserved from yeast to man strongly implies that cytosolic fumarase is not just the by-

product of inefficient protein import but likely has an important role. Until recently, cytosolic fumarase's function was assumed to be the scavenging of excess fumarate produced by two cellular sources: fumarylacetoacetate hydrolase in tyrosine catabolism, and arginosuccinate lyase in the urea cycle (Metzler 1977). HLRCC was speculated to result from loss of fumarase activity leading to elevated cytosolic fumarate, which can competitively inhibit HIF prolyl hydroxylase (HPH) and stabilize the transcriptional activator hypoxia-inducible factor (HIF) by preventing its proteasomal degradation (Koivunen et al. 2007). HIF plays an important role in regulating angiogenesis-regulated genes, such as *VEGF* (Michiels et al. 2001).

In a seminal study, Yogev and colleagues (Yogev et al. 2010) recently employed *S. cerevisiae* to unequivocally investigate the role of cytosolic fumarase. Utilizing a procedure effectively inaccessible to almost all other model organisms (Mireau et al. 2003), *S. cerevisiae* cells were re-engineered to express only mitochondria-localized fumarase. By knocking out the nuclear fumarase gene and knocking-in a mitochondrial DNA-encoded version, Yogev and colleagues discovered that cytosolic fumarase functions as part of a nuclear DNA damage response (DDR) pathway, which is normally activated following formation of double-strand breaks (DSBs). Hydroxyurea- or ionizing radiation-induced DSBs lead to the rapid translocation of cytosolic fumarase into the nucleus. Yeast mutants deficient for cytosolic fumarase displayed both delayed and abrogated activation of both the DDR-activated histone variant γH2A(X) that marks the sites of DNA breakage, and the Rad53p/CHK2 checkpoint protein. Intriguingly, addition of fumarate alone was sufficient to rescue the DNA repair deficit, whereas malate or a catalytically inactive form of fumarase was not. The DDR response function of cytosolic fumarase was found to be conserved in human HeLa cells. The precise role played by nuclear fumarate remains unclear.

iii. Ubiquinone Biosynthesis – A New Function for Coq4p—Ubiquinone (a.k.a. Coenzyme Q, CoQ10 in humans, or simply 'Q') is a mobile, one- or two-electron carrier molecule that is synthesized on the inner mitochondrial membrane and is essential for transferring reducing equivalents from complexes I and II to complex III in the ETC. Ubiquinone also serves as a direct electron acceptor for the uracil biosynthetic enzyme dihydroorotate dehydrogenase (Denis-Duphil 1989), and as an indirect electron acceptor for nine acyl-CoA dehydrogenases involved in lipid or amino acid metabolism (Ghisla and Thorpe 2004). The latter is accomplished by way of electron-transferring flavoprotein (ETF) and the CoQ10-linked mitochondrial ETF dehydrogenase (ETFDH). Ubiquinone is present in virtually all cell membranes, where it plays an important antioxidant role. Indeed, ubiquinone has both electron-scavenging and electron-donating capabilities (Santos-Ocana et al. 2002; Villalba and Navas 2000). In humans, CoQ deficiency presents with four major clinical manifestations: 1) encephalomyopathy, characterized by recurrent myoglobinuria, encephalopathy, and ragged-red fibers; 2) infantile multi-systemic disease, usually with prominent nephropathy and encephalopathy; 3) cerebellar ataxia with marked cerebellar atrophy; and 4) pure myopathy (Quinzii et al. 2008). Primary CoQ10 deficiencies due to mutations in ubiquinone biosynthetic genes (*COQ2, PDSS1, PDSS2*, and *ADCK3*) have been identified in patients with either the infantile multi-systemic or the cerebellar ataxia phenotypes (DiMauro et al. 2007; Gironi et al. 2004; Mollet et al. 2008). In contrast, secondary CoQ10 deficiencies due to mutations in genes not directly related to ubiquinone biosynthesis (*APTX, ETFDH*, and *BRAF*), have been identified in patients with either the cerebellar ataxia or pure myopathy phenotypes, as well in cardiofaciocutaneous syndrome (Aeby et al. 2007; Gempel et al. 2007; Musumeci et al. 2001).

Our knowledge of ubiquinone biosynthesis in humans is derived almost entirely from studies in the yeast *S. cerevisiae* and *Schizosaccharomyces pombe,* as well as the bacteria *Escherichia coli*. Ubiquinone is comprised of a fully-substituted benzoquinone head group

attached to a polyisoprenoid tail. The number of isoprenoid units is species specific, consisting of 10 in humans and 6 in *S. cerevisiae*. Nine complementation groups (*COQ1* through *COQ9*) define the Q-biosynthetic route in *S. cerevisiae* (Tzagoloff and Dieckmann 1990). Mammalian orthologs of yeast *COQ* genes have been identified by sequence homology and confirmed by functional complementation (Tran and Clarke 2007a). Biosynthesis of ubiquinone begins with the formation of the isoprenoid tail that is catalyzed by the polyprenyl diphosphate synthase, Coq1p (in humans, this function is undertaken by a related protein comprised of a heterodimer of PDSS1 and PDSS2 subunits). Next, the polyprenyl diphosphate tail is condensed with 4-hydroxybenzoic acid (derived from tyrosine) to form 4-hydroxy-3-polyprenylbenzoic acid (HHB) in a reaction catalyzed by Coq2p. Modification of the benzoquinone head group then follows a series of steps that remain only partially characterized and occur in an unknown order. These reactions include two *O*-methylation steps catalyzed by Coq3p, a *C*-methylation step catalyzed by Coq5p, two hydroxylation steps probably involving Coq6p, and a monoxygenase step catalyzed by Coq7p/CLK-1. Coq4p, Coq8p (a probable kinase, and orthologous to ADCK3 in humans) and Coq9p have unknown roles. An additional protein, Coq10, appears to be involved in ubiquinone trafficking, rather than its biosynthesis (Tran and Clarke 2007a).

In humans, more than half of patients that present with CoQ10 deficiency have unidentified genetic mutations (Quinzii et al. 2008). Based on studies in yeast, it is almost certain that disruption of any gene involved in ubiquinone biosynthesis will be pathogenic in people. Coq4p is a particularly good candidate since genetic disruption of this locus in *S. cerevisiae* is known to also result in the biochemical loss of Coq3p, Coq6p, Coq7p, and Coq9p (Marbois et al. 2009). The primary sequence of Coq4p was recently found to contain a putative zinc ligand motif HDxxH-$(x)_{11}$-E motif (Marbois et al. 2009) but, oddly, no enzymatic domains associated with the kind of catalytic activities necessary for modification of the benzoquinone head group. HA-tagged Coq9p can co-immunoprecipitate Coq4p, along with Coq5p, Coq6p and Coq7p. Based on these findings, Clarke and colleagues (Marbois et al. 2009) hypothesized that Coq4p forms a scaffold on the matrix side of the inner mitochondrial membrane upon which much of the ubiquinone biosynthesis machinery may be decorated.

