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Mitochondrial bioenergetics and disease in *Caenorhabditis elegans*

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1. ABSTRACT

Simple multicellular animal model systems are central to studying the complex mechanisms underlying a bewildering array of diseases involving dysfunctional mitochondria. Mutant nuclear- and mitochondrial-encoded subunits of the *Caenorhabditis elegans* mitochondrial respiratory chain (MRC) have been investigated, including GAS-1, NUO-1, NUO-6, MEV-1, SDHB-1, CLK-1, ISP-1, CTB-1, and ATP-2. 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 *C. elegans*, MRC dysfunction can mimic signs and symptoms observed in human patients with primary mitochondrial disorders, such as neuromuscular deficits, developmental delay, altered anesthetic sensitivity, and increased lactate levels. Antioxidant dietary supplements, coenzyme Q substitutes, and flavin cofactors have been explored as potential therapeutic strategies. Furthermore, mutants with altered longevity have proved useful for probing the contributions of bioenergetics, reactive oxygen species, and stress responses to the process of aging. *C. elegans* will undoubtedly continue to provide a useful system in which to explore unanswered questions in mitochondrial biology and disease.

2. INTRODUCTION

A rich, multinational history of mitochondrial research began more than a century ago. Mitochondria were first described as ubiquitous "bioblasts" in histological sections of nucleated cells in 1890 (1), shown to be stained by the redox dye Janus Green B in 1899 (2), and visualized at high resolution using electron microscopy in 1952 (3). Following these morphological observations, the biochemistry of the mitochondrial respiratory chain (MRC) was elucidated and shown to be associated with the inner mitochondrial membrane (4,5,6). In addition to the respiratory chain, mitochondria were found to be the site of the citric acid and urea cycles, fatty acid oxidation, and the biosynthesis of amino acids, nucleotides, lipids, hemes, and iron-sulfur clusters (7). Meanwhile, with the advent of molecular biology, nuclear and mitochondrial genes encoding proteins with mitochondrial functions were identified (8). Subsequent advances in understanding mitochondrial protein import, fission-fusion dynamics,

apoptosis, ion homeostasis, redox balance, and stress sensing by the mitochondrion, were due in a large part to research employing yeast model organisms (9). One topic of current investigation is the multifaceted interplay between mitochondrial metabolism and signaling pathways that control transcription and other regulatory networks (10,11,12,13,14).

In addition to uncovering fascinating biology, the study of mitochondria is motivated by its role in human diseases. It is well established that primary mitochondrial disorders are caused by defects within the organelle. Moreover, the mitochondrion plays a crucial role in a wide array of diseases including cancer, type 2 diabetes, Huntington's, Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis, and Freiderich's ataxia (15,16,17,18). The list of diseases with a possible connection to the mitochondrion is rapidly growing, and includes HIV, deafness, glaucoma, obesity, cardiovascular disease, stroke, autism, and autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (19-28). Diminishing function of mitochondria is associated with the process of aging (29). Furthermore, mitochondrial targets are involved in the therapeutic and/or adverse effects of many pharmaceuticals and toxins, such as ethanol, cyanide, arsenic, antiviral nucleoside analogues, nonsteroidal anti-inflammatory drugs, certain antibiotics, and anesthetics (30,31,32). The widespread involvement of this organelle in disease and pharmacology is due to its multitude of functions, unique risks associated with dual genetic control, its prokaryotic evolutionary origin, and its unique physicochemical characteristics (33). Thus, it has become increasingly clear in recent years that metabolism and other mitochondrial functions are important to a broad spectrum of medical research, and this is the focus of this special edition on mitochondrial bioenergetics in human health.

The worldwide prevalence of primary mitochondrial disorders is estimated at one in 5,000 (34). This group of genetic disorders usually presents in children who suffer from a heterogeneous collection of symptoms. These include neuromuscular deficit, developmental delay, and elevated lactate and pyruvate often detected in the serum. These primary mitochondrial disorders are caused by genetic mutations in either the mitochondrial genome (mtDNA) or the nuclear genome. The mtDNA encodes 13 subunits of the MRC, as well as 22 tRNAs and 2 rRNAs that translate these proteins. These are particularly vulnerable to mutation, with about 10 to 20-fold mutation rate, because they lack protective histones, are in close proximity to reactive oxygen species (ROS) generation at the MRC, and the mtDNA repair processes are generally less efficient than those in the nucleus (35). Of infants diagnosed with mitochondrial disease, mtDNA mutations can account for approximately 17% (36). Moreover, at least 900 nuclear genes have been identified with mitochondrial functions, and over 100 have mutations associated with mitochondrial disorders (37,38). These disease genes include those that encode many subunits of the MRC as well as genes involved in fusion-fission dynamics, mtDNA abundance, mitochondrial nucleoside/nucleotide pool, mitochondrial translation, protein import, and phospholipid synthesis (39). The disease ranges in severity from mild to fatal in infancy; in fact, mtDNA mutations are often associated with early spontaneous abortions and/or neonatal deaths, while 1 in 200 healthy humans carry a potentially pathological mtDNA mutation (40,41).

Mitochondrial disorders are sadly as of yet largely untreatable; they are also difficult to diagnose as symptoms vary greatly. It is not trivial to perform genetic tests to identify an underlying mutation, because the mitochondrion is under dual genetic control, and any of a number of mtDNA or nuclear disease genes can give rise to mitochondrial disease. Non-Mendelian inheritance affects the presentation of the disease, such that maternal effects can influence time of onset and mtDNA mutation heteroplasmy can influence tissue-specific pathology (42). The variable presentation and complexity of mitochondrial diseases in humans has supported a hope that animal model organisms will be useful in identifying novel therapeutic strategies in the treatment of mitochondrial disorders (43). The model organism approach is especially relevant for studying mitochondria because their functions and the MRC components are highly conserved from yeast to man (44). In this review, we focus on MRC defects investigated using the simple nematode, *Caenorhabditis elegans*, and how insights gained from this model organism have been applied to understanding the biology and potential treatment of mitochondrial disorders.

