Cell Biology of the Mitochondrion

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ABSTRACT Mitochondria are best known for harboring pathways involved in ATP synthesis through the tricarboxylic acid cycle and oxidative phosphorylation. Major advances in understanding these roles were made with *Caenorhabditis elegans* mutants affecting key components of the metabolic pathways. These mutants have not only helped elucidate some of the intricacies of metabolism pathways, but they have also served as jumping off points for pharmacology, toxicology, and aging studies. The field of mitochondria research has also undergone a renaissance, with the increased appreciation of the role of mitochondria in cell processes other than energy production. Here, we focus on discoveries that were made using *C. elegans*, with a few excursions into areas that were studied more thoroughly in other organisms, like mitochondrial protein import in yeast. Advances in mitochondrial biogenesis and membrane dynamics were made through the discoveries of novel functions in mitochondrial fission and fusion proteins. Some of these functions were only apparent through the use of diverse model systems, such as *C. elegans*. Studies of stress responses, exemplified by mitophagy and the mitochondrial unfolded protein response, have also benefitted greatly from the use of model organisms. Recent developments include the discoveries in *C. elegans* of cell autonomous and nonautonomous pathways controlling the mitochondrial unfolded protein response, as well as mechanisms for degradation of paternal mitochondria after fertilization. The evolutionary conservation of many, if not all, of these pathways ensures that results obtained with *C. elegans* are equally applicable to studies of human mitochondria in health and disease.

KEYWORDS electron transport chain; respiration, free radicals; mitochondrial morphology; biogenesis; quality control; WormBook

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THERE has been a resurgence in interest in mitochondrial structure and function in recent years (Dancy *et al.* 2015; Maglioni and Ventura 2016). This change is in part due to a growing appreciation of the role of mitochondria in multiple functions other than energy production (*e.g.*, Ca^{++} homeostasis (Sarasija and Norman 2015; Oxenoid *et al.* 2016), generation of reactive oxidation species (ROS) (Hekimi *et al.* 2016), regulation of apoptosis (Yee *et al.* 2014), activation of endoplasmic reticulum (ER)-stress response (Kim *et al.* 2016)), as well as other far-ranging sequelae of mitochondrial dysfunction (Kaufman and Crowder 2015; Fong *et al.* 2016; Melentijevic *et al.* 2017). Mitochondria are also implicated in many common diseases, like Alzheimer and Parkinson's disease (Ray *et al.* 2014; Fong *et al.* 2016), and in the process of normal aging (Wang and Hekimi 2015). In particular, *Caenorhabditis elegans* has been instrumental in outlining key relationships between mitochondrial function and aging (Munkacsy and Rea 2014; Wang and Hekimi 2015; Mishur *et al.* 2016). However, in the area of primary mitochondrial disease, *C. elegans* has also been extremely useful, as its powerful array of tools can be applied to understand basic mitochondrial physiology in a way not possible for mammalian models (Dancy *et al.* 2015). In addition, the remarkable conservation of genes and pathways that are part of mitochondrial function makes studies in nematodes highly relevant to mammalian mitochondria (Polyak *et al.* 2012; Schrier Vergano *et al.* 2014).

Human mitochondrial disease is actually a collection of hundreds of rare diseases, for changes to literally thousands of genes may affect mitochondrial function. In addition, the organelle is under the control of dual genomes, leading to a potential mosaic pattern of pathogenic mutations. Of course, the same considerations apply to the nematode. The majority of mitochondrial proteins are encoded by nuclear genes and follow the usual Mendelian patterns of inheritance. A few [two ribosomal RNAs, 22 tRNAs, and 12 subunits of the electron

transport chain (ETC)] are encoded by mitochondrial DNA (mtDNA)—a circular genome within the mitochondria that is 13,794 nucleotides in length. An excellent review of mitochondrial genetics in the nematode is presented in WormBook (Lemire 2005). Notably, mtDNA can acquire mutations such that a graded percentage of mutant mtDNA exists in different tissues and cells (heteroplasmy), from very low incidence to a very high one. The percentage of heteroplasmy often defines the phenotype, *i.e.*, how much of a defect in mitochondrial function is present in the organism.

The nuclear genes may encode structural subunits of the mitochondrial respiratory chain (MRC), as well as assembly factors, transport proteins, or unidentified proteins that are crucial for mitochondrial function. In humans, muscle, gut, and nerve cells are extremely dependent on the energy delivered by mitochondria, and therefore have low thresholds for displaying symptoms of mitochondrial disease. Thus, changes in mitochondrial proteins cause striking clinical features in those tissues types. However, the phenotypes associated with mitochondrial disease can be very difficult to recognize, since they can present as a constellation of multi-system pathologies that change over time.

In this chapter we will discuss studies that explain basic mitochondrial function, which have been pursued in the hope of contributing to our understanding of mitochondrial disease. In addition, specific analysis of mitochondrial morphology and mitophagy will be discussed. Mitochondrial functions as they relate to apoptosis and aging are discussed in other chapters of *WormBook*.

The Tricarboxylic Acid (TCA) Cycle

The TCA cycle, also called the Krebs cycle or the citric acid cycle, is carried out within the matrix of the mitochondrion. It is a series of eight enzymatic steps that consumes, and then regenerates, citrate. In doing so, it links the metabolism of carbohydrates, fats, and proteins, as catabolism of these compounds generates acetyl CoA. This key molecule enters the TCA cycle, is oxidized, producing, among other things, the reducing agents NADH and FADH. These two molecules are the electron donors that ultimately transfer electrons to the MRC to begin the process of oxidative phosphorylation (below). The TCA cycle also generates precursors to certain amino acids. This ancient pathway, common to all aerobic organisms, is highly conserved across the animal kingdom. However, nematodes may be the only member of the animal kingdom that can bypass a decarboxylation step of the TCA cycle via an alternative metabolic process—the glyoxylate pathway. The glyoxylate pathway uses a subset of the enzymes of the TCA cycle to allow small carbon compounds to be used to build macromolecules by taking isocitrate to succinate and glyoxylate, rather than α-ketoglutarate, as in the TCA cycle. The enzyme responsible for catalyzing this reaction, isocitrate lyase, is encoded by the gene *icl-1* in nematodes, and will be discussed in the section *C. elegans is a good model system for the study of mitochondrial function*. For a complete discussion of the TCA cycle in nematodes, see the WormBook chapter on Intermediary Metabolism (Braeckman *et al.* 2009).

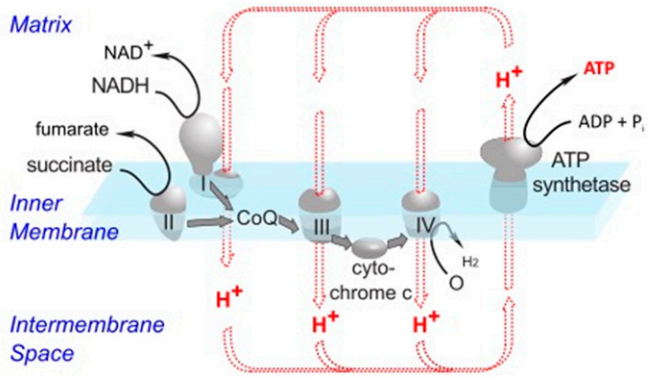

Figure 1 A schematic representation of components of oxidative phosphorylation within the inner mitochondrial membrane. Complex specific substrates like malate (complex I;CI) or succinate (complex II;CII) donate electrons that are ultimately accepted by oxygen to produce water. In the process, protons are pumped out of the mitochondrion to generate an electrochemical force that drives ATP synthesis. For clarity, each complex is shown as an individual entity. Ordinarily complexes I, III, and IV are part of a larger supercomplex that functions as a respirasome. There is little complex I found by itself on native gels from *C. elegans* mitochondria. (Figure courtesy of Ernst-Bernhard Kayser, PhD.)

Oxidative Phosphorylation/ETC

Oxidative phosphorylation refers to the process of synthesizing ATP by a stepwise series of transfer of electrons through large protein complexes embedded within the inner mitochondrial membrane. In the process, oxygen is consumed and an electro-chemical gradient is established that drives the synthesis of ATP. The individual redox active complexes, complexes I–IV, shuttle electrons to their final acceptor—oxygen—to form water (Figure 1). These protein complexes vary tremendously in composition. Complex I, which receives electrons from NADH, consists of over 40 proteins, while complex II consists of four. The subunits of complex I are encoded by both the mitochondrial and nuclear genome, while all complex II subunits are encoded by the nuclear genome. Complexes I, III, and IV exist as a supercomplex. Complex II is thought to exist as a separate entity, and represents a point of intersection between the TCA cycle and electron transport. The supercomplex containing complexes I, III, and IV has clear allosteric interactions in nematodes (below), and probably represents the functional unit of respiration (below). Mobile electron carriers, like coenzyme Q (CoQ) and cytochrome *c*, move electrons between protein complexes. Complex V is a well-studied protein complex that harnesses the energy stored by the proton gradient generated by complexes I–IV to generate ATP. Taken as a whole, ADP is phosphorylated generating ATP, and oxygen is consumed. The consumption of oxygen in respiration is a measurable parameter reflecting mitochondrial function (Figure 2).

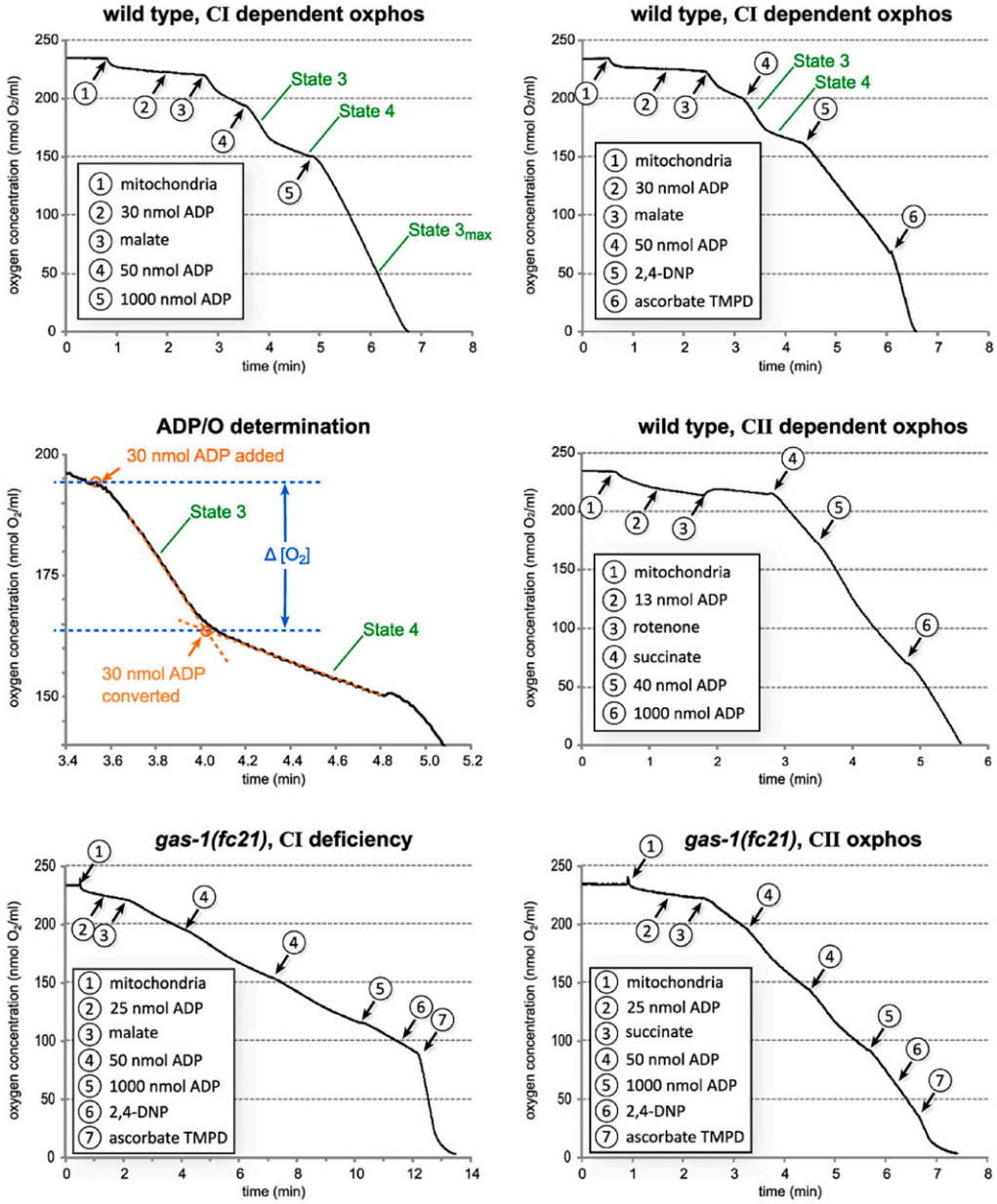

Figure 2 (A, B) Representative tracings of complex I-dependent oxidative phosphorylation in a Clark electrode. Oxygen concentration is plotted on the *y*-axis, time on the *x*-axis. In N2, (A) initially, mitochondria are placed in a chamber and allowed to respire. (1) Addition of ADP (2) does not increase the slow rate of oxygen consumption until malate is added (3) as a substrate for complex I respiration. Over time, ADP is consumed, and oxygen depletion slows until ADP is added a second time (4). Addition of DNP (5) dissipates the proton gradient normally present across the inner mitochondrial membrane, and allows oxygen to be reduced without linkage to production of ATP. This allows any complex I transport electrons at maximum rates when provided with the proper substrate. In the absence of DNP, providing a substrate for complex IV, TMPD ascorbate (6), bypasses the rate limiting step of complex I in the ETC, and rapidly depletes all oxygen within the chamber. (B) In *gas-1*, a mutant which causes a defect in the 49 kDa subunit of complex I (Kayser *et al.* 1999, 2001), it can be seen that even the addition of large amounts of ADP (5) does not increase the very slow rate of oxygen consumption when mitochondria are supplied with a complex I substrate, nor does addition of DNP. Bypassing complex I respiration completely by adding TMPD ascorbate (7) rapidly depletes oxygen. In both (A) and (B) an ADP/O ratio is calculated by measuring the amount of oxygen consumed when mitochondria are supplied with a known amount of ADP. (C) ADP is assumed to be converted to ATP when the rate of oxygen consumption abruptly slows. This "knee" in the curve is sometimes referred to as the state 3 to state 4 transition. (Figure courtesy of Ernst-Bernhard Kayser, PhD.)

Techniques

Oxidative phosphorylation: Studies of rates of oxidative phosphorylation of isolated mitochondria by their nature assess the integrated function of the entire respiratory chain (Kayser *et al.* 2001, 2004). In intact mitochondria, electron transport, as measured by oxygen consumption (respiration), and generation of ATP (phosphorylation) are tightly coupled by the proton motive force across the inner mitochondrial membrane (Puchowicz *et al.* 2004). Rates of oxygen disappearance as a function of substrate and ADP concentration can be measured in a Clark electrode, which uses a platinum electrode to measure the oxygen concentration in a solution (Figure 2). It is important to note that the rates of oxygen consumption by the mitochondria depend on the availability of substrate (such as pyruvate or succinate), and the availability of ADP to be phosphorylated. Chance and Williams defined five states of mitochondrial respiration (states 1–5); states 1, 2, and 5 represent starvation states for the mitochondria (absence of ADP, substrate, and oxygen, respectively) (Chance and Williams 1955). Generally, states 3 and 4 are studied to evaluate mitochondrial function. State 3 rates occur when oxygen, substrate, and ADP are all readily available. When ADP levels fall (having all been converted to ATP), oxygen consumption falls; this is termed state 4.