The Northeast Structural Genomics (neSG) consortium is one of four large-scale, NIH funded centers operating under the ‘Protein Structure Initiative (PSI-2)’. This program initially aimed to reduce the cost and time required to determine three-dimensional protein structures. As part of this initiative, Alr8543, a cyanobacteria protein that contains the Coq4p fold, was recently solved (Forouhar et al. 2009). In an effort to identify the function of Coq4p, and for the purposes of this review to illustrate how studies in *S. cerevisiae* can be used to further our understanding of human diseases, we have generated a homology model of Coq4p from *S. cerevisiae* using Alr8543 as a template (Schwede et al. 2003). The large evolutionary distance between Coq4p and Alr8543 permits easy identification of structurally and functionally important residues (Figure 1A). From the crystal structure, Alr8543 assembles into a homodimer. Interestingly, geranylgeranyl monophosphate co-crystallized with the protein in a deep binding pocket. This molecule is a polymer of four isoprenoid units. Also in the crystal structure, a magnesium ion (Mg^{2+}) was chelated by the putative zinc (Zn^{2+})-ligand motif (Figure 1B). The *COQ4* complementation group in *S. cerevisiae* contains 3 loss-of-function, nonsense mutations: *coq4-1* (E226K), *coq4-2* (E121K), and *coq4-3* (G120E). The quality of our modeling, as well as the reliability of using Alr8543 as a template, can be assessed by mapping these mutations onto the yeast Coq4p structural prediction. Since these are loss-of-function alleles we would expect each mutation to cause a protein defect of obvious consequence. Indeed, this appears to be the case (see details in Figure 1C). Based on these findings, we predict that Coq4p does indeed act as an anchor protein primarily for the polyisoprenoid tail of HHB. We suggest that Coq4p acts to tether

HHB to the inner mitochondrial membrane by its distal four or five isoprenoid tail units, thus allowing the benzoquinone head group to rotate freely for sequential modification by Coq5p, Coq6p, Coq7p and Coq9p. From the structure, it is also very likely that the surface of Coq4p may form a scaffold for attachment of the latter proteins.

iv. Barth Syndrome: Tafazzin and cld-1—Barth syndrome (BTHS) is an X-linked disorder caused by inactivation of Tafazzin, the protein encoded by the *Taz G4.5* gene (Bione et al. 1996). Clinically, Barth Syndrome is characterized by cardiomyopathy, neutropenia, and delayed growth (Barth et al. 1983; Spencer et al. 2006). Tafazzin mutations hamper the remodeling of cardiolipin (diphosphatidylglycerol), which is a tetra-acyl phospholipid that is almost exclusively located in the inner mitochondrial membrane where it constitutes up to 25% of the total lipid. Cardiolipin has been implicated in diverse mitochondrial functions including membrane biogenesis, electron transport chain functionality, apoptosis, and lipid-protein interface interactions (Chicco and Sparagna 2006). In eukaryotes, the final step in cardiolipin biosynthesis involves linking phosphatidylglycerol with the activated diacyl group of cytidinediphosphate diacylglycerol (Schlame et al. 1993) in a reaction catalyzed by the enzyme cardiolipin synthase (Crd1p in *S. cerevisiae*). Although the reaction of cardiolipin synthase was first demonstrated in rat liver (Tamai and Greenberg 1990), the systematic characterization of cardiolipin synthases has largely been undertaken using the yeast *S. cerevisiae*.

As there are four distinct fatty acyl groups in cardiolipin, there is enormous potential for complexity in the distribution within molecular species. For most animal tissues, however, cardiolipin contains almost exclusively C18 fatty acids, 80% of which is typically linoleic acid (18:2(n-6)). Testis cardiolipin is an exception that contains mainly palmitic acid (C16:0), while brain cardiolipin contains many different fatty acids including polyunsaturated arachidonic acid (20:4(ω-6)) and docosahexanoic acid (22:6(n-3)). In *S. cerevisiae*, cardiolipin is mainly comprised of 16:1 and 18:1 fatty acids. It appears that the ultimate fatty acid composition of cardiolipin in eukaryotes is attained by re-modeling. Such remodeling could be achieved in theory by the coenzyme A (CoA)-dependent deacylation-reacylation cycle known as the Lands cycle. However, it is now believed that the main remodeling route is via CoA-independent transacylation between different phospholipids, a reaction in which Tafazzin plays a central role (Houtkooper et al. 2009).

Barth Syndrome is marked by the presence of aberrant cardiolipin molecular species (Schlame and Ren 2006). Tetra-acylated cardiolipin levels are low, levels of tri-acylated monolysocardiolipin (MLCL) are elevated and the acyl chain composition of the remaining CL is more saturated. Tafazzin encodes a phospholipid transacylase (Neuwald 1997) that transfers an acyl chain from phosphatidylcholine (PC) to MLCL (Ma et al. 1999; Schlame and Rustow 1990; Xu et al. 2003). Identification of Tafazzin quickly set the stage for *S. cerevisiae* to become a model system for studying Barth Syndrome, since it has an orthologous gene. Mutant Δ*taz1* yeast exhibit phospholipid defects similar to those observed in Barth Syndrome cells – they accumulate aberrant cardiolipin species and have decreased levels of mature cardiolipin (Gu et al. 2004; Vaz et al. 2003). Cardiolipin remodeling in Δ*taz1* mutants was shown to be essential for the stability of mitochondrial membranes following exposure to elevated temperature or osmotic stress (Koshkin and Greenberg 2000; Koshkin and Greenberg 2002). Interestingly, when complementation assays using the human Tafazzin gene were performed in Δ*taz* mutants, only splice variants lacking exon 5 were able to fully rescue the aberrant cardiolipin profile. Constructs expressing human Tafazzin lacking exon 5 preferentially incorporated C18:1, not C18:2 as is typical of human cardiolipin. Furthermore, variants lacking exon 5 restored mitochondrial coupling to Δ*taz* mutants when challenged with hypotonic stress (Ma et al. 2004). These findings led to the realization that, in humans, Tafazzin splice variants that lack exon 5 are likely the active

variant *in vivo,* and that preferential incorporation of C18:2 likely reflects differences in substrate availability rather that substrate selectivity (Houtkooper et al. 2009).

The re-modeling reaction of Tafazzin could theoretically proceed by a cyclic mechanism - in which case only trace amounts of either lyso-phosphatidylcholine or MLCL would be required. Recently, a phospholipase specific for cardiolipin was identified in yeast (cardiolipin-specific deacylase 1, *CLD1*). This enzyme hydrolyzes newly synthesized cardiolipin with a strong substrate preference for palmitoyl acyl groups and functions upstream of tafazzin to generate MLCL (Beranek et al. 2009). Interestingly, deletion of *CLD1* in the Δ*taz* background did not decrease the total amount of cardiolipin or lead to an elevation in MLCL levels, yet still resulted in a mitochondrial pathology. This implies that in Barth Syndrome patients, dysfunction of mitochondria probably does not result from excessive MLCL accumulation but, rather, from defective cardiolipin re-modeling.

v. Mitochondria-associated Membranes (MAM)—One of the obvious advantages of using yeast as a model organism is that it provides an excellent tool for exploratory research - science that has the potential to result in unexpected insight into previously inexplicable human disorders. In this light, recent studies have shown that there are structural and functional contact points between mitochondria and endoplasmic reticulum (ER). Interaction of endoplasmic reticulum with mitochondria (termed mitochondrial-associated membranes, MAM), was first described by Copeland and Dalton in 1959 (Copeland and Dalton 1959) and has since been confirmed both biochemically and structurally (via electron microscopy) in mice (Ardail et al. 1993), rats (Camici and Corazzi 1995) and yeast (Gaigg et al. 1995; Zinser et al. 1991). How MAM relates to cell function remains an area of active exploration. Recent studies emphasize a role for MAM in the biosynthesis and trafficking of phosphatidylcholine and phosphatidylserine between ER and mitochondrial membranes (Voelker 2003), as well as in the transmission of physiological and pathological Ca^{2+} signals between both organelles (Giorgi et al. 2009; Schon and Area-Gomez 2010; Simmen et al. 2010). MAM's central role in non-vesicular inter-organelle lipid trafficking, and its direct impact on cell physiology, first came into light with a seminal paper on the neurodegenerative disorder neuronal ceroid lipofuscinosis (NCL) (Vance et al. 1997). NCLs are a group of autosomal recessive lysosomal storage diseases resulting from one of 160 known mutations in eight NCL genes (*CLN1–CLN3, CLN5–CLN7* and *CLN10*) (Lyly et al. 2009). Pathologically, NCL is characterized by the intracellular accumulation of autofluorescent lipopigment and the progressive loss of neocortical neurons. The major component of stored lysosomal material is either subunit *c* of mitochondrial ATP synthase or sphingolipid activator proteins A and D (Cooper et al. 2006). Vance and colleagues (Vance et al. 1997) used an *mdm/mdm* (*CLN8*) mutant mouse model to show aberrant biochemical separation of MAM from livers of older, but not younger, mice. Moreover, the amount of the MAM-specific protein phosphatidylethanolamine *N*-methyltransferase-2 (PEMT2) was found to be reduced by 60% in *mdm/mdm* liver homogenates of all ages, compared to control animals, despite normal levels of *PEMT2* mRNA. The activity of two additional phospholipid biosynthetic enzymes, CTP:phosphocholine cytidylyltransferase and phosphatidylserine synthase, were also reduced by 50% in *mnd/mnd* liver microsomes. Consistent with disruption of MAM enzymatic activities, 2–3 fold greater uptake of exogenously-applied phosphatidylcholine and phosphatidylserine occurred in mutant *mnd/mnd* cells relative to control cells.