3. *C. ELEGANS* MODELS FOR INVESTIGATING THE MITOCHONDRIAL RESPIRATORY CHAIN

Since the metabolic demands of various tissues are different, multicellular organisms are

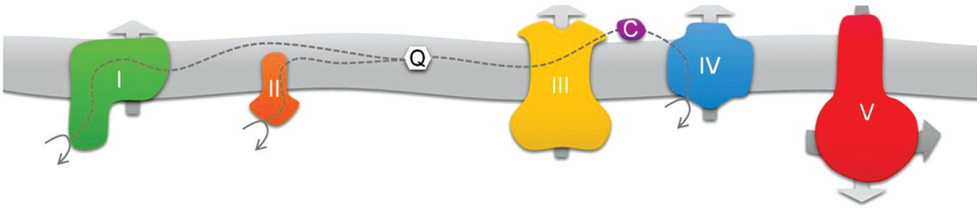
preferred for understanding tissue-specific regulation of mitochondrial functions and signaling between cell types (45). *C. elegans* is a nematode with a simple anatomy of 959 somatic cells in the adult hermaphrodite, with a mapped neural network, an epidermis, an intestine, distinct muscle groups, an excretory system, and gonads. In addition, unlike yeast or cultured cells, *C. elegans* display quantifiable behavioral phenotypes including locomotion, feeding and defecation, mating and egg-laying, mechanosensation, attraction/avoidance, and learning (46), as well as a measurable lifespan (47,48,49) and innate immunity to pathogens (50). A remarkable advantage of *C. elegans* for genetic research is the ease with which genes can be knocked down. Although this is fairly straightforward in other model organisms and cultured cells, RNAi is made especially facile in *C. elegans* because of the uptake of RNA via feeding (51). In fact, *C. elegans* readily take up diverse compounds during feeding, and are therefore a useful pharmacologic model for testing potential dietary supplements (52). Because *C. elegans* are transparent, fluorescence microscopy can be conducted on live animals throughout development, with dyes or genetically encoded reporters (53). As with any system in which bioenergetics is studied, biochemical analyses of purified mitochondria or MRC proteins are possible in *C. elegans*, and the reproducibility of this system has even led to its proposed use as a benchmark for clinical tests (54,55).

It should be noted that a number of differences have been documented between human mitochondria and *C. elegans* mitochondria. The mtDNA of *C. elegans* contains homologues of 36 of the 37 genes found in human mtDNA, lacking the ATP8 subunit of Complex V (56). Although the mtDNA of both animals is prone to mutation, these mutations seem to be generally better tolerated by *C. elegans* than humans (57,58). *C. elegans* also appear to have fewer copies of mtDNA per cell than humans (59). Unlike humans, *C. elegans* cannot accomplish *de novo* synthesis of hemes, including those found in several components of the MRC, but rather rely on dietary heme (60). Instead of coenzyme Q_{10}, which has a chain of 10 isoprenyl repeats, *C. elegans*, like rodents, primarily use coenzyme Q_9 (61). 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 (62,63). Furthermore, mitochondria isolated from *C. elegans* can respire with malate as the substrate, while malate on its own is a poor substrate for mammalian mitochondria (54,64). *C. elegans* mitochondria may be less sensitive to certain artificial uncouplers (64). Lastly, cadiolipin, a membrane phospholipid required in the inner mitochondrial membrane for proper MRC function in humans (65,66), appears to only be required in the *C. elegans* gonad and not in somatic cells (67).

However, despite these subtle differences, most biochemical properties of mitochondria purified from *C. elegans* appear to be similar to those purified from mammalian cells, including the TCA cycle, MRC activities, oxygen consumption profiles, supercomplex formation (54,64,68,69). The mtDNA of *C. elegans* (13.7. kb) is similar in size and structure to that of humans (16.6. kb) and both experience heteroplasmy, polyploidy, and maternal inheritance (56,70). Of the 91 human genes thought to encode MRC subunits, at least 72 have homologues identified in *C. elegans* (59). Many genes encoding mitochondrial proteins have been identified and characterized in *C. elegans* (see Figure 1), largely confirming substantial homology between the mitochondria of *C. elegans* and mammals (71). Furthermore, in many cases, the phenotypes observed in *C. elegans* mutants or knockdowns mirror those documented for genetic mitochondrial disorders in human patients, validating overall functional similarity between these systems. Of these, example strains bearing classical mutations are listed in Table 1, with genetic and/or biochemical evidence of loss-of-function indicated. Several additional MRC subunits have been studied using RNAi, although RNAi can act differently than classical mutations, and may depend upon the efficiency of knockdown in a given experiment (72,73). The *C. elegans* classical mutant strains can be viewed as disease models for mitochondrial disorders, and will be discussed in this context in further detail in this review.

3.1. Complex I: GAS-1, NUO-1, and NUO-6

Complex I, also known as NADH-CoQ oxidoreductase or NADH dehydrogenase, is a mega-Dalton complex of around 45 different proteins (74). It is an entry point of electrons into the MRC, with a flavin mononucleotide (FMN, also known as riboflavin-5'-phosphate) and an iron-sulphur cluster involved in electron transfer. It is the most commonly defective component of the MRC in patients with primary mitochondrial disorders. At least nineteen subunits of complex I, as well as several proteins involved in complex I assembly,



	Complex I			Complex II		Coenzyme Q		Complex III		Complex IV	ATP synthase
Example *C. elegans* genes	*gas-1*	*nuo-1*	*nuo-6*	*mev-1*	*sdhb-1*	*clk-1*	*coq-3*	*isp-1*	*ctb-1*	*cco-1*[a]	*atp-2*
C. elegans phenotypes	Mit[b,c] Mev Gas Slu SLD FEC	Mit Mev[d] Gas[a] Slu Stp Dec SLD[d] FEC[d]	Mit Slu Dec SLD FEC	Mev Slu FEC	Mev[d] Stp FEC[d]	Mit Gas Slu Stp[e] Dec SLD FEC	Stp SLD FEC	Mit Dec SLD FEC	Stp	Mit[c] Mev SLD FEC	Mit Slu Stp Dec
Human diseases	LS, MELAS, LHON			Paraganglioma, phaeochromo-cytoma		Encephalomyo-pathy, nephrosis, ataxia		Encephalo-hepatopathy, GRACILE, LS		LS, encephalopathy	Encephalo-cardiomyopathy, LS