Commonly, a state 3 rate is measured primarily to determine an approximation of the maximal respiratory capacity of the mitochondrion with a large, nonlimiting, amount of

ADP present within the chamber for a fixed amount of mitochondria and O_2. Once all ADP is consumed, a clear decrease in the rate of oxygen consumption can be seen by a "knee" in the consumption curve. This decreased rate is the state 4 rate. This represents continued electron flow that is not coupled to production of ATP. The oxygen consumption rates are also a function of the particular substrate provided a mitochondrial preparation. Electrons can enter via complexes I, II, III, or IV in experimental studies of oxidative phosphorylation, and can be used to characterize the step of respiration that is defective in a mutant (see discussions of *gas-1*, *clk-1*, and *isp-1* in this chapter) (Kayser *et al.* 2001, 2004; Suthammarak *et al.* 2010). The state 3 rate over the state 4 rate (respiratory control ratio, RCR) is a useful measure of mitochondrial membrane integrity and proton leak across the inner mitochondrial membrane. An ADP/O ratio can also be calculated, a measurement of the amount of ADP consumed relative to oxygen disappearance. This is also a characteristic of each substrate, as more ATP is made when electrons originate from NADH (complex I) than from FADH (complex II). For a review of techniques to assess mitochondrial function, see the excellent review by Brand and Nicholls (2011) (Figure 2).

Recently, development of the Seahorse Metabolic Analyzer offers advantages for measuring respiration (Dancy *et al.* 2016; Luz *et al.* 2016b). Respiration can be measured in a large number of very small samples (up to 384 well plates), rapid measurements of basal and maximal respiratory rates can be made, and automatic delivery of specific inhibitors/uncouplers is possible. The use of 96- or 384-well plates reduces the requirement for an abundance of purified mitochondria. Using 24-well plates, the Seahorse has been used to measure whole nematode respiration (Dancy *et al.* 2015; Luz *et al.* 2016b). By using a series of inhibitors of respiration, it assesses both the rates of disappearance of oxygen [oxygen consumption rate (OCR)] and the acidification of the medium [extracellular acidification rate (ECAR)] with different substrates and uncouplers. OCR is similar to oxygen consumption in the Clark electrode, and, depending on the substrates and inhibitors, can correlate to specific steps measured with that instrument. In contrast, ECAR measures acid production via glycolysis with lactate as the primary end product. An example of measurements with the Seahorse is shown in Figure 3.

ETC assays: ETC assays are commonly used to study mitochondrial function. Unlike oxidative phosphorylation studies, mitochondrial membrane integrity is purposefully compromised for ETC studies, in order to introduce electron donors and acceptors, which normally cannot cross the mitochondrial membrane, into the mitochondrial matrix. In this way, individual enzymatic steps of electron transport can be dissected. For example, maximal rates for complex I, complex II, complex III, complex IV, and certain combinations of electron transfer from one complex to the next can be determined by using donors and acceptors specific to those complexes (Kayser *et al.* 2001, 2004). An outline of the donors and acceptors available and the specific activities they interrogate is shown in Figure 4.

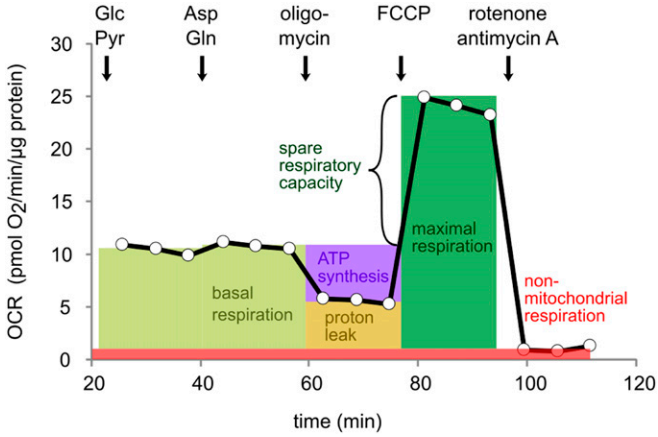

Figure 3 Representative tracing of respiration in a Seahorse Analyzer. Capable of measuring mitochondrial respiration in cells in culture, oxygen consumption can be measured in very small volumes, as in 96-well plates. OCR is measured over time, fueled often by glucose. However, whole nematode respiration, respiration of purified mitochondria, synaptosomes, *etc.*, can also be assayed. Oligomycin is a specific inhibitor of the ATPase complex V, and prevents protons from crossing the membrane through this complex to phosphorylate ADP. Oligomycin is used in these experiments to completely inhibit respiration. FCCP is a mitochondrial uncoupler (similar to DNP discussed in the legend to Figure 2) which allows protons to leak freely across the mitochondrial membrane and uncouples oxygen consumption from phosphorylation of ADP. Antimycin A is a specific inhibitor of complex III, and rotenone is a specific inhibitor of complex I. They are used together to block all mitochondrial respiration even in the presence of an uncoupler such as FCCP. At the same time the Seahorse can measure acidification of the medium, providing an estimate of glycolysis. This measurement is termed ECAR. (Figure courtesy of Ernst-Bernhard Kayser, PhD.)

ETC results have been published for many mitochondrial mutants in *C. elegans*, with striking differences seen between strains (Kayser *et al.* 2001, 2004; Miyadera *et al.* 2001; Grad and Lemire 2004; Suthammarak *et al.* 2009, 2010). In each case, a difference is seen in the enzyme activity predicted by defects in the predicted subunit. However, in some cases, surprising defects in other components of the MRC have been noted indicating allosteric interactions between complexes. For example, intrinsic complex I defects are seen in *isp-1* mutants (a complex III mutation) (Suthammarak *et al.* 2010), and also when complex IV subunits are knocked down by RNAi (Suthammarak *et al.* 2009). In *clk-1*, the defect in CoQ synthesis affects only transfer of electrons from complex I to complex III (Kayser *et al.* 2004), but not complex II to complex III. Studies have shown that the effects of complex III and IV on complex I relate to their effects on supercomplex formation (Suthammarak *et al.* 2009, 2010). In general, the functions of the complexes, and the biochemical studies used to characterize them, are extremely conserved from the nematode to humans. The ability to isolate large amounts of mitochondria, and the availability of mutants or RNAi knockdowns in specific complexes, even led to a nematode kit to use for normalization of mitochondrial studies in

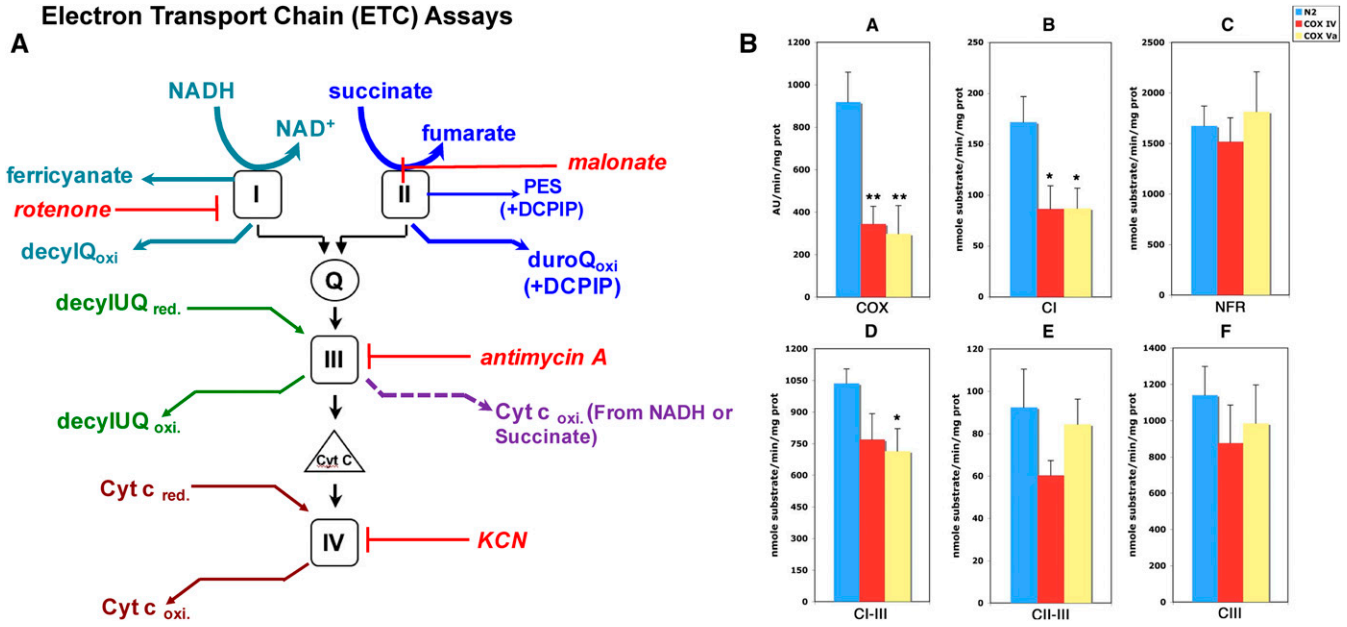

Figure 4 (A) A representation of assays of enzymatic steps within electron transfer. Electron donors and acceptors can be added to permeabilized mitochondria to isolate specific steps of the ETC. Their reduction is followed by a color change of an electron acceptor in a spectrophotometer. For example, the electron donor NADH is oxidized by complex I. All subsequent flow of electrons within the complex is blocked by rotenone. Ferricyanate is the electron acceptor for the first part of complex I (termed NFR activity and taken as a measure of the amount of complex I, rather than the activity of complex I); decylQ is the acceptor when electrons are allowed to pass through the entirety of complex I (a measure of complex I activity). However, electrons from NADH can, in the absence of rotenone, flow through Q and through complex III, to reduce the electron acceptor, oxidized cytochrome *c* (a measure of I–III activity). Further flow is blocked by potassium cyanide. Other individual steps are indicated by their respective electron donors and acceptors. (B) An example of a battery of ETC enzymatic assays used to investigate knockdown of two different subunits of complex IV. COX, flow of electrons through complex IV; CI, flow of electrons through complex I; NFR, NADH-ferricyanide reductase, the first step of NADH oxidation; I–III, flow of electrons through complex I and complex III; II–III, flow of electrons through complex II and complex III; CIII, flow of electrons through complex III. In this case, knockdown of complex IV subunits reduced enzymatic activity of complex IV, but also affected electron flow within complex I, reflecting the significance of allosteric interactions between components of respiratory chain supercomplexes. Values for N2 are in blue, knockdown of COX subunits are in red or yellow. [Figure originally published in Suthammarak *et al.* (2009)]. (Figure 4A courtesy of Ernst-Bernhard Kayser, PhD.)

humans (Chen *et al.* 2011). Using this kit allowed for normalization of biochemical studies, and to potentially standardize values between different laboratories.

MRC

C. elegans is a good model system for the study of mitochondrial function

Although many other valuable approaches are being used to understand how mitochondria function, *C. elegans* offers powerful and unique advantages. The degree of conservation of mitochondrial proteins between nematode and mammals is quite high, and indicates that knowledge gained in the nematode will be largely transferrable to mammals. The ability to label mitochondria, and follow their mobility, structure, and even function in the transparent nematode, further strengthens *C. elegans* as a model for mitochondrial studies. Assays that are used to study human mitochondria are readily applicable to the same studies in nematodes, with little modification. This is true of studies of oxidative phosphorylation, ETC enzyme assays, blue native gels (BNGs), free radical production, damage, etc. (Kayser *et al.* 2001, 2004; Suthammarak *et al.* 2009, 2010, 2013). It should be noted, however, that isolation of functional mitochondria from the nematode is a bit more difficult than from mammalian cells, largely due to having to disrupt the cuticle while preserving the intact mitochondria.

This one limitation in isolation is overshadowed by the ability to generate nuclear-encoded mitochondrial defects with RNAi. Since it is possible to titrate RNAi treatment in the nematode to generate different degrees of mitochondrial damage, one can study those many mitochondrial proteins that would be lethal in a knockout animal (Rea *et al.* 2007). In turn, this allows isolation of virtually limitless amounts of mitochondria with known defects. We view RNAi treatment as a unique method to generate, at a specific point in time, gene loss leading to mitochondrial disease in a manner simply not available in most other organisms.

Mutant nuclear-encoded and mitochondrial-encoded subunits of the *C. elegans* MRC have been investigated, including complex I subunits (GAS-1, NUO-1, and NUO-6), complex II (MEV-1), complex III (ISP-1 and CTB-1), complex IV (COXIVa and COXV), and complex V (ATP-2). In addition, mutations inhibiting the synthesis of ubiquinone (CLK-1 and COQ-3) have also been identified. These, as well as proteins that modify the

MRC indirectly, have been studied on the molecular, cellular, and organismal levels through the variety of experimental approaches that are readily achievable in *C. elegans*. In these models, MRC dysfunction can cause increased or decreased lifespan, neuromuscular deficits, developmental delay, decreased fecundity, or altered anesthetic sensitivity—all of which are phenotypes that mimic symptoms observed in human patients with primary mitochondrial disorders. MRC dysfunction also variably affects gene expression profiles (Falk *et al.* 2008; Zuryn *et al.* 2010), and ROS production (Yang *et al.* 2009; Desjardins *et al.* 2017). Antioxidant dietary supplements, such as vitamin E, have been investigated using disease and aging models (Adachi and Ishii 2000; Zou *et al.* 2007). Substitutes for depleted CoQ and flavin cofactors have also been explored as useful therapies for ETC defects (Grad and Lemire 2004; Gonzalez-Cabo *et al.* 2010). Furthermore, MRC mutants with altered longevity have proved useful for probing the contributions of energy consumption, ROS, and stress responses to the process of aging.

There are a number of differences between mammalian and *C. elegans* mitochondria. The ~14 kb circle of mtDNA of *C. elegans* contains homologs of 36 of the 37 genes found in human mtDNA, but is missing the ATP8 subunit of complex V (Okimoto *et al.* 1992). *C. elegans* also appear to have fewer copies of mtDNA per cell than humans (Tsang and Lemire 2002; Reinke *et al.* 2010). Instead of coenzyme Q10 (CoQ_{10}), which has a chain of 10 isoprenyl repeats, *C. elegans*, like rodents, primarily use coenzyme Q9 (CoQ_9) (Larsen and Clarke 2002). Whereas the glyoxylate cycle is not normally found in animals, *C. elegans* possess a malate synthase/isocitrate lyase termed ICL-1 or GEI-7, which cleaves isocitrate to form glyoxylate and succinate (Liu *et al.* 1995). In the nematode, unlike plants, this pathway is encoded by a single gene. It gives the nematode the ability to divert a TCA cycle intermediate to production of two-carbon fragments used for anabolic function. Furthermore, mitochondria isolated from *C. elegans* can respire with malate as the substrate, while malate alone is a poor substrate for mammalian mitochondria (Kayser *et al.* 2001). *C. elegans* mitochondria are also less sensitive to certain artificial uncouplers, and to thenoyltrifluoroacetone (TTFA), an inhibitor of complex II in mammals (Kayser *et al.* 2001). Lastly, cardiolipin—a membrane phospholipid required in the inner mitochondrial membrane for proper MRC function in humans—appears to only be required in the *C. elegans* gonad and not in somatic cells (Sakamoto *et al.* 2012). Despite these differences, in most ways *C. elegans* mitochondria are very similar to mammalian mitochondria, and will likely provide a useful system in which to explore unanswered questions in mitochondrial bioenergetics and dysfunction. We will discuss the mutations studied thus far in *C. elegans* organized by function.

MRCs

Complex I: It is worth noting that, in the mitochondrial literature, the naming of complex subunits can be confusing. The naming varies based on species, with subunits potentially named based on molecular weight, location, or function within the complex. For example, the nematode gene we will describe in some detail below, *gas-1*, which has a mass of 51 kDa, is the ortholog of the 49 kDa subunit of cows, and corresponds to NDUFS2 of mice and humans.

Complex I is also known as NADH-CoQ oxidoreductase, which reflects its function in the ETC. Complex I of bovine heart mitochondria has 45 structural subunits, of which 38 are encoded by nuclear DNA (nDNA), and seven are encoded by mtDNA in mammals (six are encoded by mtDNA in the nematode). Fourteen of the subunits represent the catalytic "core" of the enzyme, with presumed roles in redox and proton translocation. Although the three-dimensional (3D) structure of complex I in *Thermus thermophilus* has been solved (Baradaran *et al.* 2013), the functions of many of the accessory subunits remain unknown.