While it is unclear the extent to which MAM disruption contributes to the NCL phenotype, reduced phosphatidylcholine levels in mitochondria may directly impact the biosynthesis of cardiolipin, which in turn, would disrupt the mitochondrial electron transport chain. To what extent *S. cerevisiae* might be used as a model to investigate the consequences of MAM disruption in NCL disorders also remains an open question. *S. cerevisiae* lack an obvious

CLN8 ortholog but does have other *CLN* orthologs. Moreover, phosphatidylserine transport from MAM to mitochondria is already known to be regulated by the Met30p protein ubiquitin ligase (Voelker 2005) - indicating that the power of yeast genetics simply awaits application to an important human disorder.

IV. The Nematode *Caenhorhabditis elegans*

IVA. Worm basics

C. elegans is a multi-cellular animal that measures only a millimeter in length (Figure 2C). Adults contain 959-cells that are organized into five main tissues including gastrointestinal tract, reproductive system, muscle, nerve, and cuticle. These non-parasitic garden worms have been utilized for nearly three decades as a model animal in which to investigate neurobiology and development through the study of basic cellular processes like apoptosis, cell signaling, and aging (Brenner 1974; Hartman et al. 2001; Ishii 2001; Rea and Johnson 2003; Senoo-Matsuda et al. 2003). Its ease of manipulation is based on each animal proceeding from an egg through four larval stages to reach adulthood in less than three days (Wood 1988). Adults live for a total of three weeks (at 20°C) and are hermaphroditic, each producing approximately 300 genetically identical offspring. Males do exist, as a result of loss of an X chromosome, thereby enabling facile genetic crossing between strains. The *C. elegans* genome was among the first to be fully sequenced in 1998, sharing greater than 83% homology with the human genome (Lai et al. 2000). The worm genome can be easily manipulated to produce single gene mutants that may be studied for a wide range of cellular and whole animal phenotypes (Brenner 1974; Hoffenberg 2003; Wood 1988). Nematodes primarily feed on *E. coli* bacteria, a fact that is commonly manipulated to knock-down genes through the phenomenon of RNA interference (RNAi) - a tool first discovered in *C. elegans* (Kamath et al. 2001; Timmons et al. 2001). Their transparent nature allows for cellular localization of fluorescence-tagged genes. The *Caenorhabditis* Genetics Center (CGC, Minneapolis, MN), a public strain repository, as well as two centralized knockout consortia, facilitate study of this low-cost animal model system. Remarkably, a centralized public database, “wormbase”, provides comprehensive links to obtain access to all previously generated mutant strains, known information on each genetic mutant strain, as well as available community resources (www.wormbase.org) (Harris et al. 2010).

IVB. Mitochondria conservation between mammals and *C. elegans*

Extensive evolutionary conservation exists in mitochondrial composition and function between humans and *C. elegans* (Falk et al. 2009). Their 13,794 base pair mitochondrial DNA (mtDNA) genome is also highly conserved, encoding 12 of the 13 proteins found in mammalian mtDNA with the exception being the complex V gene, *ATP8*. Mitochondrial genome content has been shown to be essential for normal worm development, with a large increase in mtDNA content occurring at the later larval stages (Tsang and Lemire 2002). Understanding specific genetic causes of human mitochondrial dysfunction can be advanced by exploiting the high degree of evolutionary conservation present in the components of the 5 complexes of the respiratory chain (RC). Similarities between species are largely predicted *in silico* using publicly available data from multiple genome sequencing and annotation websites (http://ucsc.genome.edu; www.wormbase.org; www.genome.jp/kegg). However, the exact composition of each respiratory complex within different species still remains a matter of investigation. For example, complex I, the largest and most commonly involved complex in human respiratory chain disease, is estimated by mass spectroscopy to consist of 38 nuclear-encoded subunits and 7 mtDNA-encoded subunits (Hirst et al. 2003). Of these, all mitochondrial encoded components and at least 32 nuclear encoded components are conserved with the nematode, *C. elegans* (Falk et al. 2009). Utilizing evolutionary homology allows key components held in common across divergent classes of organisms to be

recognized and studied. Intact mitochondria can also be isolated from worm populations to permit performance of classical *in vitro* assays of mitochondrial respiratory capacity and enzyme activities (Falk et al. 2006; Grad et al. 2007).

IVC. Modeling respiratory chain dysfunction in *C. elegans*

Several well-characterized strains have been described that harbor classical gene mutations in nuclear-encoded subunits of the respiratory chain. These include missense and/or deletion mutants in subunits of complex I (*gas-1*(*fc21*)); *nuo-1*), complex II (*mev-1*(*kn1*)), complex III (*isp-1*(*qm150*)), as well as in CoQ biosynthesis (*clk-1*) (Felkai et al. 1999; Feng et al. 2001; Ishii et al. 1998; Kayser et al. 2001; Tsang et al. 2001) (Table III). Dilution of RNAi to knockdown expression of a given nuclear-encoded mitochondrial protein can also be titrated to modulate phenotypic effects (Rea et al. 2007).

Recent research in nematode models of mitochondrial dysfunction has revealed that complex I-dependent respiratory capacity mediates whole organism response to volatile anesthesia (Falk et al. 2006). Systematic knockdown of 28 highly-conserved complex I subunits and two complex I assembly factors has further revealed widely different influences on energy production, anesthetic sensitivity, and free radical generation among different components of this one megadalton complex that serves as an entry point for metabolic substrates into the respiratory chain (Falk et al. 2009). RNAi that knocked-down a complex IV subunit gene also revealed complex I activity to be impaired, apparently through disruption of respiratory chain supercomplex formation (Suthammarak et al. 2009).