Figure 1. *C. elegans* mitochondrial respiratory chain subunits of interest. Top, a drawing of the major components of the respiratory chain within the mitochondrial inner membrane. The intermembrane space is at the top, and the matrix is at the bottom. Electron transfer is represented by a dotted grey line. Proton translocation is represented by large vertical arrows. ATP generation is represented by a large horizontal arrow. The oxidation of NADH and succinate, and the reduction of oxygen, are represented by small curved arrows. Note that the complexes are stylistically drawn spaced apart and in equal stoichiometries for simplicity. Bottom, a table of commonly studied MRC components in *C. elegans*, phenotypes documented for loss-of-function classical mutation of these genes, and human diseases frequently associated with dysfunction at each step in the MRC. Mit: long-lived; Gas: hypersensitive to volatile anesthetics; Mev: hypersensitive to oxidants; Slu: slow locomotion; Stp: sterile progeny, including due to larval arrest of the parent; Dec: reduced defecation, usually also present with reduced pharynx pumping; SLD: slow larval development; FEC: low fecundity/brood size; LS: Leigh Syndrome; MELAS: mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes; LHON: Leber's hereditary optic neuropathy; GRACILE: growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death; [a]Phenotypes observed for RNAi; [b]phenotype observed at 15 degrees C; [c]phenotype observed in the presence of 5-fluoro-2'-deoxyuridine (FUdR); [d]phenotype observed in strains with point mutant transgenes; [e]phenotype observed on diet lacking coenzyme Q.

have mutations associated with Leigh Syndrome, a devastating but incurable childhood mitochondrial disorder (75,76,77). Complex I dysfunction is also associated with lactic acidosis, encephalomyopathy, cardiomyopathy, and leukodystrophy/myoclonic epilepsy (78). *C. elegans* homologues have been identified for at least 38 of the 45 human genes encoding complex I subunits (59,79). To empirically determine if these genes contribute to complex I function, complex I-dependent respiration was measured in *C. elegans* with each of 28 subunits knocked down by RNAi, and 15 of these caused a statistically significant decrease (79).

The most characterized complex I subunit in *C. elegans* is GAS-1. *gas-1(fc21)* mutant animals have dramatically decreased complex I activity, as measured by oxygen consumption of isolated mitochondria provided with glutamate or malate as substrate, and also by spectrophotometric assays of rotenone-sensitive NADH-cytochrome-c reductase activity and rotenone-sensitive NADH-decylubiquinone reductase activity, as measured by a spectrophotometric assay. The GAS-1 homologues are known as the human NDUFS2 and the bovine 49kDa subunit (54). Mutations in NDUFS2 have been identified in nine separate families with Leigh Syndrome, Leigh-like Syndrome, cardiomyopathy, and encephalomyopathy; 8 of the 12 patients described died before age 4 (80,81,82,83). Intriguingly, our laboratory first identified *gas-1* in a screen for mutants with abnormal responses to volatile anesthetics. *C. elegans* with 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 (84). 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 (85). The effect on anesthetic sensitivity requires both neuronal and muscular expression of *gas-1* and evidence that this might be linked to a pre-synaptic

Table 1. Loss-of-function mutations in *C. elegans* models of mitochondrial disease

Gene	Mutant	Sequence alteration	MRC activity	Reference
gas-1	fc21	Single substitution, Arg to Lys	Three-fold lower CI-dependent respiration	(84,96)
nuo-1	ua1	1.2.-kb deletion, with transgenic NUO-1 bearing A352V, T434M, or A443F	50% decreased respiration, 50-75% decreased CI-dependent respiration, 50-70% decreased CI activity	(110,111)
nuo-6	qm200	Single substitution, Gly to Glu	10-fold decreased CI activity and 40% decreased respiration	(72)
mev-1	kn1	Single substitution, Gly to Glu	10-fold decreased CII in isolated membranes; but unchanged ATP	(118,120)
sdhb-1	gk165	2.2.-kb deletion, with transgenic SDHB-1 bearing P211H, P211L, P211F, P211R	At least 50% decreased CII reductase activity	(125)
clk-1	qm30	590-bp deletion	No CoQ_9	(96,129,134)
	qm51	No splicing of intron 2 and an early stop codon	No CoQ_9	
	e2519	Missense leading to partial LOF	No CoQ_9; three-fold lower CI-dependent respiration	
isp-1	qm150	Single substitution, Pro to Ser	2-fold lower respiration, 6-fold lower CIII activity	(152,210)
ctb-1	qm189	Single substitution, Ala to Val	2-fold lower CIII activity	
coq-3	qm188	2.5.-kb deletion	NF	(133)
atp-2	ua2	710-bp deletion	NF	(110)
lrs-1	mg312	Early stop codon at residue 247	NF	(160)
polg-1	ok1548	2149 bp deletion	NF	(170)
sod-2	gk257	161-bp deletion	Decreased CI-dependent respiration and CI redox activity	(94,178)
	ok1030	900-bp deletion	20% decreased respiration	

CI, CII, CIII, CIV: complex I, II, III, and IV activities determined biochemically; LOF: loss-of-function; MRC: mitochondrial respiratory chain; mt: mitochondrial; NF: no published reports of MRC activity found; OCR: oxygen consumption rate

effect in motor neurons comes from an aldicarb/levamisole assay (54). 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 (79). This is further confirmed by an increase in complex I activity, through mutation of the insulin receptor *daf-2*, which results in halothane resistance (85). The link between complex I dysfunction and volatile anesthetic hypersensitivity is also observed in mice and humans (86,87). This discovery, which began with *C. elegans*, has influenced the clinical recommendations for dosing of volatile anesthetics to patients with mitochondrial disorders (88). This serves as an instructive example of bench-to-bedside research in *C. elegans*. Furthermore, these results paved the way for recent research using *C. elegans* to understand potential neurotoxic side-effects of volatile anesthetics, which is potentially a risk in surgeries on very young children (89,90,91).