Complex I subunit homolog knockdown studied by RNA interference: All seven mtDNA subunits, at least 31 nDNA subunits, and four known complex I assembly factors demonstrate extensive evolutionary conservation between humans and *C. elegans* (between 25 and 99.2% amino acid similarity). Twenty-six subunits of complex I have been confirmed via mass spectrometry analysis of complex I isolated by BNG electrophoresis from wildtype (N2) *C. elegans* mitochondria (Suthammarak *et al.* 2009).

RNAi has been used to effectively produce animals with targeted loss-of-function in each of 28 individual, nuclear-encoded, structural subunits, and two assembly factors of mitochondrial complex I. The gene-silencing pathway is not present within the mitochondrion, so mtDNA-encoded genes are not amenable to this technique. The percent knockdown of the genes by RNAi was determined in the F2 generation (mean qPCR knockdown). Mean qPCR knockdown did not qualitatively predict either degree or direction of alterations in either of the two measured phenotypes, respiratory capacity, or anesthetic sensitivity. The lack of correlation between knockdown and phenotype indicates that not all subunits equally effect these endpoints. Therefore, complex I subunits differ in their biological contributions to complex I respiratory capacity and anesthetic behavior. Three subunits of complex I have been studied individually. These are described below.

gas-1: The most characterized complex I subunit in *C. elegans* is GAS-1 (Morgan *et al.* 1990; Kayser *et al.* 1999, 2001; Van Raamsdonk and Hekimi 2011; Wernick *et al.* 2016). Along with the PSST subunit, GAS-1 is thought to partially form the CoQ binding site of complex I. The *fc21* allele of *gas-1* (hypomorphic allele; R290K) was identified (Kayser *et al.* 1999), surprisingly enough, in a screen for altered sensitivity to volatile anesthetics. *gas-1*(*fc21*) animals also have shortened lifespans at 20°, few offspring, grow slowly, and are extremely hypersensitive to oxidative stress. However, these phenotypes are variable depending on growth conditions (Van Raamsdonk and Hekimi 2011; Pujol *et al.* 2013; Desjardins *et al.* 2017).

GAS-1 orthologs are known as the human NDUFS2 or the bovine 49 kDa subunit. Mutations in NDUFS2 have been

identified in nine separate families with Leigh Syndrome, Leigh-like Syndrome, cardiomyopathy, and encephalomyopathy. Studies of rates of oxidative phosphorylation allowed assessment of the impact of *gas-1*(*fc21*) on the metabolic capacity of the whole respiratory chain (Kayser *et al.* 2001). In *gas-1* animals, complex I-dependent oxphos rates, with glutamate or malate as substrates, are ~25% of the wild type. ETC enzyme assays confirmed that complex I-dependent activity was significantly decreased in *gas-1* compared to N2. *gas-1*(*fc21*) mutant animals also have a decrease in NADH-ferricyanide reductase activity, the first enzymatic step in complex I, in which NADH is oxidized. Since GAS-1 is near the CoQ binding site within the mitochondrial membrane, and not in the mitochondrial matrix where NADH oxidation occurs, this effect may be an allosteric effect within complex I, or a decrease in the amount of complex I. As a possible compensatory change, in mitochondria from *gas-1* animals, complex II-dependent respiration (succinate as the substrate) is doubled relative to that of N2. BNGs show that the amount of complex I was decreased in *fc21* animals. However, recent work has shown that, by decreasing complex II-dependent metabolism, complex I is stabilized in *gas-1*(*fc21*), and the animals become long-lived, similar to other complex I mutants (Pujol *et al.* 2013).

C. elegans with hypomorphic loss-of-function in *gas-1* are profoundly hypersensitive to volatile anesthetics, such as halothane, enflurane, and isoflurane, and the gene therefore takes its name from *general anesthetic sensitive*. RNAi-based knockdown of *gas-1* also resulted in halothane hypersensitivity, and was dose-dependent with respect to RNAi induction, further confirming the link between *gas-1* function and volatile anesthetic sensitivity. The effect on anesthetic sensitivity requires both neuronal and muscular expression of *gas-1*, and evidence that this might be linked to a presynaptic effect in motor neurons comes from an aldicarb/levamisole assay. Furthermore, for the majority of complex I subunits, RNAi-based knockdown results in halothane hypersensitivity, indicating a general link between complex I dysfunction and volatile anesthetic hypersensitivity. This is further confirmed by an increase in complex I activity, through mutation of the insulin receptor *daf-2*, which results in halothane resistance. The link between complex I dysfunction and volatile anesthetic hypersensitivity is also observed in mice and humans. This correlation, which began with *C. elegans*, has influenced the clinical recommendations for dosing of volatile anesthetics to patients with mitochondrial disorders (Morgan *et al.* 2002; Niezgoda and Morgan 2013). It remains unclear exactly how complex I dysfunction leads to volatile anesthetic hypersensitivity. However, recent work has shown that, in a mouse model of complex I deficiency, the frequency of glutamatergic synaptic potentials is selectively susceptible to inhibition by the volatile anesthetic, isoflurane (Zimin *et al.* 2016).

Other phenotypes of the *gas-1*(*fc21*) animal include slow development, reduced locomotion, and reduced fecundity. Similar to human patients with complex I dysfunction, *gas-1*(*fc21*) animals are short-lived at room temperature, but long lived at 15°. Both the fecundity and short-lived phenotype are temperature sensitive, with virtually no offspring, and a very short adult life span if the animals are placed at 25°. *gas-1*(*fc21*) has an increased mitochondrial mass, and an increase in complex II-dependent respiration as measured by oxygen consumption of isolated mitochondria provided with succinate as substrate (Kayser *et al.* 2001). In fact, increased complex II-dependent respiration is a general characteristic when any one of a number of complex I subunits are knocked down (Falk *et al.* 2006). These phenotypes mirror those of human patients with mitochondrial disease who have elevated complex II-dependent respiration and "ragged red fibers" made up of overproduced mitochondria (Dancy *et al.* 2015).

In addition to reduced NADH oxidation and reduced proton pumping, loss-of-function in complex I can alter the production of ROS such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$). ROS damage correlates with a variety of pathologies and aging. The MRC, and, in particular, complexes I and III, is known to be the major endogenous source of ROS (Chen *et al.* 2003); cells derived from patients with loss-of-function in complex I often have increased superoxide and hydroxyl radical levels as well as increased lipid peroxidation, presumably due to inefficient electron transfer by the MRC. Increased ROS production may have an autocatalytic effect by damaging MRC components, reducing complex I levels. *gas-1*(*fc21*) animals have an increase in oxidative damage to specific, mitochondrial, proteins, and are hypersensitive to the oxidizing agent, paraquat, and also to hyperoxia (Hartman *et al.* 2001; Suthammarak *et al.* 2013). Similarly, when complex I is inhibited by rotenone, wild-type animals become hypersensitive to hyperoxia (Ishiguro *et al.* 2001). Lowering the oxygen tension in cultures of *gas-1* moves its dose–response curve for anesthetic sensitivity toward that of N2, while growing N2 in 80% oxygen heightens its sensitivity to a volatile anesthetic (Hartman *et al.* 2001). In fibroblasts cultured from a patient with mutant NDUFS2—a vitamin E derivative—reduced ROS levels to wild-type levels and riboflavin rescued ATP production. These results suggest that treatment with supplements containing antioxidants such as vitamin E may be beneficial in patients with complex I dysfunction in which ROS is elevated.

Presumably in response to an oxidative burden, *gas-1*(*fc21*) animals upregulate the expression of genes that function in detoxification of ROS and oxidative damage, such as the inducible form of superoxide dismutase (SOD), *sod-3*. Possibly because of this, overall ROS damage levels are similar in *gas-1* to wild type (Suthammarak *et al.* 2013), even though, as noted above, mitochondrial proteins have increased ROS damage. The induction of SODs in *gas-1*(*fc21*) mirrors a SOD induction observed in some patients with complex I deficiency, which correlates with decreased ROS damage. SOD induction can improve the phenotypes of complex I deficiency, as evidenced by reduced

neurodegeneration in a mouse model with virally delivered SOD. In addition to ROS scavenging, SOD-2 functions to stabilize the I:III:IV supercomplex through a direct interaction between the protein and complex I (Suthammarak *et al.* 2013)—an association also seen in mice (W. Suthammarak, personal communication). Thus cells may attempt to compensate for defective complex I function by increasing supercomplex stability, through upregulating SOD-3 levels. Each of these characteristics demonstrate the similarities between mitochondrial dysfunction and SOD function in the nematode and in mammals.

nuo-1: Grad and Lemire studied NUO-1 (the ortholog of mammalian flavoprotein NDUFV1 subunit of complex I) in *C. elegans*. The *ua1* allele has a 1.2-kb deletion that has been shown by the Lemire group to decrease complex I-dependent respiration. Since the mutation causes sterility, Lemire's group maintained *ua1* through presence of a balancer (Tsang *et al.* 2001). When a heterozygous mother produces homozygous offspring, lifespan is extended, but the animals are arrested as L3 larvae. *nuo-1* animals also had a slow pharyngeal pumping rate, defecation rate, and locomotion.

Less severe point mutants were later studied by introducing a *nuo-1* transgene, containing single specific amino acid substitutions, into the deletion strain (Grad and Lemire 2004). A352V, T434M, and A443F transgenic animals retain the decreased complex I activity, and also have decreased complex IV activity (Grad and Lemire 2006). Unlike the original deletion strain, the transgenic animals reach adulthood but are very short lived. They also have low fecundity and hypersensitivity to hyperoxia and paraquat, and A352V has slow development. Thus, while a severe defect (the deletion with the maternal effect from the balancer) causes longer lifespan, a smaller change (single substitution but without balancer) causes shorter lifespan. It may be that larval development and egg production confer energetic demands higher than those attainable by the deletion strain, especially after any maternal contribution to *nuo-1* expression.

It is interesting that the short lifespan of *nuo-1* transgenic point mutants appeared to be dependent on the reduced energy production in these animals. This was examined further by inserting an enzyme, yeast L-lactate-cytochrome *c* oxidoreductase, that makes lactate-dependent respiration possible. In addition to increasing lifespan, ATP levels, respiration rates, and fecundity were also improved (Grad *et al.* 2005). This suggested that, in these transgenic animals, the availability of energy determines lifespan.

nuo-6, nuo-2: The third complex I subunit studied in *C. elegans* via classical mutation is NUO-6. *nuo-6* encodes the complex I subunit orthologous to the mammalian complex I subunit, NDUFB4. Yang and Hekimi identified a missense mutation in *nuo-6*, *qm200* (Yang and Hekimi 2010b). As expected, *nuo-6*(*qm200*) has decreased respiration and electron transport rates, although an increase in ATP concentrations is also observed. Similar to *gas-1*(*fc21*) and *nuo-1*(*ua1*), *nuo-6*(*qm200*) exhibits slow embryonic and postembryonic development, reduced fecundity, and neuromuscular defects such as slow locomotion, pharyngeal pumping, and defecation (Yang and Hekimi 2010b). *nuo-6*(*qm200*) is long-lived, without the developmental arrest seen in *nuo-1*(*ua1*), in agreement with other mitochondrial defects that can lead to lifespan extension in *C. elegans*.

RNAi knockdowns of *nuo-6* or *nuo-2*—the NDUFS3 subunit in *C. elegans*—both of which decreased ATP levels, also extend lifespan. *nuo-6*(*qm200*), but not *nuo-2*(*RNAi*), was reported to induce SODs, and the *nuo-6*(*qm200*) animals are resistant to paraquat (Yang and Hekimi 2010b), suggesting an improved stress tolerance. Interestingly, *nuo-6*(*qm200*) appear to have increased superoxide anion ($O_2^{\bullet-}$) but unchanged overall ROS levels, and manipulation of the $O_2^{\bullet-}$ levels using antioxidants (vitamin C or N-acetyl-L-cysteine) or an oxidant (paraquat) also extended lifespan. The many paradoxes yet to be understood in the relation between mitochondrial function and life span are obvious, but the reader is referred to an excellent review on the subject (Wang and Hekimi 2015).

However, in general, RNAi knockdown caused a stress-like response (increased autophagy and activation of the heat shock response), while the missense mutation did not. Similar results were noted when comparing the missense mutation in the complex III subunit *isp-1*(*qm150*) with *isp-1*(*RNAi*) (Yang and Hekimi 2010b). The latter authors concluded that there were two separate mechanisms, brought on by mitochondrial dysfunction, that were capable of slowing development and extending lifespan (Yang and Hekimi 2010b). In support of this possibility, Pujol *et al.* (2013) did not find an interaction between complex II activity and the effect of *nuo-6* on lifespan, in contrast to interaction of complex II and the effects of *gas-1*. Yang and Hekimi (2010a) went on to show that $O_2^{\bullet-}$ generation was actually increased in the missense mutants of *isp-1* and *nuo-6*, while total ROS was unchanged or mildly decreased. The roles of $O_2^{\bullet-}$ in lifespan are beyond the scope of this chapter, but it is important to realize that $O_2^{\bullet-}$ is one of the byproducts of electron transport from complex I.

Complex II (succinate ubiquinone oxidoreductase): Complex II is the succinate-CoQ oxidoreductase, also known as succinate dehydrogenase. It is also an entry point of electrons into the MRC. It has several unique features: it is the only MRC complex encoded entirely in the nuclear genome; it is the only MRC complex that does not translocate protons across the membrane; and it is small, composed of four nuclear-encoded subunits. In *C. elegans*, defects in three of these result in embryonic lethality when knocked down, and the fourth results in a decrease in survival of the mutant, *mev-1* (Ichimiya *et al.* 2002).

mev-1: Ishii and colleagues identified a missense mutation, *mev-1(kn1)*, in the gene *mev-1* (methyl viologen sensitive) (Ishii *et al.* 1990). *mev-1* encodes the succinate dehydrogenase cytochrome *b* large subunit (SDHC subunit) of complex II (Figure 1) (Ishii *et al.* 1998). The *kn1* allele of *mev-1* was the first mutation affecting a mitochondrial protein identified

in *C. elegans*. Analogous to GAS-1, MEV-1 forms part of the quinone binding site of complex II. Ishii and colleagues showed that *mev-1* has a shortened lifespan (also similar to *gas-1*), decreased fecundity, an increase in oxidative damage, and is hypersensitive to oxidative stress (Ishii *et al.* 1998). Of the genes associated with primary mitochondrial disorders, complex II was the first direct hit as a nuclear gene encoding a MRC subunit in humans. Mitochondrial disorders have now been linked to mutations found in all four of the complex II subunit genes. Notably, mutations in complex II subunits have been linked to cancers, including cervical paraganglioma, and familial paraganglioma.

Mitochondria from *mev-1* animals have decreased complex II-dependent oxidative phosphorylation rates (succinate as the substrate), and decreased complex II activity in ETC studies. Ishii and Hartman also demonstrated that the rate of $O_2^{\bullet-}$ production and steady state $O_2^{\bullet-}$ levels are elevated in *mev-1* animals (Senoo-Matsuda *et al.* 2001). *mev-1* showed increased overall hydroxynonenal (HNE) oxidative damage but a different pattern of damaged proteins was affected than that seen in *gas-1*. This raised the question of whether the specific location of the ROS generation makes a difference to which proteins are damaged.

For comparison to the results of Ishii, Guo and Lemire constructed two different complex II mutants in yeast, both near a binding site of complex II to CoQ. One of these was modeled on the *mev-1* mutation in nematodes (Guo and Lemire 2003). These latter authors found that both mutations reduce transfer of electrons from complex II to III, and both were sensitive to oxidative stress. Thus, the *mev-1* yeast mutants share phenotypes found in *mev-1* nematode mutants. In addition, mitochondria from each can produce significant amounts of free radicals, which the authors postulated was due to release of electrons from an intermediate ubisemiquinone radical.