IVD. Investigating *in vivo* mitochondrial functions in *C. elegans*

One of the most frequently investigated phenotypes of *C. elegans* mitochondrial mutants is altered longevity (Ventura et al. 2006). Genome-wide RNAi screens have identified a wide range of mitochondrial mutations that actually prolong life (Dillin et al. 2002; Lee et al. 2003). In contrast, a well-characterized complex I subunit mutant (*gas-1*(*fc21*)) is short-lived at 20°C (Kayser et al. 2004). Extensive investigations of the potential role of oxidative stress in altered longevity of mitochondrial mutants has been performed to test “the free radical theory of aging”, which collectively suggest that oxidative damage can be experimentally dissociated from aging (Honda and Honda 2002; Ishii 2000; Lee et al. 2003; Rea et al. 2007; Van Raamsdonk and Hekimi 2010). Similarly, slow physiologic rates and prolonged lifespan were recently reported in 11 different *clk* mutants, all of which had mitochondrial dysfunction and evidence of decreased energy utilization but no systematically increased oxidative stress resistance or reduced oxidative damage, which suggests that impaired energy metabolism can cause increased lifespan without reducing reactive oxidant species (ROS) production (Van Raamsdonk et al. 2010). Interestingly, recent comparison of RNAi-mediated gene knock-down and classical mutations in the same RC subunit genes suggested that prolonged lifespan in nematodes can be induced by distinct aspects of mitochondrial biology; whereas the former induces a stress and autophagy response, the latter alters respiratory capacity and ROS metabolism (Yang and Hekimi 2010).

Mitochondria-targeted fluorescent dyes can be fed to *C. elegans* to permit quantitation of mitochondrial specific parameters, such as oxidant burden and mitochondrial membrane potential. Recent whole nematode fluorescence quantitation studies suggested that animals having prolonged lifespan have reduced mitochondrial membrane potential, which has been proposed to be mediated by relative uncoupling of oxidation from phosphorylation in these animals (Lemire et al. 2009). In addition, targeted fluorescence microscopy quantitation of fluorescence in mitochondrial-dense terminal pharyngeal bulbs (Figure 3C, right image) offers a means for targeted analysis of mitochondrial functions in living animals without interference of non-specific binding of these lipophilic dyes to other structures (Dingley et

al. 2010). Such *in vivo* studies remove confounding experimental factors inherent to *in vitro* systems, such as buffer composition, oxygen tension, etc. Studies in the short-lived complex I mutant, *gas-1*(*fc21*), suggest it has increased *in vivo* mitochondrial matrix oxidant burden and decreased mitochondrial membrane potential (Dingley et al. 2010).

Gene expression analyses in nematode models have revealed a highly specific adaptive response occurs in the setting of primary RC dysfunction (Falk et al. 2008). Specifically, RC dysfunction results in global cellular transcriptional alterations interpretable at the level of biochemical pathways, as assessed by cluster analyses such as gene set enrichment analysis (GSEA). 15 biochemical pathways were concordantly *upregulated* on transcriptional profiling between *C. elegans* models of primary RC dysfunction due to mutations in nuclear-encoded subunits of complexes I (*gas-1*), II (*mev-1*), and III (*isp-1*) and a murine model of primary RC dysfunction due to a *Pdss2* mutation that impairs CoQ biosynthesis (Peng et al. 2008). RC dysfunction stimulates the constituent components of oxidative phosphorylation (OXPHOS), TCA cycle, and many pathways (e.g., glycolysis, amino acid and fatty acid metabolism) that furnish substrate to it, as well as key cellular defense pathways such as glutathione and P450 metabolism. Such similarities spanning evolution constitute a transcriptional ‘‘signature’’ of RC disease.

IVE. Investigating mechanism and efficacy of mitochondrial therapies in *C. elegans*

Introduction of an alternative pathway for lactate oxidation was shown to markedly improve the phenotype of a *nuo-1* (*NDUFV1* homolog) complex I subunit mutant worm. Specifically, this was accomplished by introduction of the yeast *CYB2* gene, which encodes an L-lactate:cytochrome c oxidoreductase that oxidizes lactate and bypasses complex I to donate electrons directly into the RC. Cyb2p expression increased complex I mutant worm lifespan, fertility, respiration rate, and ATP content, suggesting that metabolic imbalance leading to lactic acidosis and energy depletions are central pathogenic mechanisms of mitochondrial dysfunction that can be treated by alternative lactate oxidation (Grad et al. 2005).

Many pharmacologic agents have discernible effects on variable endpoints when fed to *C. elegans*. Riboflavin (B2) but not thiamine (B1) improved animal fitness (progeny) and mitochondrial function (increased complex I and complex IV assembly, decreased lactic acid levels) following administration to the *nuo-1* complex I subunit mutant (Grad and Lemire 2004; Grad and Lemire 2006). N-acetyl-l-cysteine (NAC) and ascorbate significantly ameliorated oxidative stress when fed to *C. elegans* complex II mutants (Huang and Lemire 2009). Probucol offered significant protection from oxidative stress in a *C. elegans* model of Parkinson’s disease (PD) attributed to its antioxidant properties, although NAC had no discernible benefit (Ved et al. 2005). 3-hydroxybutyrate, which is generated by the ketogenic diet, fully rescued wild-type nematodes treated with a complex I inhibitor, rotenone, and partially rescued transgenic nematode models of multiple genetic forms of PD (Ved et al. 2005). Both resveratrol and nicotinic acid extended lifespan in wild-type *C. elegans* (Gruber et al. 2007; Hashimoto et al. 2009), but have not previously been studied in RC dysfunction. Many therapeutic agents of purported benefit in human RC disease are thus amenable to mechanistic study in *C. elegans* models of primary RC dysfunction.

V. The Fruit Fly *Drosophila melanogaster*

VA. Utility of *Drosophila* as a genetic model system to study mitochondrial biology and disease

As a genetic model system, the fruit fly, *Drosophila melanogaster*, offers many advantages (Figure 2D). The biology and genetics of *Drosophila* have been studied for over a century and have been extensively documented (Ashburner et al. 2005; Demerec 1994). There is an

extensive genetic "toolbox" for *Drosophila* that includes manipulatable transposable elements for mutagenesis and transgenesis, a GAL4/UAS bipartite system that allows differential spatiotemporal transgeneic expression, extensive genetic reagents that enable mosaic clonal analysis (i.e., the generation and analysis of homozygous mutant clones of an essential gene within heterozygous animals via mitotic recombination) in both developing and adult tissues, and a genome-comprehensive transgenic RNAi library (Bellen et al. 2004; Brand and Perrimon 1993; Dietzl et al. 2007; Perrimon 1998; Ryder and Russell 2003; Thibault et al. 2004; Xu and Harrison 1994). Over the past thirty years, studies in *Drosophila* have elucidated many fundamental mechanisms of cellular growth and development that are conserved among higher eukaryotes, including humans (Gilbert 2008). During the past decade, the utility of modeling human diseases in the fly has been repeatedly demonstrated, in particular for neurodegenerative disorders (Lu and Vogel 2009). *Drosophila* models of Parkinson's disease (Park et al. 2009), amyotrophic lateral sclerosis (Tsuda et al. 2008), and spinocerebellar ataxia (Al-Ramahi et al. 2007; Lam et al. 2006) have provided insights into pathogenetic mechanisms. The *Drosophila* models of Parkinson's disease are especially notable examples given that mitochondrial complex I deficiency plays a role in disease pathogenesis (Lu and Vogel 2009) and that mutants for the orthologs of the familial Parkinson's disease genes *PARKIN* (Greene et al. 2003; Pesah et al. 2004) and *PINK1* (PTEN-Induced Putative Kinase 1) (Clark et al. 2006; Park et al. 2006) demonstrate distinctive mitochondrial phenotypes, suggesting a role for these genes in modulating mitochondrial morphology and function. The power of *Drosophila* models to provide novel insights into human disease pathogenesis is also exemplified by a genetic screen for modifiers of neurodegeneration in a model for spinocerebellar ataxia that, in addition to chaperones and the proteasome, implicated multiple other cellular processes including RNA processing, transcriptional regulation, and cellular detoxification in modulating disease severity (Fernandez-Funez et al. 2000). In summary, extensive conservation of fundamental cellular processes with mammalian systems, an established track record of modeling human diseases, short generation time, and the availability of many sophisticated genetic tools make *Drosophila* an attractive model system for performing genetic analyses and screens to model human mitochondrial diseases.