These findings also add insight into the elusive question of how these chemicals induce animal anesthesia (92). Direct measurement of complex I enzyme kinetics of isolated *C. elegans* mitochondria was dose-dependent with respect to isoflurane concentration, with an IC_{50} comparable to the ED_{50} for animal anesthesia. As expected, complex I with the *gas-1(fc21)* mutation was not inhibited by isoflurane *in vitro*, nor were wild-type complexes II, III, or IV. This is consistent with the hypothesis that complex I may be a direct target for this anesthetic. The V_{max} of rotenone-sensitive NADH-decylubiquinone reductase activity decreased to 65% in the presence of 5.7.% isoflurane, while the K_m with respect to decylubiquinone remained unchanged. The Hill coefficient of around 2, which indicates cooperativity of CoQ binding, was not significantly changed by isoflurane. No change to I:III:IV supercomplex stability was observed by 2-dimensional native gel electrophoresis in the presence and absence of isoflurane. These

data ruled out competitive binding at the CoQ site or disruption of CoQ channeling as potential mechanisms of inhibition (93). Further investigation is needed to reveal the mechanism by which volatile anesthetics reduce complex I activity.

Other phenotypes of the *gas-1(fc21)* animal include slow development, reduced locomotion, and reduced fecundity (94). Similar to human patients with complex I dysfunction, *gas-1(fc21)* animals are short-lived. The lifespan phenotype is less pronounced at cold temperatures, and exacerbated at high temperatures, indicating a potential temperature-dependence in this point mutant (95). *gas-1(fc21)* has an increase in mitochondrial mass, as a possible compensatory mechanism (71). They also have a decrease in NADH-ferricyanide reductase activity, possibly through an allosteric effect within complex I (54). *gas-1(fc21)* also has an increase in complex II-dependent respiration, as measured by oxygen consumption of isolated mitochondria provided with succinate as substrate (96). In fact, increased complex II-dependent respiration is a general characteristic when any one of a number of complex I subunits are knocked down (79). These phenotypes mirror those of human patients with mitochondrial disease who have elevated complex II-dependent respiration (97) and "ragged red fibers" made up of overproduced mitochondria (98).

In addition to reduced NADH oxidation and reduced proton pumping, loss-of-function in complex I can alter the production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH). The MRC, and in particular complexes I, II, and III, is known to be major endogenous source of ROS, in addition to at least 6 or 7 dehydrogenase enzymes in the mitochondrial matrix (99). ROS and the damage it causes to the cell correlate with a variety of pathologies and aging (100). Furthermore, elevated ROS generation by and oxidative damage to complex I correlates with ischemia reperfusion injury (101), and 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 (102,103). Increased ROS may have a downward spiral effect by damaging MRC components and signaling to reduce complex I levels (104). *gas-1(fc21)* animals have an increase in oxidative damage to proteins (96). *gas-1(fc21)* animals are hypersensitive to the oxidizing agent, paraquat, and hypersensitive to hyperoxia (95). Similarly, when complex I is inhibited by rotenone, wild-type animals become hypersensitive to hyperoxia (105). In fibroblasts cultured from a patient with mutant NDUFS2, a vitamin E derivative reduced ROS to wild-type levels and riboflavin rescued ATP production (104,106). These results suggest that treatment with supplements containing antioxidants such as vitamin E may be beneficial in patients with complex I dysfunction causing elevated ROS.

Presumably in response to this oxidative burden, *gas-1(fc21)* animals upregulate the expression of genes that function in detoxification of ROS and oxidative damage, such as superoxide dismutase *sod-3*, and possibly because of this, their superoxide anion levels are similar to wild-type (94,95). 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 (107). SOD induction can improve the phenotypes of complex I deficiency, as evidenced by reduced neurodegeneration in a mouse model with virally delivered SOD (108). In addition to ROS scavenging, SOD-2 functions to stabilize the I:III:IV supercomplex through a direct protein-protein interaction. Thus cells may attempt to compensate defective complex I function by increasing supercomplex stability, through upregulating SOD-2 levels. However, disruption to *sod-2* can be life-extending in *gas-1(fc21)* (94,109).

Another subunit of complex I that has been studied in some detail in *C. elegans* is NUO-1, the flavoprotein NDUFV1 subunit, named with reference to NADH-ubiquinone oxidoreductase. The loss-of-function *nuo-1(ua1)* mutation appears more detrimental than *gas-1(fc21)* as it causes developmental arrest at the third larval stage, in homozygous mutant animals with a heterozygous mother. Interestingly, these arrested animals are long-lived and have neuromuscular defects of decreased pharynx pumping and defecation and impaired locomotion (110). Like *gas-1(fc21)* and *gas-1* RNAi, *nuo-1* RNAi results in halothane hypersensitivity (79).

In order to generate strains that could be propagated, transgenic *nuo-1(ua1)* animals were created that express *nuo-1* bearing point mutations A352V, T434M, or A443F. These mutations are designed to mimic those in humans with mitochondrial disease, and the transgenic *C. elegans* all still have decreased respiration and complex I activity, as well as decreased complex IV

activity, suggesting a crosstalk between complex I and IV, possibly through interactions within the I:III:IV supercomplex (111,112). They all display low brood size, decreased lifespan, increased lactate and lactate:pyruvate ratio, and hypersensitivity to hyperoxia and paraquat, and the A352V mutant has slow development (111). A similar acidosis is seen in RNAi of *nuo-1*, and is reminiscent of acidosis observed in some patients with mitochondrial disorders (113), and could be partially rescued in the A443F mutant by the activator of pyruvate dehydrogenase, sodium dichloroacetate (111). Interestingly, allowing the animals to efficiently undergo lactate-dependent respiration, by transgenic expression of the yeast L-lactate-cytochrome c oxidoreductase, rescues ATP levels, respiration rates, fecundity, and lifespan of the A352V and T434M point mutants (114). Since the NDUFV1 subunit is predicted to interact with the cofactor flavin mononucleotide, riboflavin supplementation was examined in the *nuo-1* point mutants. Riboflavin rescues complex I stability and partially rescues complex I activity, complex IV activity, lactate and lactate:pyruvate, and brood size (111,112). These data from *C. elegans* suggest that dietary riboflavin may be of use to patients with NDUFV1 defects, and possibly also those with defects in the other flavoproteins, NDUFV2 and NDUFV3.