In addition to paraquat, *mev-1*(*kn1*) animals are also hypersensitive to increased oxygen concentration, much like animals treated with the complex II inhibitor, TTFA. It is possible that these hypersensitivities are due in part to a decrease in SOD activity that is detected in the mutant. *mev-1*(*kn1*) animals have measurably increased levels of superoxide anion ($O_2^{\bullet-}$) production, especially under hyperoxia; decreased levels of reduced glutathione; increased protein oxidation; increased DNA mutation rate; and increased expression of *sod-3*. *mev-1*(*kn1*) animals have decreased fecundity and shortened lifespan, the latter of which could be rescued by treatment with a SOD/catalase-mimetic compound under certain conditions (Ishii *et al.* 1990; Hartman *et al.* 2001; Ishiguro *et al.* 2001; Senoo-Matsuda *et al.* 2001). This suggests that similar SOD/catalase-mimetic compounds could be developed for use in human patients with complex II deficiency, and possibly complex I deficiency as well.

The paraquat and hyperoxia sensitivity, ROS increase, decreased fecundity, and short lifespan phenotypes of *mev-1*(*kn1*) are reminiscent of *gas-1*(*fc1*). It should be noted that it was confirmed that complex II-dependent respiration (with succinate as substrate) is reduced in *mev-1*(*kn1*), while complex I-dependent respiration (with glutamate or malate as substrate) remains unchanged (Kayser *et al.* 2004). This suggests that disrupting either entry point of electrons to the MRC can have similar effects, and it should be noted that disrupting both simultaneously, in a *mev-1*(*kn1*);*gas-1*(*fc21*) double mutant, is lethal. In addition to reduced respiration, *mev-1*(*kn1*) animals exhibit a twofold increase in lactate and lactate/pyruvate ratio, which mirrors the lactic acidosis seen in patients with mitochondrial disorders. However, *mev-1*(*kn1*) does not result in anesthetic hypersensitivity, including to halothane and isoflurane, implying that the mechanism of action of these volatile anesthetics is not due to a general MRC defect or increase in ROS.

CoQ: *clk-1:* The investigations of the function of *clk-1* illustrate many of the issues in studying mitochondria in *C. elegans*. First studied as a long-lived animal, its phenotype included poor synchrony in development, rates of pharyngeal pumping, and defecation (Wong *et al.* 1995; Felkai *et al.* 1999). When cloned, it was found to be an enzyme that is involved in the synthesis of CoQ, the mobile electron carrier that shuttles electrons from either NADH derived electrons from complex I, or succinate-derived electrons from complex II, to complex III (Ewbank *et al.* 1997; Miyadera *et al.* 2001). These molecules contain an isoprenyl tail of varying length that is species specific, and embeds the molecule into the inner mitochondrial membrane. Quinones also contain a polar head group that is thought to be the mobile element receiving electrons from either complex I or complex II. In nematodes, as in rodents, the dominant quinone of the electron transport chain is CoQ_9, denoting nine repeats within the isoprenyl tail. In humans CoQ_{10} is the functional form, while in bacteria it is CoQ_8. Loss of *clk-1* in nematodes results in the accumulation of an intermediate molecule of CoQ_9 synthesis, called DMQ_9—a molecule lacking a methoxy group on the polar head (Miyadera *et al.* 2001). Both N2 and *clk-1* animals contain small amounts of bacterial CoQ_8 when fed a normal bacterial diet. Dietary CoQ_8 is necessary for survival of the *clk-1* mutant (Jonassen *et al.* 2001). Growing nematodes on Q-less bacteria causes developmental arrest of *clk-1* and prolonged lifespan for N2 (Larsen and Clarke 2002).

Initial studies of *clk-1* revealed no measurable mitochondrial defects—puzzling, given its key role in electron transport (Braeckman *et al.* 1999, 2002; Felkai *et al.* 1999). However, further studies of this mutant yielded some key findings. When grown on bacteria that contain no CoQ, *clk-1* is developmentally arrested as L2 larvae (Jonassen *et al.* 2001, 2002). If switched from a normal bacterial diet to that of a Q-less strain, no effect is seen after L4—a time at which mitochondrial biogenesis for egg production has already occurred. Later studies with isolated mitochondria showed that when multiple aspects of mitochondrial function are examined, *clk-1* animals are severely impaired, even when grown on K12 bacteria which contain CoQ_8 (Kayser *et al.* 2004). Under these growth conditions, *clk-1* animals have

normal enzymatic activity of complexes I, II, and III. However, their ability to transfer electrons from I to III is specifically and severely impaired, while electron transfer between complexes II and III is normal. Studies with exogenously added CoQ indicated these results are likely explained by the inability of exogenous CoQ to access the I–III–IV supercomplex at the quinone binding site (Yang *et al.* 2009, 2011).

Complex I is composed of ~46 subunits in nematodes (Falk *et al.* 2006), and is part of a supercomplex that consists of complexes I, III, and IV (Suthammarak *et al.* 2009). Together, these represent the functional entity for oxidative phosphorylation, and are linked together as one very large entity called the respirasome. Measurements in nematodes and mammals have shown that the enzymatic activity of complex I (transfer of electrons through the complex) is significantly higher when it is part of the entire supercomplex than when it is isolated further (Schafer *et al.* 2006; Suthammarak *et al.* 2009; Lopez-Fabuel *et al.* 2017). In nematodes, unlike mice, for example, very little complex I exists in isolation; the majority of complex I that separates on digitonin containing BNGs exists as a supercomplex: either I:III_2:IV_n or I:III_2 (Suthammarak *et al.* 2009). Complex II is made up of four subunits, and is not part of a supercomplex, but rather represents the intersection of the TCA cycle with the ETC. This has led to the hypothesis that there are two pools of quinones in nematodes, one that is incorporated into the respirasome during its formation, and one that is not (Yang *et al.* 2011). This free fraction may consist of the bacterial CoQ_8 that will be available to the hatched nematode, and allows for escape of the embryonic lethality phenotype of *clk-1* by virtue of normal passage of electrons from complex II to complex III. The predominant species, DMQ_9, may be sequestered into the supercomplex during embryonic development. Since few new mitochondria are made until gonadogenesis, it may be that the supercomplex only possesses the nonfunctional DMQ_9, while the functional, newly acquired, bacterial quinone obtained through ingestion, CoQ_8, is only available to complex II. Interestingly, the accumulation of DMQ_9, even though rendering nematodes incapable of normal mitochondrial respiration, may contribute to the longevity phenotype *of clk-1* (Yang *et al.* 2009). *clk-1* fed CoQ8-containing bacteria displays negligible ROS damage to mitochondrial proteins when assayed by HNE damage, much less than wild-type nematodes or *clk-1* animals grown on a bacteria synthesizing a range of quinones ($CoQ_{5,6,7,8,9,10}$). Thus, it has been postulated to serve as an efficient scavenger of mitochondrially produced ROS.

Complex III: Complex III is the CoQ-cytochrome *c* oxidoreductase, also known as the cytochrome bc_1 complex. Within complex III, the iron-sulfur cluster of the Rieske protein, the two hemes of cytochrome *b*, and the heme of cytochrome c_1 are involved in electron transfer during the Q cycle. Mutations in complex III subunits are associated with several mitochondrial diseases in humans. Two of these are infantile syndrome GRACILE, named for the symptoms of growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death and Leigh syndrome, discussed earlier with complex I. In *C. elegans*, homologs of at least 8 of the 11 human genes encoding complex III subunits have been identified. Three well-characterized complex III subunits are encoded by two nuclear genes, *isp-1* and *cyc-1* (Feng *et al.* 2001; Dillin *et al.* 2002), and the mtDNA gene *ctb-1* (Feng *et al.* 2001); they take their names from their mammalian orthologs, the Rieske iron sulfur protein, cytochrome c_1, and cytochrome *b*, respectively.

isp-1: isp-1(*qm150*) was originally isolated in a screen for animals that are slow to develop, and have decreased defecation as a measure of neuromuscular impairment (Feng *et al.* 2001). *isp-1*(*qm150*) is a perfect example of a "slow" phenotype, having both slower embryonic and postembryonic development, but long lived. Consistent with these results, RNAi against the complex III subunit *cyc-1* or treatment of wild-type animals with Antimycin A, a complex III inhibitor mimic the phenotypes of *isp-1* (Dillin *et al.* 2002).

isp-1(*qm150*) has a profound decrease in complex III-dependent oxidative phosphorylation and electron transport (Suthammarak *et al.* 2010). In addition, the I:III:IV supercomplex is severely disrupted. This suggests that the ISP-1 subunit of complex III is a direct or indirect stabilizer of the supercomplex. Complex I activity, as well as complex I–III activity, is dramatically impaired in *isp-1*(*qm150*), most likely as an allosteric consequence of disrupted supercomplex-mediated CoQ channeling (Suthammarak *et al.* 2010). Other smaller changes were detected in *isp-1*(*qm150*), such as increased complex II activity, decreased NADH ferricyanide reductase activity, and decreased complex IV activity staining on a native gel. In summary, the *isp-1*(*qm150*) mutation causes not only defective complex III activity but also indirect decreases in complex I and IV activity, probably due to supercomplex disruption. Interestingly, Jafari *et al.* collected multiple suppressors of the *qm150* allele and found them all to be intragenic in a six-amino-acid region of the ISP-1 protein (Jafari *et al.* 2015, 2016). Similar mutations in the yeast homolog of ISP-1had similar effects, clearly demonstrating the high level of conservation of ISP-1 across a wide phylogenetic range. Furthermore, this work in *C. elegans* established the importance of a tether region in the protein that allows the electron transporting region of the protein to move during a switch from acceptor to donor functions. Thus, work in the nematode has established a functional role undoubtedly conserved throughout phylogeny.

ctb-1: ctb-1(*qm189*) was identified by the Hekimi laboratory as a spontaneous suppressor of the slow rate of development but not the long lifespan of *isp-1*(*qm150*) (Feng *et al.* 2001). As noted above, *ctb-1* is encoded by a mitochondrial gene. The mutation is generally present as a homoplasmic mutation, *i.e.*, all copies of the mtDNA contain the mutant allele. Presumably, strong selective pressure in an *isp-1*(*qm150*) background drove the mutation to the homoplasmic state.

A detailed biochemical analysis was undertaken to uncover the molecular mechanisms behind the phenotypes of these mutations. On its own, *ctb-1*(*qm189*) has decreased complex III and complex I–III activity, albeit not as severe as *isp-1*(*qm150*) (Suthammarak *et al.* 2010). The I:III:IV supercomplex was not disrupted in *ctb-1*(*qm189*). Compared to *isp-1*(*qm150*), the complex I activity of *ctb-1*(*qm189*) is affected to a lesser extent [~30% in *ctb-1*(*qm189*) *vs.* 70% in *isp-1*(*qm150*)], and no change is detected for NADH ferricyanide reductase activity.

When *isp-1*(*qm150*) and *ctb-1*(*qm189*) are both present in a double-mutant strain, *ctb-1*(*qm189*) is epistatic to *isp-1*(*qm150*) for all of these biochemical characteristics, except activities involving complex III, which were still severely reduced (Suthammarak *et al.* 2010). *ctb-1*(*qm189*) rescued the supercomplex instability and complex I defect of *isp-1*(*qm150*), as well as the developmental and neuromuscular defects. Surprisingly, *ctb-1*(*qm189*) did not rescue the complex III defect, and no reversal was observed in the lifespan extension phenotype of *isp-1*(*qm150*). This suggests that the lifespan is controlled by complex III, and we can assign the "slow" phenotypes to the indirect effect on complex I. These data are consistent with the findings using direct disruption of complex I, via *gas-1*(*fc21*), which causes "slow" phenotypes but no lifespan extension. This also means that the *ctb-1*(*qm189*) mutation, which causes loss-of-function in complex III, also has a gain-of-function in stabilizing the I:III:IV supercomplex to counteract the destabilizing effects of *isp-1*(*qm150*).

Complex IV: Complex IV is also known as cytochrome *c* oxidase or COX. Complex IV terminates the flow of electrons through the ETC, reducing oxygen to water. Electron transfer involves two hemes, cytochrome *a* and cytochrome a_3, and two copper centers, the Cu_A and Cu_B centers. Complex IV activity can be regulated by signals including the intramitochondrial ATP/ADP ratio (Ludwig *et al.* 2001). Mitochondrial disorders are associated with mutations in at least eight genes involved in complex IV assembly and function, resulting in Leigh Syndrome, keto-acidotic coma, hepatopathy, hypertrophic cardiomyopathy, tubulopathy, and leukodystrophy. In *C. elegans*, homologs of 11 of the 16 human genes encoding complex IV subunits have been identified (Tsang and Lemire 2003a,b). When *C. elegans* mitochondrial complexes were separated by native polyacrylamide gel electrophoresis, and bands were identified as containing complex IV by an in-gel-activity stain, mass spectrometry of those bands confirmed the presence of at least six proteins whose closest human homologs are known to be part of complex IV (Suthammarak *et al.* 2009). Several of these, including homologs of subunits COXVB/*cco-1*, COXIV, COXVA, and COXVIIC, have been investigated using RNAi, with a combination of qPCR, reduced ATP levels, reduced respiration, and/or reduced complex IV activity validating knockdown (Dillin *et al.* 2002; Lee *et al.* 2003; Suthammarak *et al.* 2009; Maxwell *et al.* 2013).

RNAi against COXIV, COXVB/*cco-1*, and COXVA subunits decreases fecundity and slows development, while RNAi against COXVIIC causes arrest at the second larval stage. Stress resistance was variably altered for RNAi of complex IV subunits: COXVB/*cco*-1, COXIV, or COXVIIC knockdown caused paraquat hypersensitivity and hydrogen peroxide resistance, while only COXIV knockdown caused tolerance for high temperatures (Lee *et al.* 2003). RNAi knockdown of COXVB/*cco-1*, COXIV, COXVA, and COXVIIC all extend lifespan, although this appears to only be true when 5-fluoro-2′-deoxyuridine (FUdR) is used in the medium to prevent reproduction, suggesting that complex IV dysfunction is worsened by the energy demands of oogenesis (Suthammarak *et al.* 2009). Complex IV may also play a role in mitochondrial fission-fusion dynamics, as knockdown of any of three subunits (COXVB/*cco*-1, COXIV, or COXVIIC) causes fused mitochondrial morphology (Lee *et al.* 2003).

Both COXIV and COXVA RNAi causes a decrease in complex I-dependent respiration and complex I activity, as measured by a spectrophotometric assay of rotenone-sensitive NADH-decylubiquinone oxidoreductase rate, while the NADH-ferricyanide reductase (NFR) activity of complex I and the activity of complex II are not altered (Suthammarak *et al.* 2009). NFR measurements follow the first step of NADH oxidation, which is thought to be dependent on a few complex I subunits that reside in the matrix arm, and are less likely to affected by levels of the I:III:IV supercomplex levels. Supercomplex levels are diminished when these complex IV subunits are knocked down. Thus, although the levels of complex I are unchanged, the complex I-dependent activities are reduced presumably by an allosteric effect from decreased complex IV. These data from *C. elegans* suggest that human patients that present with reduced activities in both complex I and IV, a not uncommon finding, may in fact, have a single genetic defect in complex IV.

Complex V: *ATP-2:* Complex V of the MRC is the F_0F_1-ATP synthase. Unlike the other complexes and redox carriers mentioned above, it is not directly involved in electron transport; rather it is responsible for ATP phosphorylation. Defects in at least two proteins in complex V assembly are associated with mitochondrial disorders. Defects in complex V cause severe disease and are often fatal in early childhood, with symptoms including lactic acidosis, hypertrophic cardiomyopathy, and 3-methylglutaconic aciduria. Complex V function may also be indirectly disrupted by mutations in other complexes via the unique ROS sensitivity of certain complex V subunits. In *C. elegans*, homologs for at least 14 of the 16 human genes encoding complex V subunits have been identified (Tsang and Lemire 2003a,b). ATP-2 in *C. elegans* is homologous to the human ATP5B. The loss-of-function mutation of *atp-2*(*ua2*) causes developmental arrest at L3 followed by a long lifespan in homozygous mutant animals with a heterozygous mother. These mutant animals also have neuromuscular defects of decreased pharynx pumping and defecation and impaired locomotion (Tsang *et al.* 2001). Mosaic analysis suggests that it is loss of *atp-2* in muscle that is the major contributor to the developmental arrest observed,

and does so in a noncell-autonomous fashion (Tsang and Lemire 2003a,b). RNAi knockdown of *atp-3*, the *C. elegans* homolog of human ATP5O, predictably decreases ATP levels and, like *atp-2*(*ua2*), lifespan is extended (Rea *et al.* 2007).