VB. Forward genetic screens in fruit flies identified mutants in mitochondrial genes

In the 1960s and 1970s, classical forward genetic screens were performed in *Drosophila* to isolate mutants that exhibit abnormal behaviors or responses to environmental stress (Benzer 1971; Homyk 1977; Homyk et al. 1980). Three of the mutants exhibiting temporary paralysis when exposed to mechanical stress (increased "bang" sensitivity), *stress sensitive B* (*sesB*), *knockdown* (*kdn*), and *technical knockout* (*tko*), have subsequently been determined to be mutants of nuclear-encoded mitochondrial genes. *sesB* encodes the fly ortholog of the mitochondrial adenine nucleotide translocator (ANT). Studies of *sesB* mutants have demonstrated abnormalities in synaptic vesicle recycling under conditions of high frequency stimulation as well as dysfunction of the Malpighian tubules (Rikhy et al. 2003; Terhzaz et al.; Trotta et al. 2004). In humans, *ANT1* mutations have been associated with autosomal dominant progressive external ophthalmoplegia with mitochondrial DNA deletions (Kaukonen et al. 2000) as well as autosomal recessive hypertrophic cardiomyopathy, myopathy, and lactic acidosis (Palmieri et al. 2005). *kdn* encodes the Kreb cycle enzyme, citrate synthase. Flies mutant for *kdn* exhibit increased bang sensitivity and seizure-like phenotypes (Fergestad et al. 2006). *tko* encodes the S12 subunit of the mitochondrial ribosome (MRPS12) (Royden et al. 1987). *tko* mutants exhibit increased bang sensitivity, abnormal responses to auditory stimulation, and fertility defects, thus providing a model of mitochondrial disease with perturbations of mitochondrial protein translation (Toivonen et al. 2001). A recent transcriptome analysis of *tko* mutants demonstrated upregulation of several pathways including catabolic pathways, pathways involved in gut

transport and absorption of lipids and proteins, and alterations of oxidative metabolism. In addition, many genes involved in gametogenesis and courtship behaviors were downregulated. Overall, the authors hypothesized that these gene expression changes represent a programmed response to cellular energy starvation (Fernandez-Ayala et al. 2010).

A more recent genetic screen resulting in the identification of multiple mitochondrial mutants involved screening for mutants that exhibit deficiencies of eye development secondary to abnormal cell cycle arrest in the developing eye imaginal disc (Liao et al. 2006) (Figure 2D). Ten of 23 complementation groups isolated were determined to be genes that encode mitochondrial proteins, including components of complexes I, III, IV and V of the respiratory chain, mitochondrial ribosomal subunits, and mitochondrial tRNA synthetases (Liao et al. 2006). Further analyses of two of the mutants of the respiratory chain, *pdsw* (*NDUFB10* ortholog) and *cytochrome c oxidase subunit Va* (*CoVa, COX5A* ortholog) demonstrated that these mutants lead to cell cycle arrest via two separate retrograde signaling pathways affecting the G1-S checkpoint regulator, cyclin E (Owusu-Ansah et al. 2008). The complex I subunit (*pdsw*) mutant exhibited increased oxidative stress and activation of the JNK stress pathway leading to activation of the cyclin E inhibitor Dacapo. The complex IV subunit (*CoVa*) mutant demonstrated decreased ATP content with relatively increased AMP causing AMP kinase-dependent downregulation of cyclin E (Owusu-Ansah et al. 2008).

VC. Additional models of mitochondrial dysfunction and disease in *D. melanogaster*

i. Mutants involving components of the respiratory chain—Drosophila mutants of components of complex II (succinate dehydrogenase) have been reported. Mutant alleles of *SdhA* have been isolated in a genetic screen designed to identify abnormal vision-dependent behaviors (i.e., abnormal phototaxis). These mutants are recessive lethal, but eye-specific mosaics exhibit retinal degeneration that can be partially suppressed by transgenic ectopic expression of CuZn-superoxide dismutase or by exposure to the antioxidant vitamin E, suggesting that reactive oxygen species play a role in pathogenesis (Mast et al. 2008). Additionally, a screen designed to isolate mutants exhibiting increased sensitivity to hyperoxia led to the identification of a transposable element (P element) insertion allele of *SdhB*. This mutant exhibits complex II deficiency, impaired succinate-dependent mitochondrial respiration, decreased lifespan, abnormal muscle mitochondrial morphology, and increased production of hydrogen peroxide (Walker et al. 2006). Also, a temperature-sensitive paralytic mutant was determined to harbor a mutant in *levy*, the ortholog of *COX6A1* (complex IV subunit). This mutant also exhibits many mutant phenotypes reminiscent of mitochondrial disease including partial complex IV deficiency, neurologic dysfunction (increased bang sensitivity), and progressive neurodegeneration (Liu et al. 2007).

ii. Mutants of mitochondrial DNA (mtDNA) and mtDNA replication—Analysis of the *sesB* mutant resulted in the identification and isolation of a spontaneous maternally inherited enhancer of increased bang sensitivity. Sequencing of the mtDNA revealed a nearly homoplasmic mutation in *ATP6* (complex V/ATP synthase subunit) that mutates a glycine residue conserved between flies and human. This mutant exhibits pathological features that model Leigh disease (subacute necrotizing encephalopathy) including vacuolization of the brain, myodegeneration, abnormal mitochondrial morphology and deficiency of ATP synthase (Celotto et al. 2006). Fly models of mtDNA maintenance involving the mitochondrial DNA polymerase γ have also been described. A mutant that exhibits an abnormal larval phototaxis behavior was determined to have a mutation in *tamas*, the catalytic subunit of DNA polymerase γ. This mutant exhibits larval locomotion defects

and abnormal development of the adult compound eye (Iyengar et al. 1999). In addition, overexpression of the catalytic subunit of DNA polymerase γ in transgenic flies causes mtDNA depletion and developmental defects (Lefai et al. 2000). Subsequently, mutants of the accessory subunit of DNA polymerase γ were identified that also exhibit mtDNA depletion and developmental defects/lethality (Iyengar et al. 2002).

iii. Mutants involving genes that regulate mitochondrial dynamics—There is a relatively recently recognized class of mitochondrial disease involving perturbation of mitochondrial dynamics and/or morphology that leads to abnormal distribution of mitochondria in cells and disease. For example, the axonal form of Charcot-Marie-Tooth disease (hereditary motor and sensory neuropathy type 2A) is caused by heterozygous mutations in *MITOFUSIN-2* (*MFN2*), one of two isoforms that mediate fusion of the mitochondrial outer membrane (MOM) (Liesa et al. 2009). Flies mutant for *Dynamin related protein 1* (*Drp1*), which is required for mitochondrial fission, exhibit increased bang sensitivity, defects in synaptic neurotransmission, and defective transport of mitochondria to motor neuron presynaptic termini (Verstreken et al. 2005). Similarly, mutants of orthologs of genes that regulate mitochondrial fusion, *Rhomboid-7* and *opa1-like* (ortholog of *OPA1*, a cause of autosomal dominant optic atrophy), exhibit defects of mitochondrial fusion (McQuibban et al. 2006). *Rhomboid-7* mutants also exhibit muscle defects with a droopy wing phenotype and impaired retinal function exhibited by electroretinogram abnormalities (McQuibban et al. 2006). *Opa1-like* mutants exhibit abnormal eye development in adult eye mosaics associated with mitochondrial fragmentation and increased reactive oxygen species, which is partially rescued by both transgenic and exogenous antioxidant treatment (Yarosh et al. 2008).