The third complex I subunit studied in *C. elegans* via classical mutation is NUO-6, the NDUFB4 subunit. 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)* has slow embryonic and postembryonic development, reduced fecundity, neuromuscular defects such as slow locomotion, pharynx pumping, and defecation (72). *nuo-6(qm200)* is long-lived, without the developmental arrest seen in *nuo-1(ua1)*, suggesting that complex I dysfunction can lead to lifespan extension in *C. elegans*. Consistent with this, RNAi against *nuo-6* or *nuo-2*, the NDUFS3 subunit in *C. elegans,* both of which decreased ATP levels, also extends lifespan (72,115). As with *gas-1(fc21)*, *nuo-6(qm200)* have induced SODs, but the *nuo-6(qm200)* animals are resistant to paraquat (72), suggesting an improved stress tolerance in these long-lived animals. Interestingly, *nuo-6(qm200)* appear to have increased superoxide but unchanged overall ROS levels (94,116) and manipulation of the superoxide levels using antioxidants (vitamin C or N-acetyl-L-cysteine) or an oxidant (paraquat) also influenced lifespan (116). Although RNAi against *nuo-6* does effectively knock down *nuo-6* mRNA and impair complex I activity, it does not change halothane sensitivity. This is consistent with the observation that subunits of the lambda subcomplex, such as GAS-1 and NUO-1, are more directly involved in the anesthetic response than subunits of the rest of complex I, such as NUO-6 (79).

3.2. Complex II: MEV-1 and SDHB-1

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, is the only MRC complex that does not translocate protons across the membrane, and is small, being composed of four subunits, named a, b, c, and d. A flavin adenine dinucleotide (FAD) and an iron-sulphur cluster are involved in electron transfer. Of the genes associated with primary mitochondrial disorders, complex II was the first direct hit in a nuclear gene encoding a MRC subunit in humans (117). Mitochondrial disorders have now been linked to mutations found in all four of the complex II subunit genes, and include Leigh Syndrome, phaeochromocytoma, cervical paraganglioma, and familial paraganglioma (78). *C. elegans* possess homologues of all four of the complex II subunits: subunit a is encoded by *sdha-1* and *sdha-2* (and possibly F48E8.3.), subunit b by *sdhb-1*, subunit c by *sdhc-1/mev-1*, and subunit d by *sdhd-1*.

MEV-1 is the succinate dehydrogenase subunit c, or cytochrome b large subunit. It takes its name from a screen that identified the mutant, *mev-1(kn1)*, based on hypersensitivity to methyl viologen, also known as paraquat, a pro-oxidant (118,119). In addition to paraquat, these animals are also hypersensitive to increased oxygen concentration, much like animals treated with the complex II inhibitor, thenoyltrifluoroacetone (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 (94,95,105,118,119,120,121,122).

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 (96). 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 (96). In addition to reduced respiration, *mev-1(kn1)* animals exhibit a two-fold 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 (95,118,120).

Another complex II subunit that has been investigated in *C. elegans* is SDHB-1, which is homologous to the B subunit of succinate dehydrogenase. This protein has an iron-sulphur center that mediates ubiquinone reduction and is mutated in human patients with paraganglioma and pheochromocytoma (123,124). Several point mutants were designed by the Lemire group to mimic known mutations associated with paraganglioma and pheochromocytoma: human residues P197 and H132 correspond to *C. elegans* P211 and H146 in SDHB-1 (125). Some are likely inviable (H146P, P211Q, and P211N), while others are able to rescue the lethal *sdhb-1(gk165)* deletion. The viable point mutants (P211R, P211H, P211L, P211F) all have reduced succinate-dependent reductase activities, as measured by spectrophotometric assays, and two of the four (P211F and P211H) have reduced live-animal polarographic oxygen consumption rates. All four are short-lived and lay dead embryos; all of their lifespans are extended by treatment with ascorbate. They are also all hypersensitive to paraquat. Furthermore, three of the four are hypersensitive to hyperoxia, and this is rescued by ascorbate treatment and *N*-acetyl-L-cysteine. Furthermore, succinate-dependent generation of superoxide was increased in mitochondria of three mutant strains (P211R, P211L, P211F), as measured by oxidation of a dihydroethidium dye. These studies support the hypothesis that reduced activity of complex II can result in increased ROS generation, and that this is associated with short lifespan, much like *mev-1(kn1)*. Furthermore, these data shed light on human patients with P197 mutations, where an increase in ROS may underlie tumorigenesis, and treatment with antioxidants may be beneficial.

When Ishii and colleagues knocked down individual complex II subunits, they found that embryonic lethality resulted from three of the four (subunits b, c, and d) result, and the fourth (subunit a) results in a decrease in survival of *mev-1(kn1)* (126). Kuang and Ebert were able to grow all four knockdowns, and they observed that three of the four (subunits b, c, and d) result in decreased number of eggs laid and increased sensitivity to the uncoupler FCCP, while all four have decreased live-animal oxygen consumption rates and decreased steady-state ATP levels (127). Intriguingly, knocking down subunits b and d increased the expression of ICL-1 (in the glyoxylate cycle) and ACS-2 (in fatty acid beta-oxidation), potentially as compensatory mechanisms for the complex II defect. In summary, unlike any of the other MRC complexes, no complex II subunit loss-of-function mutation or RNAi has been observed to lengthen lifespan; disrupting complex II is almost always life-shortening.