Modeling for Mammals

Toxicology

The same advantages that make *C. elegans* useful for developmental and mitochondrial studies make the nematode useful for toxicologic investigations. There have been many reports in the past decade demonstrating the usefulness of *C. elegans* for studying neurotoxicity from exposure to chemicals (for a review, see Avila *et al.* 2012). During the past decade, mitochondrial toxicity in the nematode has received increased attention. In two reports from 2008, the Ebert laboratory studied toxicity of the insecticide, phosphine. Using *C. elegans*, they found that phosphine caused loss of the mitochondrial membrane potential, and that resistance to phosphine resulted from defects in the mitochondrial ETC (Zuryn *et al.* 2008). Further, the authors showed that phosphine and mitochondrial uncouplers functioned in an additive manner in causing nematode lethality (Valmas *et al.* 2008). They suggested that the additivity with uncouplers and phosphine represented a way to address the increasing resistance to the drug being observed in insects.

A report in 2015 from the van der Spek laboratory developed the use of *C. elegans* for the study of mitochondrial toxicity with antiretroviral drugs (de Boer *et al.* 2015). They studied toxicity of antiretroviral drugs using mitochondrial morphology, mtDNA content, quinone reduction, and RNA expression. Work from the Meyer laboratory has also helped establish *C. elegans* as a model for mitochondrial toxicity (Bodhicharla *et al.* 2014; Gonzalez-Hunt *et al.* 2014; Rooney *et al.* 2014). They studied the effects of lowering mtDNA levels with ethidium bromide on toxic effects of aflatoxin B1, arsenite, paraquat, rotenone, and ultraviolet radiation (Gonzalez-Hunt *et al.* 2014). The results were mixed with sensitization to arsenite and ultraviolet radiation, protection from rotenone, and no effects on the toxicity of other compounds. Closely related work measuring the effects of organic and inorganic mercury on *C. elegans* documented that mitochondrial endpoints (mtDNA copy number and ATP levels) were susceptible to mercury (Wyatt *et al.* 2017). The precise changes depended on coexposure to other secondary toxins (UVC and hydrogen peroxide), and whether the mercury was presented in organic or inorganic form. Caito and Aschner also showed that organic mercury (MeHg) caused mitochondrial dysfunction, decreased mitochondrial mass and mtDNA copy number (Caito *et al.* 2012, 2013). Interestingly, they also showed that the effects were NAD^+ dependent and could be alleviated by pretreatment of the nematodes with exogenous NAD^+. In closely related work, Luz and colleagues showed that arsenite caused mitochondrial dysfunction, and that arsenite toxicity was increased in the presence of some, but not all, genetic defects in mitochondrial function (Luz *et al.* 2016a,c; Luz and Meyer 2016). They concluded that the interaction between arsenite toxicity and mitochondrial function was a novel class of interactions first identified in the nematode that needed study in humans. Lagido *et al.*, in the Glover and Pettitt laboratories, developed a luciferase expressing strain that allowed *in vivo* assessment of relative ATP levels in *C. elegans* (Lagido *et al.* 2008). They later described the use of this strain for monitoring toxicant effects on mitochondrial function (McLaggan *et al.* 2012; Lagido *et al.* 2015). Members from the Meyer laboratory extended the use of this strain to allow rapid evaluation of the effects of toxicants on mitochondrial function, particularly the electron transport chain, glycolysis and fatty acid oxidation (Luz *et al.* 2016b).

Studying whole nematodes, Fitsanakis and colleagues showed that Mn^{++} exposure led to defective mitochondrial complex I function, with an increase in hydrogen peroxide concentrations (Bailey *et al.* 2016; Todt *et al.* 2016). In response to the increase in hydrogen peroxide, the expression of a glutathione S-transferase reporter (*Pgst-4*::*gfp*) was also increased in the exposed animals. The importance of oxidative stress on Mn^{++} toxicity was further demonstrated in *C. elegans* by Ijomone *et al.* (2016). The latter authors studied the effects of *trt-1* [telomerase reverse transcriptase (TERT)] on Mn^{++} toxicity. TERT has been shown to translocate to the mitochondrial matrix and play a role in resistance to oxidative stress (Singhapol *et al.* 2013). Surprisingly, loss of the *trt-1* gene led to *decreased* sensitivity to Mn^{++} exposure. The reason for this unexpected finding was not clear, although the authors suggested that *trt-1* animals had a resistance to apoptosis, which may have protected dopaminergic neurons from neurodegeneration. While the precise mechanisms of Mn^{++} toxicity remain to be elucidated, the above studies show the breadth of value for *C. elegans* in those studies. The strengths of the nematode are well known to the nematode community, but these attributes were less well known to the toxicology community. The appreciation of these attributes has led to a striking increase in understanding the roles of Mn^{++} in oxidative stress and mitochondrial dysfunction.

Other isolated and unrelated studies have also made use of the nematode for toxicology. Behl *et al.* (2016) used *C. elegans* to study toxicologic effects of organophosphate exposure. Using a mitochondrial membrane permeabilization assay, they found that several organophosphate compounds used in flame retardants caused defects in mitochondrial function in the nematode. Kamal *et al.* (2016) showed an interaction between *hif-1* and the mitochondrial dysfunction caused by ethidium bromide in the nematode. While these studies are of divergent compounds, they again show the usefulness of a strong genetic model such as the nematode for performing initial studies of the interactions between genetics and environmental toxicology.

Anoxia

C. elegans has been used to study resistance to profound hypoxia by several authors. Kim *et al.* (2011) showed that anoxia induced *Drp1*-dependent mitochondrial fission in

mouse fibroblasts, and that loss of *drp-1* in nematodes shortened lifespan. Others followed that lead, and reported that the nematode entered a "reversible suspended animation state of locomotory arrest" after exposure to <0.1% O_2 (Park *et al.* 2012; Ghose *et al.* 2013). In agreement with the mouse data, mitochondria underwent DRP-1 dependent fission when hypoxic, and, when reoxygenated, the mitochondria recovered and underwent SKN-1 dependent fusion. The recovery was regulated by EGL-9 and HIF-1, and suggested that a conserved response to anoxic stress was in play. Kaufman and Crowder (2015) showed that resistance to anoxia involves mitochondrial proteostasis, and that manipulation of the mitochondrial unfolded protein response could be used to protect the nematode from anoxic lethality. Pena *et al.* (2016) confirmed and extended this finding, showing that activation of the UPR^{mt} either by drugs (ethidium bromide) or genetically [*spg-7 and atfs-1*(*gf*)] was sufficient to reduce lethality in response to anoxia in a reperfusion protocol. In what seemed to be unrelated studies, overexpression of nicotinamide mononucleotide adenylyltransferase type 1 in mice was previously shown to reduce brain injury resulting from hypoxia (Verghese *et al.* 2011). Crowder and colleagues followed these data by showing that heterologous expression of the mouse nicotinamide mononucleotide adenylyltransferase type 1 (*Nmnat1*) protected *C. elegans* from anoxia (Mao *et al.* 2016). This approach was intended to serve as an approach for a suppressor screen to remove the protection afforded by *Nmnat1* in the nematode. Their screen showed that loss of UPR^{mt} factors removed the protection afforded by overexpression of *Nmnat1*, and that *Nmnat1* activated the UPR^{mt}. The authors favored improved mitochondrial proteostasis as the mechanism underlying the effect of *Nmnat1* on hypoxic survival. Whatever the mechanism, it is clear from these data that the UPR^{mt} is crucial in protection from anoxia, functions in the same pathway as *Nmnat1*, and that similar mechanisms extend from the nematode to mammals.

Mitochondrial Morphology

Shapes in different cell types, fluorescence, EM, motility

The general organization of *C. elegans* mitochondria is very similar to that in other multicellular animals, with some adaptations for different cell types. Body wall muscles have relatively long mitochondria, usually aligned with the muscle filaments. Because these cells are flat, the mitochondria are easily brought into focus in a single plane by light microscopy. Larval body wall muscles have relatively long tubular mitochondria, while mitochondria in muscles of young adults are intermediate, and mitochondria of old animals are more fragmented. Hypodermal cells are also flat with relatively long mitochondria, but these mitochondria are arranged in a more erratic pattern with many branches. Intestinal cells occupy a larger space with depth, and their mitochondria crisscross this space. As a result, their mitochondria are difficult to capture in an image without 3D reconstruction. Neuronal mitochondria are smaller with fewer branches, most likely due to spatial constraints. Gonad mitochondria are relatively small, but highly abundant, visible as punctae surrounding the nuclei along the rim of the gonad. Examples are shown in Figure 5.

Distinct mitochondrial distributions and shapes in different cell types suggests that they are adapted to specific cellular architectures or functions. Relatively short mitochondria in neurons may facilitate their transport along axons. Longer mitochondria in other cell types could be the default state for mitochondrial lengths in metabolically active tissues, while the distinct spatial arrangements in different cell types could be dictated by their cellular architectures. Large variations in mitochondrial lengths have been observed in mammalian cells, where lengths are influenced by metabolic demand (Liesa and Shirihai 2013), but these types of metabolic adaptations have not yet been studied in *C. elegans*. Striking morphological changes have, nevertheless, been observed during the aging process in *C. elegans* (Regmi *et al.* 2014; Palikaras *et al.* 2015). Mitochondria are generally more elongated in younger nematodes, and tend to become fragmented and swollen in older animals. Mitochondria traveling along neuronal projections are unusual; they are short in larvae, somewhat longer in young adults, and short again in aging animals (Morsci *et al.* 2016). These age-dependent differences in length likely reflect changes in the rates of mitochondrial biogenesis during the life of a nematode. *C. elegans* is an ideal model for uncovering trends like this.

Cristae morphologies

Mitochondrial cristae greatly increase the surface area of the inner membrane, thus providing large areas for membrane-dependent processes, such as oxidative phosphorylation. High degrees of membrane curvature are needed to accommodate this expansion of membrane. Curvature is induced by dimers of the ATP synthase complex. The two ATP synthase complexes in a dimer are joined at an angle, causing the membrane to bend (Hahn *et al.* 2016). These dimers can also concatamerize into chains that bend membrane over longer distances.

Mitochondrial cristae are often depicted as baffles or folds of the mitochondrial inner membrane with no other purpose than to enlarge the surface area available for oxidative phosphorylation. This picture was upended with the advent of EM tomography, a technique in which relatively thick sections containing whole mitochondria are imaged at different angles, followed by mathematical reconstruction of a 3D model. These models show that cristae in most organisms are tubes and sacks with thin tubular connections to mitochondrial inner membrane at the surface (the rim) (Frey *et al.* 2002). Tubular connections with the rim are called cristae junctions. These junctions usually have a diameter of ~30 nm. In recent years, the proteins that generate these cristae junctions have been identified (Figure 6A). They include large inner membrane proteins called Mitofilins (IMMT-1 and IMMT-2 in *C. elegans*), intermembrane space proteins (M176.3 in *C. elegans*), and a lipid binding protein (MOMA-1 in *C. elegans*)

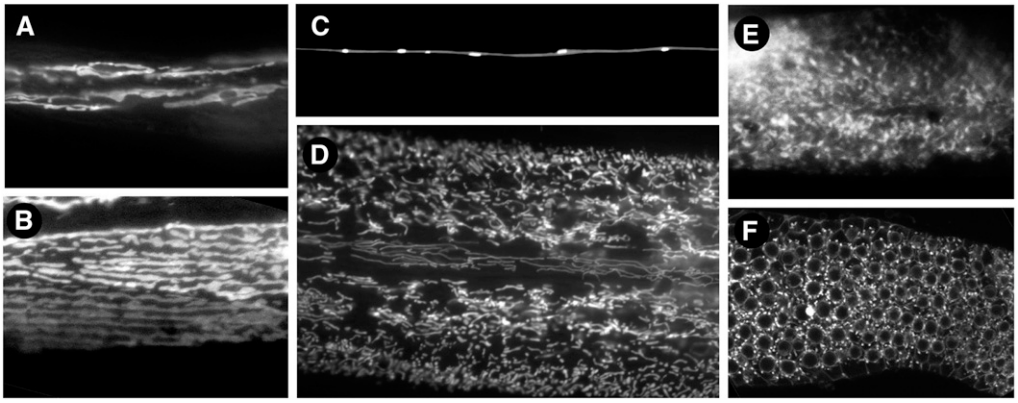

Figure 5 Mitochondrial morphologies in different *C. elegans* cell types. (A) Mitochondria in larval body wall muscles detected with mitochondrial GFP under control of the myo-3 promoter. (B) The same, but in a young adult animal. (C) Mitochondria in a neuronal process detected with mitochondrial GFP under control of the mec-7 promoter. (D) Mitochondria in hypodermal cells detected with mitochondrial GFP under control of the col-12 promoter. (E) Mitochondria in intestinal cells detected with mitochondrial GFP under control of the ges-1 promoter. (F) Mitochondria in dissected gonad detected by staining with Mitotracker. (A–E) are conventional epifluorescence images. (F) is a confocal image.

(Head *et al.* 2011). Similar proteins were discovered in yeast and mammals (reviewed in Kozjak-Pavlovic 2017) and a common nomenclature was agreed upon. The cristae junction protein complex was renamed the MICOS (Mitochondrial Contact Site) complex (Pfanner *et al.* 2014). Mitofilins are now referred to as Mic60, M176.3 is Mic19, and MOMA-1 is Mic26. Deletions in yeast mitofilin Fcj1/Mic60 sever cristae from the rim membrane, while overexpression widens the cristae junctions (Rabl *et al.* 2009). These results suggest that the MICOS complex helps keep the cristae junctions open and prevents them from collapsing. Similar effects were observed with electron microscopy (EM) tomography of the *C. elegans* *immt-1* and *immt-2* mutants (Mun *et al.* 2010). The overall mitochondrial morphologies of *C. elegans* MICOS mutants are also abnormal (Head *et al.* 2011). These mitochondria have alternating thinner and thicker diameters, as if they undergo localized swelling. It therefore seems likely that loss of cristae junctions affects the structural integrity of mitochondria.

The outer and inner membranes of mitochondria are evolutionarily related to the outer and inner membranes of the ancestral gram negative bacteria. The outer membranes of bacteria and mitochondria both have numerous pores formed by β-barrel transmembrane proteins called Porins in bacteria, or VDAC in mitochondria (Zeth 2010). These proteins form relatively nonselective channels that allow most small molecules and ions to diffuse freely across their outer membranes. In contrast, their inner membranes are impermeable to ions. Instead, selective carrier proteins regulate transport across those membranes. *C. elegans* mitochondrial outer membranes have only one VDAC, encoded by the *vdac-1* gene, unlike mammals, which have three VDAC isoforms, but their membrane compositions are otherwise very similar (Messina *et al.* 2012). *C. elegans* and other higher eukaryotes have similar complements of mitochondrial inner membrane transporter proteins.

Fission and fusion proteins

The classic image of mitochondria as sausage-shaped organelles floating in a sea of cytoplasm changed with the advent of vital dyes and fluorescent proteins for live cell imaging. *C. elegans* mitochondria move along microtubules and they fuse and divide, depending on the age and metabolic state of the cell, similar to the dynamics of mitochondria in other organisms (Mishra and Chan 2016). Fission helps distribute mitochondria during cell division. Fusion is beneficial by allowing minor defects in proteins or DNA of one mitochondrion to be complemented by functional proteins or DNA from another mitochondrion. The roles of these processes in mitochondrial turnover will be discussed in the next section. Here, we will focus on the mechanics of fission and fusion.