iv. Fly model of Barth syndrome provides pathogenic insights—To develop a fly model of Barth disease, hypomorphic alleles of *tafazzin* were generated by imprecise excision of a P element insertion. These mutant animals exhibit normal rates of *de novo* cardiolipin synthesis but demonstrate defects of cardiolipin remodeling resulting in relative cardiolipin depletion in combination with accumulation of cardiolipin deacylated intermediates, monolyso-cardiolipins (Malhotra et al. 2009; Xu et al. 2006). In addition, these mutants exhibit abnormal mitochondrial morphology in muscle, locomotor defects, and male infertility (Malhotra et al. 2009; Xu et al. 2006). Using a candidate gene approach, the male infertility phenotype was suppressed by inactivation of the gene encoding a calcium-independent phospholipase A_2, $iPLA_2$-VIA, which mediates cardiolipin deacylation (Malhotra et al. 2009). Furthermore, treatment of lymphoblasts derived from Barth patients with an $iPLA_2$ inhibitor, bromoenol lactone, partially restores cardiolipin homeostasis (Malhotra et al. 2009). Therefore, this fly model of Barth syndrome has provided new insight into disease pathogenesis as well as identified a potential therapeutic target.

v. *Drosophila* porin mutant models mitochondrial dysfunction and disease—The voltage-dependent anion channel (VDAC or porin) is an integral membrane protein present in the mitochondrial outer membrane (MOM). VDAC is a monomeric, voltage-gated channel that allows passage of molecules up to 5 kDa. Multiple isoforms have been identified in numerous eukaryotic species. VDAC provides the predominant pathway for small metabolites such as ATP, ADP, phosphocreatine, and small ions across the MOM. While classically viewed as the major determinant of MOM permeability since the 1970s, studies over the past 15 years have clearly demonstrated that VDAC also interacts with both cytosolic and MOM proteins (reviewed in (Shoshan-Barmatz et al. 2010). For example, VDAC integrates mitochondrial and cytoplasmic energy metabolism by binding hexokinases to effectively link glycolysis with mitochondrial oxidative phosphorylation, and is also the mitochondrial docking site for glycerol kinase and creatine kinase (Shoshan-Barmatz et al.

2010). *D. melanogaster* contains a cluster of four genes (*porin, CG17137* [*Porin2*], *CG17139,* and *CG17140*), which encode proteins that are homologous to known VDACs (Graham and Craigen 2005). *porin* exhibits the greatest homology to mammalian VDACs and is ubiquitously expressed in the fruit fly, while the other three fly VDACs have a more spatially restricted expression pattern predominantly in the male reproductive tract (Graham and Craigen 2005). Using imprecise excision of a P element inserted in the 5' UTR of *porin*, a series of hypomorphic alleles has been generated and characterized (Graham et al. 2010). Flies deficient for *porin* exhibit a range of mutant phenotypes including partial developmental lethality, defective mitochondrial respiration, partial complex I deficiency, fertility defects, skeletal muscle abnormalities, and neurological dysfunction. The fertility defects include reduced fertility and fecundity in females and infertility in males associated with sperm immotility. The muscle phenotype consists of abnormal mitochondrial morphology with unusual inclusions by electron microscopy, as well as a functional deficit manifested as a defect in a climbing assay. The neurological dysfunction in porin-deficient flies is demonstrated by an increased sensitivity to mechanical stress ("bang sensitivity"), progressive retinal dysfunction demonstrated by abnormal ERGs, and an abnormal electrophysiological response at the larval neuromuscular junction (NMJ). The neurological dysfunction at the larval NMJ is also associated with abnormal distribution of mitochondria within the motor neuron. Using a mitochondria-targeted GFP transgene expressed in the motor neuron, a relative paucity of mitochondria in the presynaptic termini and axons with concomitant accumulation of mitochondria in the motor neuronal cell bodies was demonstrated (Graham et al. 2010). The phenotypes of defective energy metabolism, male infertility with sperm immotility, and neurological dysfunction in porin-deficient fruit flies are reminiscent of abnormal phenotypes seen in mitochondrial diseases. These studies suggest that porin deficiency in the fly is a valid model relevant both for primary mitochondrial diseases and for common adult-onset neurological and metabolic diseases in which mitochondrial dysfunction has been implicated to play a pathogenic role.

VD. Future of modeling mitochondrial disease in *D. melanogaster*

As described in this review, over the past ten years mutants have been described in several *Drosophila* orthologs of human mitochondrial disease genes or genes critical to fundamental mitochondrial functions. Characterizations of these mutant lines have provided many insights into the pathogenesis of mitochondrial diseases, as well as identified some potential therapeutic targets and strategies. Additional insights into disease pathogenesis and potential therapeutic strategies will undoubtedly come from the continued utilization of *Drosophila*'s greatest strength as a model system: genetic screens. Future forward genetic screens as well as modifier screens in models of mitochondrial disease that are designed to identify suppressors of mutant phenotypes promise to advance understanding of fundamental mitochondrial functions as well as identify new therapeutic targets and strategies for human mitochondrial diseases.

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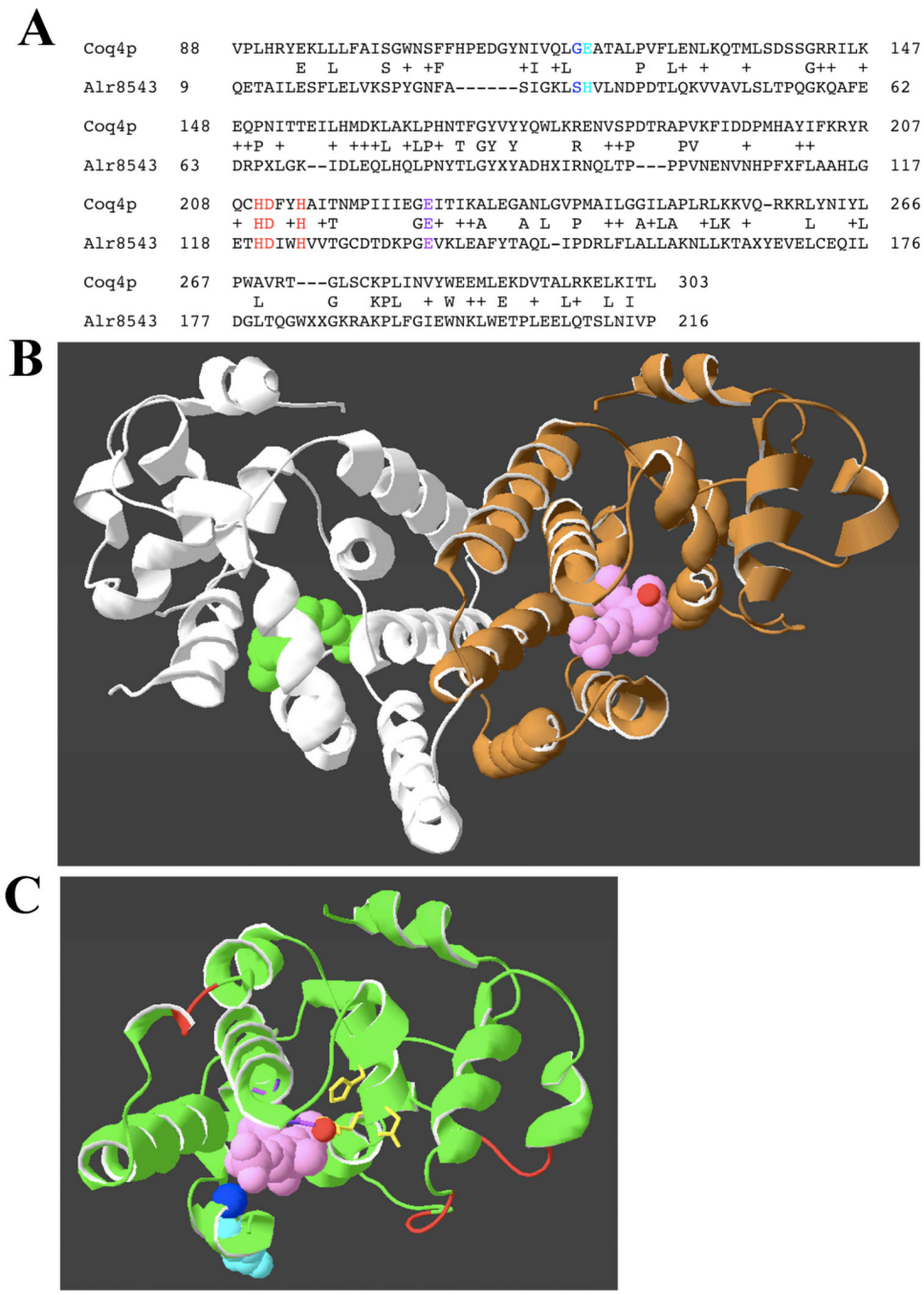