3.3. Coenzyme Q: CLK-1 and COQ-3

Coenzyme Q (CoQ), also known as ubiquinone (oxidized) or ubiquinol (reduced), is a small-molecule isoprenoid that acts as carrier of electrons from complex I or II to complex III. It can also accept electrons derived from fatty acids or branched-chain amino acids. Defects in the biosynthesis of CoQ can cause severe MRC blockade. Human CoQ_{10} deficiency can present in infants as encephalomyopathies, nephrosis, ataxic syndromes, and isolated myopathy (128). In *C. elegans*, CoQ_9 synthesis requires the *clk-1* gene and, by homology prediction, the *coq-3* gene. *clk-1* was named because its loss of function results in a biological clock abnormal phenotype (129,130). Great interest in *clk-1* was sparked when it was observed to extend lifespan (129,130). When the gene was cloned, it was surprising to find that a loss in something as essential as CoQ would extend life.

clk-1 animals have slow embryonic and postembryonic development, reduced fecundity, and

have neuromuscular defects of decreased pharynx pumping, defecation, and locomotion (130), which are rescued when animals are supplemented with a soluble form of CoQ_{10} (131). Interestingly, these slow phenotypes were also present in wild-type animals fed a metal chelator, which may be due to chelating iron required by CLK-1 (132). Similarly, *coq-3(qm188)* animals with heterozygous mothers are slow to develop and have lower fecundity, while those with homozygous mutant mothers have developmental arrest in the first larval stage and die thereafter (133). As would be expected, *clk-1(qm30), clk-1(qm51),* and *clk-1(e2519)* mutants lack detectable CoQ_9 and accumulate the precursor demethoxy-Q_9 (DMQ_9) and the related rhodoquinone (RQ_9) (134). As possible compensatory mechanisms, *clk-1(qm30)* has increased mtDNA levels and induces ICL-1 of the glyoxylate pathway (135,136). In fact, the transcriptional profile of *clk-1(qm30)* is strikingly different than wild type, and reversing these transcriptional changes through RNAi of the regulator, *fstr-1/2*, partially rescues the slow development, locomotion, pharynx pumping, and lifespan effects without altering CoQ_9 or DMQ_9 levels (136). *clk-1(e2519)* and *clk-1(qm30)* have a dramatically decreased complex I-dependent respiration (85,96). This is expected, since CoQ is required for complex I to transfer electrons to complex III. Interestingly, *clk-1(e2519)* and *clk-1(qm30)* are hypersensitive to halothane, much like *gas-1(fc21)*, possibly due to this decrease in complex I activity (96).

However, the true mechanism behind the phenotypes in *clk-1* is complex for several reasons. More than one pathway can be inhibited in *clk-1* mutants, as CLK-1 has another function in binding mtDNA, which may regulate its replication and/or transcription (137), and CoQ has antioxidant functions outside of the MRC, and possibly outside of the mitochondrion (138). Consistent with this, overexpression of *clk-1* in wild-type animals causes shortening of lifespan, which cannot be ascribed solely to a possible increase in CoQ in the MRC (139). Furthermore, the DMQ_9 and RQ_9 that accumulate in place of CoQ could potentially act as a substitute for CoQ in some cases, or have other unique effects on the cell, as could the flux through the glyoxylate pathway (61,134,135,140,141). Another confounding aspect of studying CoQ deficiency in *C. elegans* is that they take up CoQ_8 from their bacterial food, especially during larval stages (140).

It is not surprising, in retrospect, that the true phenotype of *clk-1* was unmasked when the mutant *C. elegans* were fed bacteria that lack CoQ_8: these animals arrested at the second larval stage, much like *nuo-1(ua1)* (61), while wild-type animals fed this bacterial strain are slightly longer-lived (142). It should be noted that this bacterial strain may have changes to other metabolite levels in response to its own respiration defects, and thus when *C. elegans* feed upon it, more than the CoQ may be changed in the diet. Interestingly, *coq-3(qm188)* animals display the same phenotype regardless of dietary CoQ (133). Since COQ-3 is upstream in the CoQ biosynthesis pathway, it is possible that *coq-3(qm188)* accumulate a different set of precursors, accounting for the differences in diet-dependent phenotype versus *clk-1*.

Counter-intuitively, *clk-1(e2519)* has no change to complex II-dependent respiration (96). This surprising difference between complex I and complex II in their response to *clk-1* mutation is also present when the bacterial food are engineered to produce CoQ with chain lengths of 6, 7, 8, 9, or 10 (143). One possible interpretation is that dietary CoQ cannot be accessed by complex I, and is preferentially utilized by complex II. Assuming this is true, it is still not clear how endogenous CoQ can be utilized by complex I when dietary CoQ cannot, but it may be related to extremely large size of the I:III:IV supercomplex or the nature of CoQ channeling. Perhaps the supercomplex sequesters a separate CoQ pool that somehow is obligated to be endogenously made, perhaps through direct deposition by the CoQ synthesizing machinery. Another explanation is that the DMQ_9 precursor, which accumulates in the *clk-1* mutant, can substitute for CoQ in complex II but not in complex I-dependent respiration. This would imply that the change in chemical structure between DMQ_9 and CoQ_9 can be uniquely discriminated by complex I and/or supercomplex I:III:IV, possibly because a greater number of contacts are made between the coenzyme and this large multi-subunit complex. To distinguish between these hypotheses, *clk-1* mutants could be grown on food lacking CoQ_8, which should decrease complex II-dependent respiration if the first hypothesis is true. Another way to discriminate between these hypotheses is by examining *coq-3*, which should have decreased complex II-dependent respiration if the second hypothesis is true. It may also be interesting to determine whether wild-type animals on a diet lacking CoQ_8 have unaffected complex I-dependent respiration (since endogenous CoQ is present) but decreased complex II-dependent respiration

(since dietary CoQ_8 is lacking, and no DMQ_9 is accumulated). A further surprise is that although complex I-dependent respiration, but not complex II-dependent respiration, is defective in both *gas-1(fc21)* and *clk-1(e2519)*, the double mutant is sterile, with an extended lifespan (96).