C. elegans mitochondria, like those in other higher eukaryotes, have lost all remnants of the bacterial fission apparatus including FtsZ. Instead, mitochondrial fission and fusion are mediated by members of the dynamin protein family (Figure 6B). These proteins form large homomeric assemblies that drive fission or fusion events through GTP hydrolysis. Mitochondrial outer membrane fission is mediated by the dynamin-related protein DRP-1 (Labrousse *et al.* 1999), which is, in many respects, very similar to classic dynamin (DYN-1 in *C. elegans*) (Clark *et al.* 1997), which severs endocytic vesicles from the plasma membrane. DRP-1 and DYN-1 proteins are both primarily cytosolic, with only a small fraction assembling on their respective target membranes to mediate fission. Once on their target membranes, these proteins form multimeric spirals that wrap around constricted membrane tubules. The spirals then further constrict until they sever the membrane, using energy from GTP hydrolysis to drive this process. Whether DRP-1 alone is enough to sever both mitochondrial outer and inner membranes, or if other proteins are required, is unclear. A hint at such a requirement comes from *C. elegans* muscle cells where mutations in DRP-1 prevent severing of the mitochondrial outer membrane, but the inner membrane is often still severed to generate a series of separate matrix compartments connected by outer membrane tubules (Labrousse *et al.* 1999), suggestive of peas in a pod, but alternative explanations, such as mechanical stress from muscle contractions, have not yet been ruled out. These studies

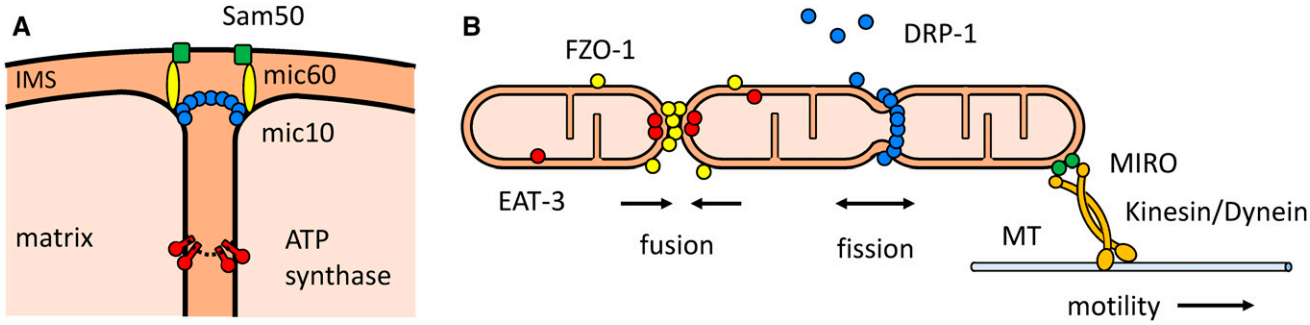

Figure 6 Control of mitochondrial inner and outer membrane morphologies. (A) Cristae shapes are determined by the MICOS protein complex, which provide negative curvature at cristae junctions and dimers of ATP-synthase, which have pair at an angle providing positive curvature for tube shaped cristae. (B) Mitochondrial fusion is mediated by FZO-1 on the outer membranes of opposing mitochondria, and by the inner membrane protein EAT-3. Mitochondrial fission is mediated by DRP-1, which is largely cytosolic but can be recruited to mitochondria, where it forms a large spiral that constricts mitochondrial membranes for fission. Transport along microtubules is mediated by kinesins and dynein, which are coupled to mitochondria through interactions with MIRO-1, -2 or -3.

with *C. elegans* were the first to show that dynamin family members mediate mitochondrial fission in multicellular organisms, and they showed for the first time that this function is specific for the mitochondrial outer membrane.

DRP-1 is recruited from the cytosol to mitochondria through binding interactions with mitochondrial outer membrane proteins. In yeast, this recruitment is mediated by a cytosolic Trp-Asp (WD) protein and the mitochondrial outer membrane protein Fis1 (Mozdy *et al.* 2000). *C. elegans* and other higher eukaryotes also have Fis1 homologs (FIS-1 and FIS-2 in *C. elegans*), but their Fis1 homologs are not required for fission (Shen *et al.* 2014), nor do they have WD protein adaptors for binding to Fis1. Instead, multicellular animals, like *C. elegans*, use other proteins to recruit DRP-1 from the cytosol to mitochondria. The main receptor is Mff, which is anchored in the mitochondrial outer membrane. *C. elegans* has two genes encoding Mff homologs (MFF-1 and MFF-2) while vertebrates have only one. Vertebrates do have additional recruitment factors, named MID49 and MID51, which are absent from *C. elegans*. There must, nevertheless, be other DRP-1 recruitment factors, because the *C. elegans mff-1 mff-2* double mutant phenotype is considerably weaker than the *drp-1* single mutant phenotype (Shen *et al.* 2014). The apoptotic proteins EGL-1 and CED-9 (reviewed in Conradt *et al.* 2016) may also affect mitochondrial fission and fusion in *C. elegans* (Jagasia *et al.* 2005; Rolland and Conradt 2006). It was even proposed that the EGL-1-CED-9 complex promotes mitochondrial fission by acting as an alternative receptor for DRP-1 (Lu *et al.* 2011), while CED-9 overexpression promotes mitochondrial fusion through the actions of FZO-1 and EAT-3 proteins, suggesting direct connections to apoptotic pathways (Rolland *et al.* 2009).

One other possible recruitment factor is FUNDC1, which has been shown to recruit Drp1 to mitochondria in mammalian cells during hypoxia (Wu *et al.* 2016), but the functions of the *C. elegans* FUNDC1 homolog T06D8.7 have not yet been tested. In addition to these DRP-1 receptors, several ER-shaping proteins also affect mitochondrial fission, presumably by modifying contacts between mitochondria and ER (Ackema *et al.* 2014, 2016), which would be analogous to the effects of these contacts on mitochondrial fission in yeast and in mammals (Rowland and Voeltz 2012).

Dynamin family members are also important for fusion between mitochondria. Outer membrane fusion is mediated by FZO-1 in *C. elegans* (similar to Fzo1 in yeast and the Mitofusins Mfn1 and Mfn2 in mammals) (Ohba *et al.* 2013), while inner membrane fusion is mediated by EAT-3 (similar to Mgm1 in yeast and Opa1 in mammals) (Kanazawa *et al.* 2008). FZO-1 is anchored in the membrane by two transmembrane segments in the C-terminal half of the protein, while EAT-3 has a putative lipid-binding domain in the same place, and it has an N-terminal transmembrane segment. These proteins therefore do not cycle on and off of their target membranes. Instead, they remain anchored in the membrane after the fusion events. The EAT-3 protein, which mediates inner membrane fusion, has an N-terminal mitochondrial targeting sequence that is removed by proteolytic cleavage during the import process. Like yeast Mgm1 and mammalian Opa1 (MacVicar and Langer 2016), a large fraction of EAT-3 proteins is cleaved once more after import, removing the transmembrane segment between the first and second cleavage sites. The presence or absence of this second cleavage gives rise to short and long forms of this protein. Both short and long forms seem to be necessary for fusion, although their exact functions during the fusion process are still debated.

In yeast, the second cleavage is mediated by the rhomboid protease Pcp1, which is necessary for fusion (Herlan *et al.* 2004). In mammals, some splice variants of Opa1 are constitutively cleaved by the AAA protease Yme1L, while the other variants are cleaved by the zinc metalloprotease Oma1, but only when mitochondria lose their membrane potential (MacVicar and Langer 2016). *C. elegans* does not have an Oma1 homolog, and cleavage is not induced by loss of membrane potential. It seems likely that instead a fraction of EAT-3 proteins is cleaved by a rhomboid protease similar to the cleavage of yeast Mgm1 by the mitochondrial rhomboid protease

Pcp1. In addition to the dynamin-related fusion proteins, there may also be a role for lipid modification during fusion. Like in mammals, where a mitochondrial phospholipase D acts in concert with the Mitofusins to promote fusion (Huang *et al.* 2011), *C. elegans* has a lipid modifying enzyme (glycerol-3-phosphate acyltransferase) on the mitochondrial outer membrane, where it promotes fusion in concert with FZO-1 (Ohba *et al.* 2013). The finding that *C. elegans* and mammals use different lipid modifying enzymes to promote fusion hints at further complexities in this process.

The phenotypes of *C. elegans* fission and fusion mutants are similar to the mutant phenotypes observed in yeast and mammalian cells (Pernas and Scorrano 2016). Fission defects lead to more interconnected mitochondria, while fusion defects give rise to fragmented mitochondria. EM images of *C. elegans eat-3* mutants also show that mitochondrial outer membranes can still fuse when inner membrane fusion is blocked, giving rise to multiple separate matrix compartments contained within a single outer membrane enclosure (Kanazawa *et al.* 2008). The brood sizes, growth rates, and lifespan of *C. elegans drp-1* and *fzo-1* mutants are not very different from wild-type animals, but *eat-3* mutants develop slowly, stay small, have low brood sizes, and live longer than wild-type or the other fission and fusion mutants (Kanazawa *et al.* 2008). Moreover, eat-3 mutants have far fewer cristae than wild-type animals, and they are more sensitive to ROS-inducing agents like paraquat, consistent with disruptions in inner membrane function (Kanazawa *et al.* 2008).

C. elegans eat-3 and *fzo-1* mutants are both more sensitive to arsenite (Luz *et al.* 2017), and both mutants acidify the cytoplasms of their intestinal cell, similar to the acidification caused by mutations in ETC proteins (Johnson and Nehrke 2010). This could be a form of lactic acidosis caused by increased reliance on glycolysis. Some differences between *eat-3* and *fzo-1* mutants were noted as well. The acidification of cytoplasm is much stronger in the cytoplasms of neurons and muscles in *eat-3* mutants than it is in *fzo-1* mutants (Johnson and Nehrke 2010). The *eat-3* mutants also inhibit axonal regeneration while *fzo-1* mutants do not, suggesting that EAT-3 has more profound effects than FZO-1 on mitochondrial function (Knowlton *et al.* 2017). The effects of EAT-3 on cristae are similar to those observed with mammalian Opa1, consistent with a general role in cristae maintenance alongside the well-defined role in mitochondrial fusion (Pernas and Scorrano 2016). *C. elegans* is therefore an excellent model for studying the pathological effects of *eat-3* (Opa1) mutations on neuronal function.

Motility and anchoring

As in other multicellular animals, *C. elegans* mitochondria move along microtubules so they can be distributed throughout the cell (Figure 6B). Anterograde transport of mitochondria is mediated by kinesins, while retrograde transport is mediated by dynein. Of the 21 kinesins in *C. elegans* (Siddiqui 2002), two have been shown to affect mitochondria. RNAi for the kinesin KLP-6 alters *C. elegans* mitochondrial morphology in muscle cells (Tanaka *et al.* 2011), while UNC-116 is needed for anterograde movement of mitochondria in *C. elegans* neurons (Rawson *et al.* 2014). The mammalian homolog of UNC-116 is KIF5, which also mediates mitochondrial transport in mammals. Transport of mitochondria along axons by UNC-116 is necessary for neuronal regeneration after injury (Han *et al.* 2016), but it is also necessary for maintaining axonal integrity (Rawson *et al.* 2014).

Kinesins and dynein interact with mitochondria through Miro and Milton proteins, which are known adaptor proteins in other organisms. *C. elegans* has three Miro genes (*miro-1*, *-2*, and *-3*), but *miro-2* and *miro-3* have deletions in conserved sequences, while *C. briggsae* only has *miro-1*, suggesting that *miro-2* and *miro-3* may have divergent functions. A deletion in *C. elegans miro-1* increases lifespan and reduces the numbers of mitochondria in hypodermal and muscle cells, but the effects on neuronal function are modest (Shen *et al.* 2016). It is conceivable that MIRO-2 and -3 affect motility, while MIRO-1 is responsible for one of the other known functions of Miro proteins, such as maintaining contacts between ER and mitochondria (Lee *et al.* 2016).

Mitochondria can also be anchored to other parts of the cytoskeleton. Mutations in the *anc-1* gene were shown to affect mitochondrial and nuclear positioning in *C. elegans* hypodermal cells (Hedgecock and Thomson 1982). The ANC-1 protein was later found to have an actin binding domain, suggesting that mitochondria are anchored by interactions between ANC-1 and the actin cytoskeleton (Starr and Han 2002). The discovery of mitochondrial and nuclear positioning by the *C. elegans* ANC-1 protein therefore helped uncover new mechanisms for organelle positioning through connections with the actin cytoskeleton (Bone and Starr 2016).

Mitochondrial Biogenesis

As in other eukaryotes, most mitochondrial proteins in *C. elegans* are encoded by the nucleus. Elaborate mechanisms exist for targeting those proteins to their proper destinations in mitochondria, and for regulating the expression of nuclear DNA and mtDNA encoded proteins in accordance with the energetic needs of the cell. In mammals, mitochondrial biogenesis is primarily regulated by the transcription factor PGC-1α, which itself is under control of AMP-kinase and Sirt1 (Friedman and Nunnari 2014). *C. elegans* does not have an obvious PGC-1α homolog. Instead, biogenesis is, at least in part, regulated by the SKN-1 transcription factor (Nrf2 in mammals) (Palikaras *et al.* 2015), along with the *C. elegans* Sirt1 homolog SIR-2.1, which controls gene expression through the FOXO transcription factor DAF-16 (Berdichevsky *et al.* 2006), and AAK-2, which acts through the transcription factors CEP-1/p53 and CEH-23 (Chang *et al.* 2017). These pathways affect longevity and stress responses in different ways and may co-operatively control mitochondrial biogenesis during normal growth and development in *C. elegans*. Expression of the nuclear encoded

mitochondrial genes is complemented by the transcription and translation of the handful of proteins encoded by genes in mitochondrial DNA. *C. elegans* mtDNA is compact (14 kb), encoding rRNAs, tRNAs, and a small number of ETC and ATP synthase proteins, with little or no space between coding sequences, similar to the mtDNAs of other multicellular animals. The organization of these genes, and other aspects of the *C. elegans* genome, have been reviewed (Lemire 2005). Here, we will briefly discuss some new advances in this area, and the general mechanisms of protein targeting to mitochondria.

Mitochondrial DNA, transcription, and translation

An analysis of mtDNA copy numbers and replication showed a surprising tolerance for different copy numbers (Bratic *et al.* 2009). More importantly, almost all mtDNA replication appears to occur in the gonad. Loss of the mtDNA polymerase POLG-1 during development has only a limited effect on the adult animal. DNA replication in the gonad stocks embryos with enough mtDNA for the next generation. *C. elegans* mtDNA has a short noncoding stretch, which was previously thought to act like a displacement loop similar to the D-loops that serve as origins of replication in mammals. A new study with 2D gel analysis of replication intermediates in *C. elegans* gonad mitochondria shows that there is no displacement loop (Lewis *et al.* 2015). Instead, DNA replication utilizes a rolling circle intermediate, like the replication of bacteriophage DNA and the DNAs of fungal and plant mitochondria. Although the replication machinery of *C. elegans* has not been studied in detail, the replication proteins in other organisms are similar to bacteriophage replication proteins (the DNA-helicase and DNA-polymerases) as well, suggesting that the machinery for mtDNA replication was co-opted from a bacteriophage. This bacteriophage may have been smuggled into the host cell during the original endosymbiosis event.

Along with DNA replication proteins, *C. elegans* has a TFAM-like DNA compaction protein HMG-5, which has been shown to affect mtDNA replication and transcription (Sumitani *et al.* 2011). There are few other studies on mtDNA transcription in *C. elegans*. However, some things can be inferred from the organization of genes. Unlike mammalian mtDNA, the protein-coding, tRNA and rRNA genes are all on one strand, suggesting that transcription is unidirectional, and the compactness suggests that there is only one start site within a unique noncoding segment (Lemire 2005). The genes are most likely transcribed as a polycistronic unit, similar to mammalian mitochondria, and then process by cleavages releasing the tRNAs that punctuate the genes. An RNA polymerase (RPOM-1) that is similar to a bacteriophage polymerase mediates mitochondrial transcription. As with the mitochondrial helicase and polymerase mentioned earlier, the gene coding for this polymerase may have snuck in with the original endosymbiont. The helicase and polymerase enzymes were then co-opted for their current functions in mitochondrial DNA replication and transcription.