Figure 1. Molecular Modeling of *S. cerevisiae* Coq4p

(A) Structure-based alignment of Alr8543, from *Nostoc sp. PCC7120,* and Coq4p, from *S. cerevisiae*. BLAST analysis yielded an almost identical alignment (E-value, 3e-36, not shown). Colored residues correspond to HDxxH-$(x)_{11}$-E motif residues (*red*) and loss-of-function *coq4* point mutations (see below for details, *blue residues*). **(B)** Crystal structure of Alr8543 (PDB ID: 3KB4) (Forouhar et al. 2009). Monomers are colored *white* and *brown*. Geranylgeranyl monophosphate is shown in *green* and *pink*. Chelated Mg^{2+} is highlighted in *red*. Dimer is orientated so the base would be sitting on a putative membrane surface, orientated 90° to the plane of the paper. **(C)** Homology model of Coq4p (only residues 116–

302 were modeled). Red loops mark sites of amino acid insertions relative to Alr8543. The following features are highlighted: Geranylgeranyl monophosphate (*pink*); chelated Mg^{2+} (*red ball*); E226K (*coq4-1*), E121K *(coq2-4)* and G120E *(coq4-3)* point mutations (*purple, azure and navy blue, respectively*); H, D and H of the predicted Zinc-ligand binding HDxxH-$(x)_{11}$-E motif (*yellow*).

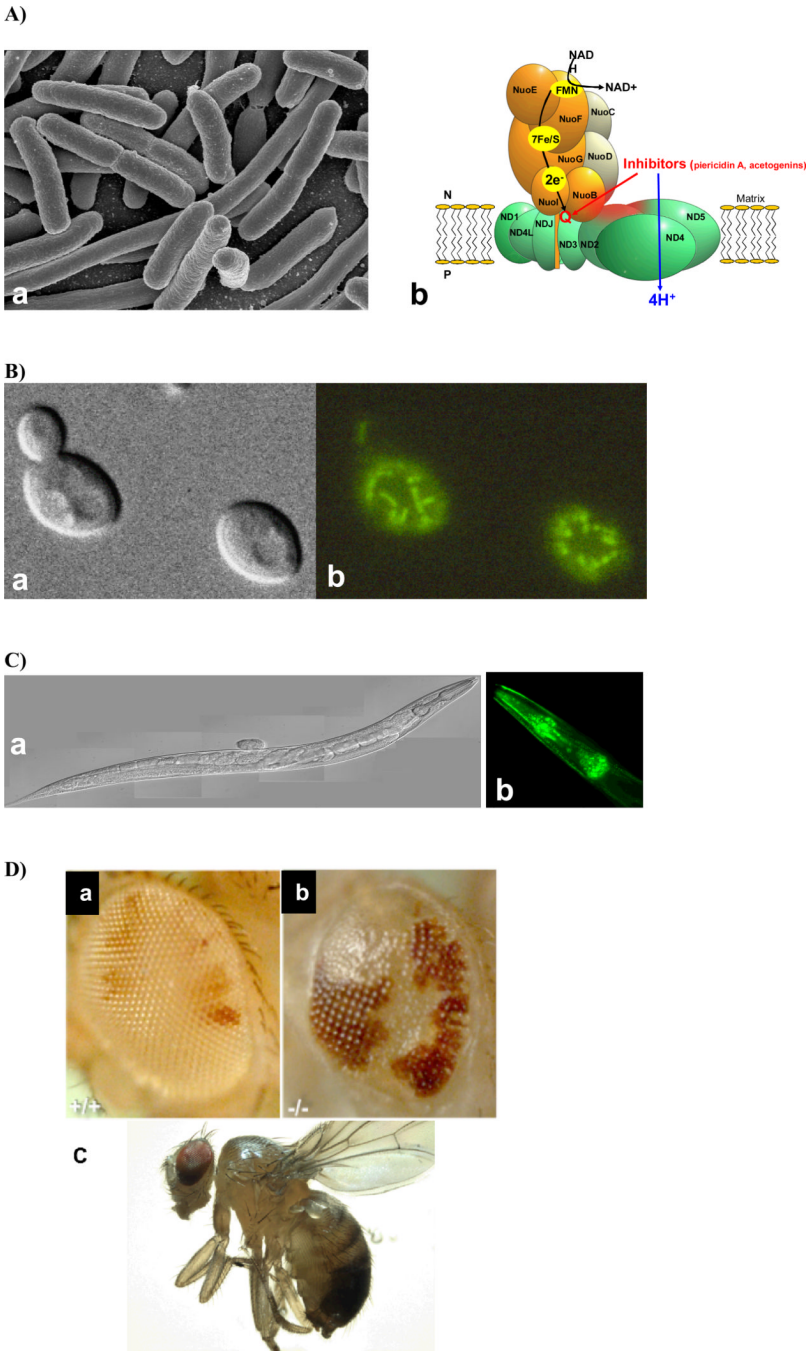

Figure 2. Images of simple model organisms relevant to the study of mitochondrial disease. [A] The Bacteria, *E. coli*

(a, left panel), Scanning electron micrograph image of *E. coli* (15,000 ×) grown in culture and adhered to a cover slip. Image is cited from http://en.wikipedia.org/wiki/Image:EscherichiaColi_NIAID.jpg (Credit: Rocky Mountain Laboratories, NIAID). (b, right panel), Subunit arrangement of *E. coli* complex I. All 14 subunits have homologs in mitochondrial complex I. The hydrophilic peripheral arm consists of NuoE (NDUFV2 in human complex I), NuoF (NDUFV1), NuoG (NDUFS1), NuoC (NDUFS3), NuoD (NDUFS2), NuoI (NDUFS8), NuoB (NDUFS7). The hydrophobic membrane arm consists of NuoH (ND1), NuoK (ND4L), NuoJ (ND6), NuoA (ND3), NuoN(ND2), NuoM (ND4),

and NuoL (ND5). **[B] The yeast, *S. cerevisiae***. Nomaski (a, left panel) and fluorescence (b, right panel) images of two cells (one is in the process of budding) that contain a recombinant GFP construct that stains the mitochondrial matrix (Cohen and Fox 2001). **[C] The nematode, *C. elegans*.** (a) The left panel depicts a single adult nematode (50× magnification). (b) The right panel illustrates the high density of mitochondria in the pharynx of animals where the *NDUFS2* complex I subunit homolog (*gas-1*) is labeled in green fluorescence protein. **[D] The fruit fly, *D. melanogaster***. Mosaic analysis of complex I mutant in the adult fly compound eye. White clones are homozygous wild type (a, left panel) and homozygous null mutant (b, right panel) for *NDUFS3* ortholog. The mutant white clones demonstrate abnormal development of ommatidia, including deficiency of lens formation that gives a “rough glass” eye phenotype similar to other mitochondrial mutants. The lower panel (c) shows an adult wild type fruit fly (Canton-S).