From experiments exploring the effects of different CoQ repeat lengths on respiration and ROS, it is interesting to note that the lifespan is more closely related to the ROS-induced damage than to the respiration levels (143). This further emphasizes the role of ROS-induced damage in determining the lifespan of MRC mutants. In contrast to *gas-1(fc1)* and *mev-1(kn1)*, *clk-1* mutants have decreased levels of oxidative damage to proteins, are resistant to UV-induced stress, and are resistant to the anti-mitotic hemiasterlin (96,144,145), although submitochondrial particles from *clk-1(qm30)* have higher levels of hydrogen peroxide generation than wild-type (143). One potential explanation for this difference in oxidative stress and stress tolerance between *gas-1* or *mev-1,* and *clk-1* could be because of the nature of the disruption to the MRC: perhaps loss of CoQ results in lower MRC activity without decreased efficiency (96). An alternate explanation is that they accumulate DMQ_9, which can serve as an antioxidant to counteract ROS production and protect against stress-induced damage. Since several pieces of evidence from the *clk-1* mutant model imply a potential benefit of DMQ_9, this may be a beneficial supplement in patients with CoQ deficiencies.

3.4. Complex III: ISP-1 and CTB-1

Complex III is the CoQ-cytochrome c oxidoreductase, also known as the cytochrome bc_1 complex. Within complex III, the iron-sulphur 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 can cause hepatopathy, septo-optic dysplasia, and multisystem disorder (146,147,148), and defects in complex III assembly are associated with several diseases. One of these is a devastating infantile syndrome GRACILE, named for the symptoms of growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death (149). Other diseases of complex III assembly include less severe infantile encephalomyopathies (150) and, in adults, Bjornstad syndrome (151). In *C. elegans*, homologues of at least 8 of the 11 human genes encoding complex III subunits have been identified (59).

Three well-characterized complex III subunits are encoded by two nuclear genes, *isp-1* and *cyc-1*, and the mtDNA gene *ctb-1*; they take their names from their mammalian homologues, Rieske iron sulphur protein, cytochrome c_1, and cytochrome b, respectively. *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 (152). *isp-1(qm150)* is a perfect example of a “slow” phenotype. They have slower embryonic and postembryonic development, decreased defecation, reduced number of eggs laid, and lower fecundity. *isp-1(qm150)* mutant animals are 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, also extend lifespan (115), and for *cyc-1* RNAi, the lifespan extension is suppressed by inactivating the glyoxylate pathway through *icl-1(ok531)* (136). *isp-1(qm150)* induce ICL-1 (135), induce the superoxide dismutase, SOD-3 (152), and have increased mtDNA levels (136) as possible compensatory mechanisms. Despite the higher SOD activity, *isp-1(qm150)* have increased superoxide and unchanged overall ROS levels. Manipulation of the superoxide levels using antioxidants (vitamin C or N-acetyl-L-cysteine) and an oxidant (paraquat) also influenced lifespan (115). Furthermore, *isp-1(qm150)* are resistant to the anti-mitotic, hemiasterlin (145), suggesting general stress-tolerance.

As expected, *isp-1(qm150)* has a dramatically defective complex III function. In addition, the I:III:IV supercomplex is severely disrupted. This suggests that the ISP-1 subunit of complex III could act as a direct or indirect stabilizer of the supercomplex through protein-protein interactions or protein conformational changes. Complex I activity, as well as complex I-III activity, is also dramatically impaired in *isp-1(qm150)*, most likely as a direct consequence of disrupted supercomplex-mediated CoQ channeling. 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 a profound decrease to complex III and an indirect decrease to complex I activity, the latter due to supercomplex disruption (153). However, it is interesting to note that *isp-1(qm150)* animals are not hypersensitive to halothane, despite their loss of complex I activity (85).

Serendipitously, *ctb-1* was identified as a spontaneous suppressor of the slow rate of development in *isp-1(qm150)*. *ctb-1(qm189)* suppresses the slow phenotypes but not the long lifespan of *isp-1(qm150)* (152). A detailed biochemical analysis was undertaken to uncover the molecular mechanisms behind the phenotypes of these mutations (153). On its own, *ctb-1(qm189)* has decreased complex III and complex I-III activity, albeit not as severe as *isp-1(qm150)*. 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 (around 30% in *ctb-1(qm189)* versus 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. *ctb-1(qm189)* rescued the supercomplex instability and complex I defect of *isp-1(qm150)*, as well as the developmental and neuromuscular defects. At the same time, *ctb-1(qm189)* did not rescue the complex III defect, and no reversal was observed in the lifespan extension phenotype of *isp-1(qm150)* (153). This suggests that the lifespan is controlled by complex III, and we can assign the "slow" phenotypes to the indirect affect on complex I. These data are consistent with the findings using direct disruption of complex I, via *gas-1(fc21)*, which causes "slow" phenotypes and 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)*.

The acceptor of electrons from complex III is cytochrome c. Cytochrome c is a single water-soluble protein that resides in the intermembrane space and shuttles electrons between complex III and complex IV. In addition to its function in the MRC, cytochrome c functions in programmed cell death, where its release from the mitochondrion during direct activation of apoptosis triggers the caspase cascade. While decreased apoptosis is broadly associated with cancer, increased cytochrome c levels, translocation of cytochrome c to the cytosol, and its accumulation in protein aggregates have been implicated in neurodegenerative disorders (154,155). In *C. elegans*, cytochrome c is encoded by two genes, *cyc-2.1.* and *cyc-2.2.* It is worth noting that although these genes have not yet been thoroughly investigated, the *C. elegans* cytochromes c have similar biochemical and biophysical properties as their mammalian homologues (156). While cytochrome c is being explored as a drug target to induce apoptosis in cancer, or reduce apoptosis in neurodegenerative disease, animal models such as *C. elegans* may prove useful in evaluating the effects of these compounds on the MRC (157).