Spontaneous mutations in *C. elegans* mtDNA are infrequent (10^{-7}/site/generation), but the large numbers of copies that are transmitted through oocytes suggest that acquisition of a new mutation is a common event (Konrad *et al.* 2017). These mutations are generally lost through natural selection, but they are also weeded out by mitophagy. Elimination of mtDNA mutations by mitophagy was first shown to work in mammalian cells (Suen *et al.* 2010), but has since then been replicated in a whole animal using *C. elegans* as a model system (Valenci *et al.* 2015). Once in a while, seemingly deleterious mutations are, nevertheless, maintained in a heteroplasmic state. These types of mutations may persist because they confer an unexpected advantage. An interesting example is provided by the *ctb-1*(*qm189*) allele, which has a mutation in the gene coding for cytochrome *b* of the ETC complex III (Suthammarak *et al.* 2010). As discussed in the section *Complex III*, this *ctb-1* allele reverses the destabilizing effects of mutations in *isp-1* (another complex III protein) on complex I. In this case, there is a clear selective advantage. There are, however, also examples of heteroplasmy without these kinds of advantages. These heteroplasmic states persist through a newly discovered mechanism relying on the mitochondrial unfolded protein response (UPR^{mt}), as discussed in more detail in *Mitochondrial Quality Control*.

Protein import machineries

Little research has been done on mitochondrial protein import in *C. elegans*, perhaps because the import machineries are generally similar to those in yeast and mammalian cells, for which a wealth of literature already exists. We will summarize the basic import pathways with some references to *C. elegans* studies, while referring to other reviews for an in depth discussion of the proteins that are involved in yeast and mammalian pathways (Harbauer *et al.* 2014; Wiedemann and Pfanner 2017). The vast majority of mitochondrial proteins (~1500 different ones, as determined with mammalian cells and yeast) are encoded by nuclear DNA, synthesized in the cytosol and imported post-translationally. The import machineries are tasked with accurately delivering proteins to the different submitochondrial destinations (Figure 7). Mitochondrial targeting sequences mark proteins for these different destinations. The most common targeting sequences consist of 20–30 amino acids at the amino terminus, sending proteins to the mitochondrial matrix. These sequences form an amphipathic helix, which is recognized by a larger protein import apparatus called the TOM complex, which includes a transmembrane channel protein TOM40.

The N-terminal targeting sequence of a protein destined for mitochondrial matrix first emerges from the TOM complex on the side of the mitochondrial intermembrane space. Once there, it is recognized by proteins of the TIM23 complex, which translocates proteins from the intermembrane space into the matrix. Proteins entering the matrix are then folded into their native conformation with help from mitochondrial HSP70 and an HSP10/HSP60 protein-folding

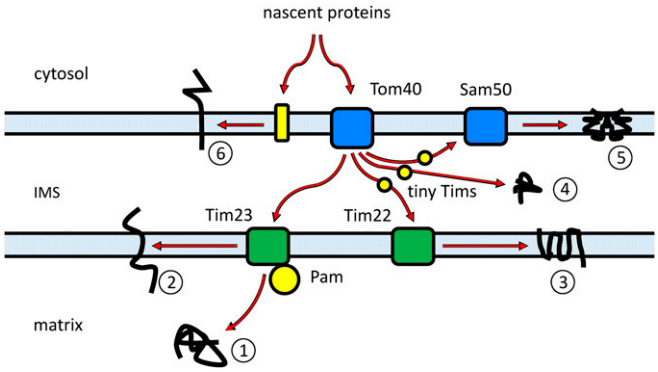

Figure 7 Overview of mitochondrial protein import pathways. Most proteins enter the intermembrane space through the Tom40 complex, followed by further targeting to their final destination. Proteins with an N-terminal targeting sequence (1) are sent to the mitochondrial matrix through the Tim23 complex, or if they have a single transmembrane segment they become anchored in the inner membrane (2). Multispanning proteins are also first translocated into the intermembrane space, but, once inside, they are sequestered by small TIM proteins and then inserted into the inner membrane via the Tim22 complex (3). Some remain in the intermembrane space, where they can be trapped by protein modifications, such as disulfide bonds or covalent heme attachment (4). Other proteins are returned to the outer membrane for folding into β-barrel proteins through the SAM pathway (5). Mitochondrial outer membrane proteins with α−helical transmembrane segments are inserted through other pathways outside of the Tom40 complex (6).

complex. Knockdown of the *C. elegans* mitochondrial Hsp70 protein (HSP-6) disrupts mitochondrial morphology, reduces ATP levels, shortens lifespan, and slows movement, consistent with generally impaired mitochondrial function (Kimura *et al.* 2007).

Proteins targeted to other destinations use variations on this theme (Figure 7). Proteins with multiple transmembrane segments, such as transport proteins in the mitochondrial inner membrane have internal hydrophobic segments that function both as targeting sequences and as transmembrane segments in the mature proteins. These proteins enter mitochondria through the TOM40 translocase, but are then sequestered by small chaperones, the tiny TIMs, in the intermembrane space. These tiny TIMs transfer the newly translocated protein to a separate inner membrane import channel called TIM22. A number of these import proteins were studied in *C. elegans* with RNAi (Curran *et al.* 2004). RNAi of *tomm-7* and *ddp-1* (a Tom40 complex subunit, and one of the tiny Tim proteins) resulted in mitochondria with an interconnected morphology *in vivo*, presumably due to defects in the assembly of outer membrane fission/fusion components. Knockdown of the tiny TIM proteins TIN-9.1 and TIN-10 impairs import of the ADP/ATP carrier into isolated *C. elegans* mitochondria (Curran *et al.* 2004). RNAi of these small Tim proteins also resulted in a small body size, reduced number of progeny produced, and partial embryonic lethality. Surprisingly, however, these treatments did not affect lifespan, unlike results obtained with HSP-6, suggesting that there are phenotypic differences between proteins that affect import alone and HSP-6, which is also important for proteostasis (discussed in the previous paragraph).

Mitochondrial Quality Control

A small but significant fraction of electrons passing through the mitochondrial electron transport chain are dislocated, producing ROS, which can then damage lipids and proteins inside mitochondria. As a first line of defense, ROS can be converted to peroxide (H_2O_2) by the enzyme SOD. *C. elegans* SOD-2 and -3 both have mitochondrial targeting sequences, although only SOD-2 is necessary to suppress the ROS-sensitivity of *eat-3* mutants, suggesting that SOD-2 is the dominant isoform (Kanazawa *et al.* 2008). Possible damaging effects of H_2O_2 can be mitigated by mitochondrial peroxiredoxins and the peroxisome enzyme catalase, which converts H_2O_2 into H_2O and O_2. ROS, nevertheless, often does cause some damage, and this damage is exacerbated when ROS production increases in mitochondria subjected to stress, for example from toxic chemicals, or in mitochondria with mutations in the ETC. Surprisingly, mutations in SOD-2 extend lifespan (Schaar *et al.* 2015), similar to other mitochondrial mutants, such as *clk-1* (Jonassen *et al.* 2002; Liu *et al.* 2017), suggesting that some damage can be tolerated and a general slowing of mitochondrial function promotes longevity (Van Raamsdonk and Hekimi 2009).

Proteases

Mitochondria cope with damage in various ways. Primary responses and some of the genes involved are reviewed in Kirstein-Miles and Morimoto (2010). Mitochondrial Hsp70 heat shock proteins prevent denatured proteins from forming protein aggregates. A mitochondrial folding complex, consisting of Hsp10 and Hsp60 subunits ,also helps refold proteins. Proteins that are damaged beyond repair can be degraded by mitochondrial proteases. The mitochondrial matrix has a proteasome-like complex (LON-complex), and another ATP-driven protease complex (ClpP), which degrade soluble proteins. RNAi for one of the ClpP subunits induces a mitochondrial unfolded protein response UPR^{mt}), which will be discussed in more detail in a later section (Haynes *et al.* 2007). Proteases in the mitochondrial inner membrane, such as the AAA-Proteases YMEL-1 (YME1L in mammals) facing the intermembrane space, and SPG-7 (AFG3L2 in mammals) or PPGN-1 (Paraplegin in mammals) facing the matrix degrade membrane proteins. Fusion between mitochondria also helps maintain mitochondrial function by promoting the exchange of functional components between partially damaged mitochondria. This type of complementation can offset low levels of damage. Increased fusion is also used to preserve mitochondrial function when cells are subjected to low levels of stress caused by toxic chemicals or when they are starved of nutrients. Starved cells cannibalize many internal components through bulk

digestion of cytoplasmic proteins in a process called macro-autophagy. However, the starved cells protect their mitochondria from macro-autophagy by converting them into elongated tubules that are resistant to autophagy by inhibiting fission and promoting fusion.

Mitophagy

Mitochondria that lose their membrane potential can no longer fuse with other mitochondria, thus preventing the poisoning of those other mitochondria with their damaged components. In essence, those damaged mitochondria are quarantined for a short time as a form of quality control (Youle and van der Bliek 2012). Some mitochondria recover their membrane potential through new protein synthesis, but the damage in others can be prohibitive. Failure to recover targets defective mitochondria for degradation through a specialized form of autophagy, called mitophagy. Mitochondrial fission contributes to this form of quality control, because fission is often asymmetric, giving rise to two daughter mitochondria with different membrane potentials (Twig *et al.* 2008). Beneficial effects of mitophagy have been demonstrated in *C. elegans* by showing that it helps to remove damaged mitochondrial DNA from nematodes (Bess *et al.* 2012). Mitophagy in concert with mitochondrial fission and fusion also helps to reduce the levels of heteroplasmy (Valenci *et al.* 2015). Recently, a chemical inducer of mitophagy was discovered using *C. elegans* as a model organism (Ryu *et al.* 2016). This inducer, Urolithin A, is a small molecule derived from pomegranates. Urolithin A increases the rates of mitophagy, prolongs the lifespan of *C. elegans* and improves muscle function in rodents (Ryu *et al.* 2016).

Key proteins involved in mitophagy were first discovered through mutations in patients with hereditary forms of Parkinson's disease. Cellular pathways connecting some of these mutations with mitochondria were first discovered with genetic analysis of *Drosophila* mutants and further deciphered with cultured mammalian cells (Pickrell and Youle 2015). Loss of mitochondrial membrane potential in damaged mitochondria is signaled by the protein kinase PINK-1. This protein has a mitochondrial targeting sequence and is normally degraded, but import stops when the mitochondrial membrane potential drops below a certain level. PINK-1 then accumulates on the outer membrane, where it recruits the E3-ligase Parkin from the cytosol, which, in turn, triggers mitophagy by ubiquitinating and degrading the mitochondrial outer membrane proteins Miro (Hsieh *et al.* 2016) (Figure 8A). Lack of energy is also sensed by AMP-kinase, which activates ULK1 (*C. elegans* UNC-51) to activate parts of the autophagy pathway (Egan *et al.* 2011).

Advances made with *C. elegans* include studies of genetic interactions between PINK-1 and the Lrrk2 homolog LRK-1—two of the main proteins implicated in Parkinson's disease. These proteins have antagonistic effects on *C. elegans* stress response and neurite outgrowth (Samann *et al.* 2009). PINK-1 directly affects mitochondrial stress responses, and LRK-1 affects the ER stress response. This antagonism is consistent with the genetics of Parkinson's disease, because the disease-causing LRRK2 mutations are gain-of-function, while Pink1 mutations are loss-of-function. It was, nevertheless, shown that LRRK2 mutations also affect mitochondrial function in *C. elegans*, possibly as an indirect effect of ER stress (Saha *et al.* 2009). Another major Parkinson's protein, α-synuclein, is also toxic through gain of function. Overexpression of this protein in *C. elegans* inhibits mitochondrial fusion, and eventually affects mitochondrial function; phenotypes that can be suppressed by overexpressing proteins that promote mitochondrial clearance, such as *PINK-1* and the Parkin-homolog *PDR-1* (Kamp *et al.* 2010).

Although PINK-1 and Parkin/PDR-1 can promote mitophagy in *C. elegans* (Ryu *et al.* 2016), other pathways might also induce mitophagy. *C. elegans* does not have a Mul1 homolog, which is a mitochondrial E3 ligase that acts in parallel with Pink1 and Parkin in mammals and *Drosophila* (Yun *et al.* 2014). *C. elegans* does have several other E3 ligases, which could affect mitochondrial turnover. These include Huwe1 (*C. elegans* Y92H12A.2), March5 (*C. elegans* M110.3), Rnf185 (*C. elegans rnf-5*) (Kuang *et al.* 2012) and Gp78 [*C. elegans hrdl-1*(*gk28*)] (Sasagawa *et al.* 2007). The extent of their redundancies with PINK-1 and Parkin/PDR-1 in *C. elegans* remains to be determined.

C. elegans has provided new insights into the connections between mitochondrial fission and mitophagy. Mutations in *drp-1* and *fis-2* (one of the two Fis1 homologs in *C. elegans*) act independently to promote clearance of mitochondria during apoptosis (Breckenridge *et al.* 2008). Although Drp1 and Fis1 act independently during clearance, these proteins do bind to each during hypoxia-induced mitochondrial fission and clearance (Kim *et al.* 2011). Nevertheless, mutations in mammalian fis1 do not affect fission under normal circumstances (Otera *et al.* 2010). An explanation for these seemingly contradictory functions of Fis1 was finally provided by several different lines of research, starting with the discovery that Fis1 mutants disrupt disposal of defective mitochondria in *C. elegans* and accumulate large amounts of unproductive autophagosome membranes (Shen *et al.* 2014). An explanation for this phenomenon came from additional findings that Fis1 binds to the Rab7 GAP TBC1D15 (Onoue *et al.* 2013) and that mutations in TBC1D15 phenocopy the effects of Fis1 mutations (Yamano *et al.* 2014). Fis1 and TBC1D15 therefore act in concert to attenuate Rab7 function during mitophagy, most likely providing a mechanism for turning the autophagy machinery off once defective mitochondria are fully encapsulated by isolation membrane.

In the wild, *C. elegans* may encounter chemicals that induce mitochondrial stress, but they are also often subjected to hypoxic conditions, which induce mitophagy as well. It was shown that anoxia and reoxygenation regulates mitochondrial dynamics through a hypoxia response pathway that includes the stress-regulated transcription factor SKN-1 (Nrf2 in mammals) and other proteins that affect mitochondrial dynamics under stress conditions (Ghose *et al.* 2013). SKN-1 is not only required for recovery during reoxygenation,

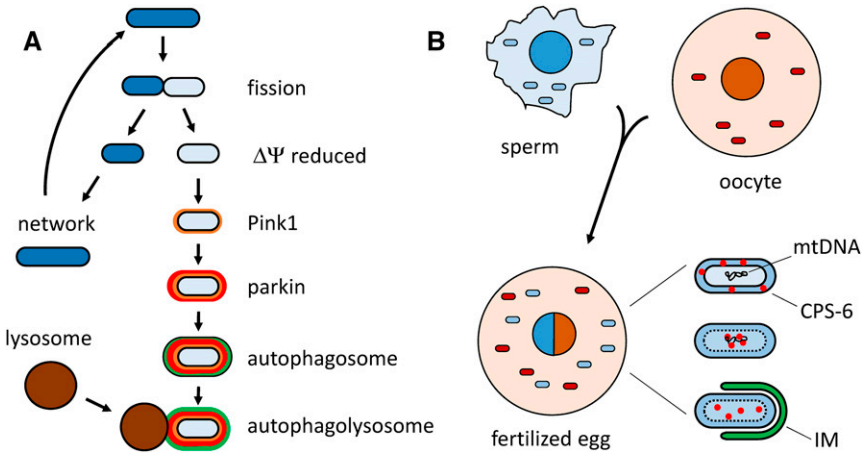

Figure 8 Outline of mitophagy pathways. (A) Mitochondria with low membrane potential, as might result from mutations in mitochondrial DNA or by damage from ROS, accumulate PINK-1 on their surface. PINK-1 then recruits the E3 ubiquitin ligase parkin (PDR-1) from the cytosol. Parkin ubiquitinates mitochondrial membrane proteins, such as Miro, and triggers autophagy through Ulk1 (*C. elegans* UNC-51). These events lead to the formation of an autophagosome encapsulating the damaged mitochondrion with membrane, followed by fusion with lysosomes. This specialized form of autophagy ensures that damaged mitochondria are quickly removed from the cell. (B) Paternal mitochondria are eliminated from zygotes by mitophagy. Soon after fertilization, the mitochondrial inner membrane becomes permeable, allowing entry of the intermembrane space endonuclease, CPS-6, into the matrix, where it degrades mtDNA. Further degradation of paternal mitochondria is mediated PINK-1 and PDR-1/parkin induced mitophagy.

it also helps coordinate mitophagy and mitochondrial biogenesis, which are important factors in *C. elegans* longevity (Palikaras *et al.* 2015). Tomatidine, a substance in green tomatoes, was recently shown to promote longevity in *C. elegans* by inducing mitophagy via the SKN-1 pathway (Fang *et al.* 2017), similar to the effects that were observed with urolithin (Ryu *et al.* 2016). SKN-1 can control the expression of genes needed for mitochondrial biogenesis and the expression of DCT-1, which is a mitochondrial outer membrane protein and a downstream effector of Pink1 and Parkin (Palikaras *et al.* 2015).