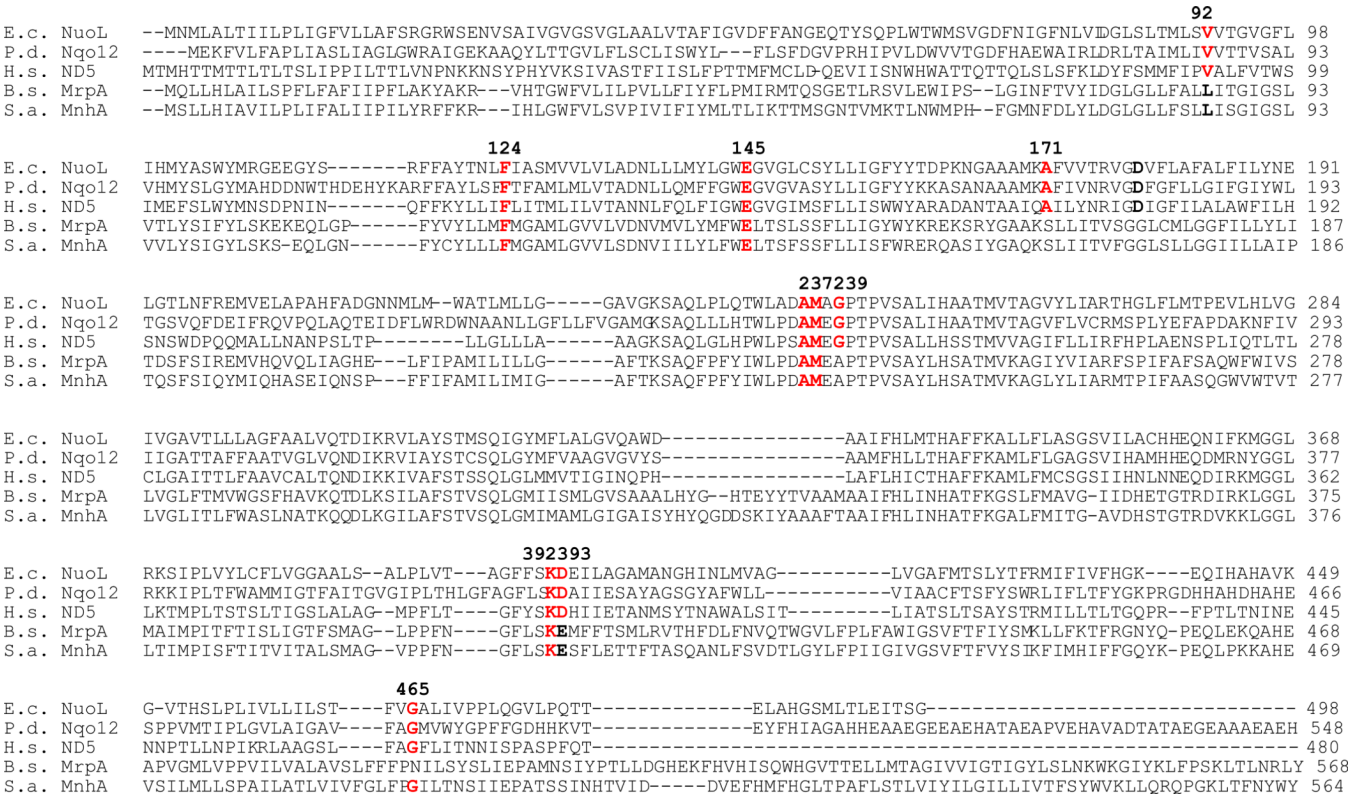

Figure 3. Multiple sequence alignments of the N-terminal region of the NuoL subunit (total 613 AA)

E. coli complex I is aligned with *P. denitrificans*, human ND5, and MrpA of multisubunit cation antiporter complexes. The highlighted amino acids and their respective numbers (based on human sequences) are disease-related mutations found in human complex I. *E.c., Escherichia coli* (GenBank Accession Number AAC75343); *P.d., Paracoccus denitrificans* (AAA25587); *H.s., Homo sapiens* (P03915); *B.s., Bacillus subtilis* (Q9K2S2); *S.a., Staphylococcus aureus* (BAA35095).

Table I

Yeast mitochondrial databases

Excellent online resources are available to facilitate study of mitochondria in the yeast *S. cerevisiae*.

Database	Description	Website
MitoP2 (Mitochondrial Proteome)	Comprehensive list of mitochondrial proteins of *S. cerevisiae*, mouse, *Arabidopsis thaliana*, neurospora and human	http://www.mitop.de
Yeast Mitochondrial Protein Database	List of *S. cerevisiae* mtDNA-encoded proteins, nDNA-encoded proteins, and proteins that play a role in mitochondrial functionality	http://bmerc-www.bu.edu/projects/mito/
YDPM (Yeast Deletion Project & Mitochondria)	Database supporting proteomic, mRNA expression, and deletion studies relevant to mitochondrial function in *S. cerevisiae*	http://www.deletion.stanford.edu/

Table II

S. cerevisiae models of human mitochondrial diseases

Representative examples of human mitochondrial diseases that have been successfully modeled using the yeast *S. cerevisiae*.

Human Disease	Disrupted Mitochondrial Protein or Function	Yeast Model *	Human Ortholog *
Mohr-Tranebjaerg Syndrome	Protein Import Machinery	TIM8	DDP1
Autosomal-dominant Progressive External Ophthalmoplegia (adPEO)	ANT (Adenine nucleotide transporter) - results in nucleotide ratio imbalance leading to mtDNA lesions	AAC2	ANT1
Friedreich Ataxia	Fe-S cluster assembly and Fe storage	YFH1	FXN
Leigh Syndrome	Complex II Activity	SDHA **	SDH Fp
GRACILE Syndrome (Growth retardation, amino aciduria, cholestasis, iron overload, lactic acidosis, and early death)	Complex III Assembly	BCS1	BCS1L
Barth Syndrome	Cardiolipin re-modeling	TAZ1	TAZ
Anemia, Sideroblastic, and Spinocerebellar Ataxia	ABC transporter (mediates Fe-S Cluster Export)	ATM1	ABC7

* All genes of nuclear origin,

** Complex II component - *S. cerevisiae* lacks a proton-pumping complex I

Table III

C. elegans models of mitochondrial respiratory chain dysfunction

Several *C. elegans* strains harboring classical missense mutations in highly conserved subunits of the mitochondrial respiratory chain are available from the public *C. elegans* strain repository (CGC). Newer deletion strains for mitochondrial proteins are continually being generated by one of several knockout consortia, including the tricarboxylic acid cycle enzyme, *idh-1*. 'Homology' indicates protein similarity by length as determined in www.wormbase.org.

C. elegans Strain Name	Mitochondrial Component	Mutation Type	Human Gene Homolog	Protein Homology (Human-Worm)
gas-1(*fc21*)	Complex I subunit	Missense	*NDUFS2*	83.4%
mev-1(*kn1*)	Complex II subunit	Missense	*SDH-C*	75.3%
isp-1(*qm150*)	Complex III subunit	Missense	*UQCRFS1*	97.5%
clk-1(qm30)	Coenzyme Q biosynthesis	Partial Deletion	*COQ7*	91.4%
idh-1(ok2832)	Kreb Cycle Enzyme (Isocitrate Dehydrogenase)	Deletion	*IDH1*	99.0%