DCT-1 is similar in sequence to Bnip3 and NIX in mammals, although they seem to play somewhat different roles during mitophagy. Bnip3 and NIX proteins can trigger developmentally programmed removal of mitochondria, for example, during erythropoiesis where mitochondria are degraded by Pink1/Parkin-independent mitophagy followed by expulsion through exocytosis (Ney 2015). A comparable pathway for programmed removal of mitochondria from somatic cells does not appear to exist in *C. elegans*. There may, nevertheless, be some shared features with a newly discovered pathway for the removal of damaged mitochondria from *C. elegans* neurons (Melentijevic *et al.* 2017). When stress is induced by impaired quality control mechanisms, such as chaperone expression, autophagy, or proteasome function, *C. elegans* neurons are able to extrude large membrane enclosed vesicles, called exophers, which can include defective mitochondria. These "jettisoned" vesicles transit through surrounding tissue. Nondegraded material from these exophers is even observed in remote cells, suggesting the occurrence of secondary transfer throughout the body. Mutations in canonical mitophagy genes (*pdr-1*, *pink-1*, and *dct-1*). This novel disposal mechanism, first discovered in *C. elegans*, promises to become an exciting new area of research.

Elimination of paternal mitochondria

Perhaps the most important example of deliberately removing functional mitochondria occurs during fertilization. Sperm cells have mitochondria, but those are selectively degraded after fertilization. As a result of this degradation, the mitochondrial DNA in sexually dimorphic eukaryotes is almost always inherited from the mother through the oocyte. This type of maternal inheritance depends on the ability to selectively remove sperm mitochondria or, to be more precise, their DNAs. Recent studies with *C. elegans* and *Drosophila* have helped uncover the mechanisms for the selective removal of paternal mitochondria (van der Bliek 2016).

The first break came with *C. elegans*, where it was shown that autophagy proteins are required for the degradation paternal mitochondria (Al Rawi *et al.* 2011; Sato and Sato 2011; Zhou *et al.* 2011; Djeddi *et al.* 2015). This specialized form of mitophagy occurs within minutes after fertilization. Autophagy mutants allow paternal mitochondria to persist throughout embryogenesis. Mitochondrial fission and fusion proteins affect this process as well (Wang *et al.* 2016). A defect in fission in the sperm delays degradation of paternal mitochondria, while a defect in fusion of accelerates this process, as would be expected for removal through mitophagy. Fission and fusion mutations in oocytes have the opposite effects, presumably because maternal mitochondria need protection from degradation.

A second break came with the discovery that the *C. elegans* endonuclease G homolog, CPS-6, which normally resides in the mitochondrial intermembrane space, is needed for the postfertilization degradation of mitochondrial DNA (Figure 8B). *C. elegans* CPS-6 was previously shown to degrade nuclear DNA during apoptosis (Parrish *et al.* 2001), but then, in *Drosophila*, it was shown that endonuclease G degrades mitochondrial DNA in sperm cells before fertilization (DeLuca and O'Farrell 2012). The twist came with *C. elegans*, where it was shown that CPS-6 degrades paternal mitochondrial DNA shortly after fertilization (Zhou *et al.* 2016). The mitochondrial inner membranes somehow lose their integrity, allowing the CPS-6 protein to enter the matrix compartment and degrade the paternal mitochondrial DNA before the

autophagosomes fuse with lysosomes. Mutations in *cps-6* cause an accumulation of autophagy intermediates. Interestingly, crosses between different genetic backgrounds showed that the retention of heterologous DNA in *cps-6* mutants is embryonic lethal, suggesting that oocytes are somehow adapted to accommodate their own mitochondrial DNA, but not DNA from heterologous strains. This type of adaptation mirrors the physiological effects of matching mitochondrial DNA haplotypes with different nuclear DNAs, as recently observed in mice (Latorre-Pellicer *et al.* 2016).

What is the mechanism for making holes in the mitochondrial inner membrane, so that CPS-6 can enter the matrix compartment? Permeability transition pores that cause necrotic cell death are too small for proteins. Osmotic swelling and rupturing of the membrane is possible, but that has not been observed. Moreover, it was recently shown that the mammalian mitochondrial inner membrane protein prohibitin 2 becomes exposed to the cytosol during mitophagy, and that this protein then acts as a receptor for binding to components of the autophagic membrane (Wei *et al.* 2017). RNAi knockdown of the *C. elegans* prohibitin 2 homolog *phb-2* prevented the clearance of paternal mitochondria after fertilization (Wei *et al.* 2017), suggesting a similar role as a mitophagy receptor in *C. elegans*. If that is the case, then there might also be holes in the mitochondrial outer membrane along with the holes in the inner membrane that are needed for CPS-6 entry into the matrix. We are left with two main questions: what makes holes in the membranes of paternal mitochondria after fertilization? What triggers the formation of these holes? Answers may come from future genetic studies with *C. elegans*.

UPR^{mt} stress response and signaling between mitochondria and the nucleus

The mitochondrial unfolded protein response (UPR^{mt}) is a cellular program in which certain nuclear genes are transcriptionally activated to counter the effects of unfolded proteins in mitochondria. This program was first discovered in mammalian cells, where the relevant transcription factors and a number of responsive genes with their regulatory elements have been identified (Hoogenraad 2017). Genes that are activated encode proteins responsible for proteolysis (ClpP, YME1L1), protein import (Tim17A, MPP), chaperonins (Cpn60, Cpn10), and other functions that mitigate problems caused by unfolded proteins (Aldridge *et al.* 2007). Although there are differences in the activation mechanisms and identities of transcription factors, much has also been learned from the discovery of UPR^{mt} pathways in *C. elegans* (Fiorese and Haynes 2017) (Figure 9A).

C. elegans UPR^{mt} is triggered by the actions of the mitochondrial matrix protease CLPP-1, suggesting that proteolysis of unfolded proteins is part of the signaling process (Haynes *et al.* 2007). This idea is supported by the discovery of a role for the mitochondrial peptide exporting protein HAF-1 in this process (Haynes *et al.* 2010). RNAi for HAF-1 suppresses UPR^{mt} and it prevents translocation of a downstream transcription factor ATFS-1 (formerly named ZC376.7) to the nucleus. ATFS-1 binds to promoters of genes induced by UPR^{mt}, but it also binds directly to OXPHOS gene promoters limiting OXPHOS transcripts during mitochondrial stress (Nargund *et al.* 2015). Whether peptides generated by CLPP-1 and exported by HAF-1 are able to directly activate ATFS-1 is unclear, but ATFS-1 does have some unusual targeting properties that may be connected with this process. ATFS-1 has dual targeting with an N-terminal mitochondrial leader sequence and a C-terminal nuclear localization signal. This protein is usually imported and degraded in mitochondria, except during UPR^{mt} when it is no longer imported into mitochondria (Nargund *et al.* 2012). It then goes to the nucleus, where it activates the expression of genes needed for UPR^{mt} (Haynes *et al.* 2010). It is therefore possible that the exported peptides somehow interfere with the mitochondrial import pathway or they redirect ATFS-1 to the nucleus in a different way.

Several other signaling proteins that are involved in UPR^{mt} were discovered with genetic screens in *C. elegans*. Along with ATFS-1, another transcription factor, DVE-1, plays a role downstream of CLPP-1 and HAF-1. DVE-1 forms a complex with the small ubiquitin-like protein UBL-5, binding to the promoters of mitochondrial chaperone genes (Haynes *et al.* 2007). Genetic screens also revealed that the histone demethylases LIN-65 and MET-2 are required for DVE-1 and ATFS-1 binding to the promoter elements of stress-induced genes (Tian *et al.* 2016). Several other histone demethylases also affect UPR^{mt}, revealing even more complexity (Merkwirth *et al.* 2016). Lastly, an eIF2α kinase named GCN-2 that modulates cytosolic protein synthesis, functions in a complementary pathway. ROS produced by defective mitochondria triggers GCN-2-dependent phosphorylation of eIF2α, but not ATFS-1 activation. Deleting *atfs-1* and *gcn-2* genes together exacerbates the developmental defects from either mutation, suggesting that translational control and chaperone induction are parts of complementary UPR^{mt} pathways (Baker *et al.* 2012).

The GCN-2-dependent ROS-induced UPR^{mt} mentioned above is inhibited by the MAP-kinase KGB-1 (Runkel *et al.* 2013). This kinase controls the response to invading pathogens and xenobiotic compounds, suggesting that these other stress responses complement ROS-induced UPR^{mt}, similar to the complementary effects observed with *atfs-1* and *gcn-2* mutants. Indeed, ATFS-1 also induces innate immune genes (Pellegrino *et al.* 2014). Mutants lacking ATFS-1 are susceptible to *Pseudomonas aeruginosa* infections, whereas hyperactivation of ATFS-1 improved *P. aeruginosa* clearance from the intestine. Thus, UPR^{mt} induced by ATFS-1 provides a mechanism for pathogen-response, countering the disruptions of mitochondrial function when *C. elegans* is grown with different pathogenic bacteria and combating those bacteria themselves (Liu *et al.* 2014).

UPR^{mt} also has a downside, because it can inadvertently help maintain copies of deleterious mtDNA in heteroplasmic *C. elegans* strains (Gitschlag *et al.* 2016; Lin *et al.* 2016;

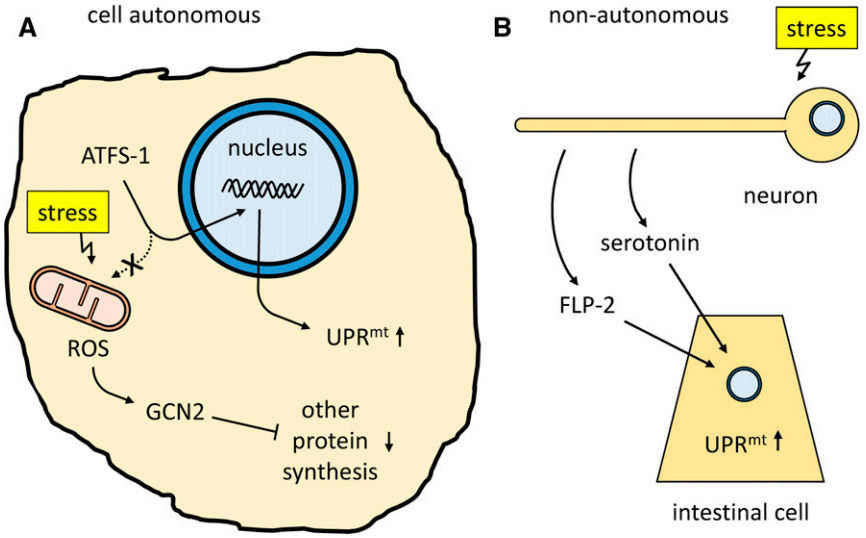

Figure 9 Outline of pathways controlling the mitochondrial unfolded protein response in *C. elegans*. (A) Cell autonomous pathways controlling UPR^{mt} within a cell include signaling through the ATFS-1 transcription factor, which is normally targeted to mitochondria and degraded, but can get routed to the nucleus when mitochondrial protein import is blocked. The other pathway involves activation of GCN-2, which inhibits cytosolic protein synthesis. (B) Cell nonautonomous UPR^{mt} is triggered in intestinal cells by neurotransmitters (serotonin and FLP-2) that are secreted by stressed neurons.

Tian *et al.* 2016). ATFS-1 plays a role in this process. An *atfs-1* mutant with the heteroplasmic *uaDf5* mtDNA deletion has almost tenfold less total mtDNA than a heteroplasmic strain with wild-type *atfs-1* (Lin *et al.* 2016). The latter strain has mild mitochondrial dysfunction and constitutive UPR^{mt} activation, while the former strain has severely disrupted mitochondria. It seems likely that OXPHOS defects caused by deletions in mtDNA activate UPR^{mt} and increase mitochondrial biogenesis in an attempt to recover OXPHOS activity, thereby propagating deleterious mtDNA (Lin *et al.* 2016). Unlike wild-type mtDNA, the genomes with deletions lack copy number control, which would suggest that they tend to populate mitochondria because of a replication advantage. However, strains with different sizes of mtDNA deletions have similar ranges of copy numbers, so other factors such as the competing effects of UPR^{mt} and clearance through PINK-1 and Parkin/PDR-1 pathways must be more important (Gitschlag *et al.* 2016).

An exciting new development with broad implications came from longevity studies with *C. elegans*. The effects on longevity were monitored in nematodes that expressed hairpin RNAi for a mitochondrial OXPHOS protein under control of various tissue specific promoters (Durieux *et al.* 2011). It was discovered that mitochondrial stress in neurons is acted upon by UPR^{mt} in other tissues, even when they have no physical connections to the RNAi-expressing cells. This cell-nonautonomous response helps control the rate of aging for these other tissues, and indeed for the whole organism, through diffusible signals that come from limited numbers of cell types (Durieux *et al.* 2011). The nature of these signals, termed Mitokines, their production by stressed cells, and their detection by peripheral tissues, has generated a lot of interest because of their potential therapeutic value (Deng and Haynes 2016).

Clues to the mechanisms for cell nonautonomous activation of UPR^{mt} came from recent studies in which the potential contributions of different neurotransmitters to this process were evaluated in *C. elegans* (Figure 9B). In one study, cytosolic expression of polyglutamine (polyQ40) was used to induce mitochondrial stress in neurons (Berendzen *et al.* 2016). The effects on UPR^{mt} gene expression was monitored in intestinal cells with a P_{hsp-6}::GFP reporter construct. It was found that cell nonautonomous activation of UPR^{mt} requires functional UNC-31 (calcium activator for serotonin secretion) and the tryptophan hydroxylase TPH-1, which is required for serotonin synthesis (Berendzen *et al.* 2016). In a second study, CRISPR-Cas9 was used to disrupt mitochondrial function in neurons together with a systematic analysis of the contributions of different *C. elegans* neuropeptides to cell nonautonomous activation of UPR^{mt} (Shao *et al.* 2016). Mutations in genes encoding a number of neuropeptides affected nonautonomous activation of UPR^{mt}, but only one (FLP-2) was also able to induce this response by overexpression alone. Further analysis revealed that a subcircuit of three *C. elegans* sensory neurons and one interneuron was necessary for this response (Shao *et al.* 2016). Both studies show that UPR^{mt} genes are needed in the signal providing neuronal cells and in the signal responsive intestinal cells, but the signals are different and the receptors on the intestinal cells are unknown. There is still much to be learned about this highly intriguing process.

Concluding remarks: *C. elegans* has provided exciting new insights into diverse aspects of mitochondrial function. The field of bioenergetics received a boost from *C. elegans*, because this genetically tractable organism shares many properties with other multicellular animals, including similarly organized ETC, toxicological properties, and downstream effects on aging. The mechanisms controlling mitochondrial dynamics in *C. elegans* are also much more similar to the mechanisms in complex animals than for example in yeast. Recent developments that were pioneered in *C. elegans* include the discovery of mechanisms for eliminating paternal mitochondria after fertilization, new mechanisms controlling

UPR^{mt} and the discovery of mitokines. Progress in this field has been rapid. We therefore fully anticipate many more discoveries like this with *C. elegans*.

Acknowledgments

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