3.5. Complex IV

Complex IV, also known as cytochrome c oxidase or COX, terminates the flow of electrons through the MRC by 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 (158). Mitochondrial disorders such as Leigh Syndrome are associated with mutations in at least one subunit of complex IV (159). Additionally, at least 7 genes involved in complex IV assembly are mutated in humans with Leigh Syndrome, keto-acidotic coma, hepatopathy, hypertrophic cardiomyopathy, tubulopathy, and leukodystrophy (39,78). In *C. elegans*, homologues of at least 11 of the 16 human genes, encoding complex IV subunits, have been identified (59). Several of these, including homologues 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 (68,115,160). Numerous classical mutant strains exist for complex IV subunits, and it will be interesting to discover whether the observations made with RNAi are phenocopied by loss-of-function mutations.

RNAi against COXIV, COXVB/*cco*-1, and COXVA subunits decreases fecundity and slows development (68,160), while RNAi against COXVIIC causes arrest at the second larval stage (160). Stress resistance was variable 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 thermotolerance in high heat (160). RNAi against COXVB/*cco-1*, COXIV, COXVA, and COXVIIC all extend lifespan (115,160), 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 (68).

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 hyperfused mitochondrial morphology (160). Interestingly, RNAi against COXVB/*cco-1* causes lipid depletion, as does treatment of *C. elegans* with the complex IV inhibitor, sodium azide, and this may be related to human lipodystrophy caused by mitochondrial side-effects of pharmaceuticals (161).

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. This is likely because the I:III:IV supercomplex levels are diminished, as evidenced by native gels (68). Thus, although the levels of complex I are unchanged, the complex I-dependent activities are reduced due to a lack of CoQ channeling. Despite this decrease in complex I activity, however, RNAi against COXIV and COXVA does not change anesthetic sensitivity, suggesting that the level of complex I, and not its activity, is responsible for the anesthetic response. These data from *C. elegans* also suggest that human patients that present with reduced activities in complex I and IV may, in fact, have a single genetic defect in complex IV (68).

3.6. Complex V: ATP-2

Finally, 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. Defects in at least two proteins in complex V assembly are associated with mitochondrial disorders, including a mutation that is found at high incidence in the Roma population. 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 (162,163). Complex V function may also be indirectly disrupted by mutations in other complexes via the unique ROS sensitivity of certain complex V subunits (164). In *C. elegans*, homologues for at least 14 of the 16 human genes, encoding complex V subunits, have been identified (59). ATP-2 in *C. elegans* is homologous to the human ATP5B. The loss-of-function mutation of *atp-2(ua2)* causes developmental arrest at the third larval stage followed by long life, in homozygous mutant animals with a heterozygous mother. These mutant animals also have neuromuscular defects of decreased pharynx pumping and defecation and impaired locomotion (110). Mosaic analysis suggests that it is loss of *atp-2* in muscle that is the major contributor to the developmental arrest observed, likely because muscle is energy-demanding (165). RNAi knockdown of *atp-3*, the *C. elegans* homologue of human ATP5O, predictably decreases ATP levels and, like *atp-2(ua2),* lifespan is extended (115). Further experiments using RNAi against *atp-2* suggest a role for complex V, apart from its role in ATP production, in mediating polycystin signaling, the disruption of which causes polycystic kidney disease (166).

3.7. Indirect modifiers of the mitochondrial respiratory chain: LRS-2, POLG-1, and SOD-2

The mitochondrion is the site of a variety of metabolic and signaling pathways that are disrupted in numerous diseases. A full list of all of these genes and their *C. elegans* homologues is not within the scope of this review. In this section, we discuss select examples that indirectly affect the MRC through mitochondrial tRNA synthesis, mtDNA replication, and superoxide scavenging. There are 19 nuclear-encoded mitochondrial tRNA synthetases that are required for the translation of mtDNA-encoded genes, all of which function in the MRC. Mutation of at least 8 mitochondrial tRNA synthetases causes nervous-system dysfunction, cardiomyopathy, exercise intolerance, anemia, and kidney failure (167). HARS2 and LARS2, the mitochondrial histidyl- and leucyl-tRNA synthetases, can be mutated to cause Perrault syndrome. This syndrome, which can also be caused by mutations in the mitochondrial protease CLPP or peroxisomal HSD17B4, is characterized by premature ovarian failure and deafness. A loss-of-function mutation in the *C. elegans* homologue of LARS2, *lrs-2(mg312)*, causes complete sterility. These animals fail to produce oocytes, indicating germline arrest, and are therefore an excellent model for ovarian failure in Perrault syndrome (168). *lrs-2(mg312)* animals have reduced mitochondrial function, with disorganized and swollen mitochondrial morphology. They are small, slow-growing, and longer-lived (160). Although no change was detected to ROS levels, *lrs-2(mg312)* are hypersensitive to paraquat, which is similar to *gas-1(fc21)* and *mev-1(kn1)* but opposite to *clk-1.*

In addition to the mtDNA mutations mentioned previously, the copy number of mtDNA in each mitochondrion can be altered in disease, particularly in mtDNA depletion syndrome.

Regulating the copy number of mtDNA involves both the efficient, faithful replication of the mtDNA and the segregation of the mtDNA circles when the mitochondrion undergoes fission. *C. elegans* is an ideal organism to study the replication, transmission, and heteroplasmy of mtDNA during development and across different tissues (59). In *C. elegans*, the copy number per animal remains relatively constant early in development, when most somatic cell divisions occur. However, the copy number increases 5-fold as the animals enter the fourth larval stage, and 6-fold at the start of adulthood during oogenesis. Further studies with *C. elegans* have revealed that mtDNA copy number is regulated by germline development and reproductive function: mutants that lack oogenesis do not greatly increase their mtDNA copy number at the start of adulthood (170,169). Although mammalian oogenesis occurs in the fetus and not in the fertile adult, much like *C. elegans*, mammalian mtDNA is replicated to produce mature oocytes with a high mtDNA copy number (171).

DNA polymerase gamma is responsible for the replication of the mtDNA in humans and *C. elegans.* Polymerase gamma is mutated in a variety of human diseases including progressive external opthalmoplegia, infantile hepatocerebral syndromes, ataxia-neuropathy syndromes, male infertility, and testicular cancer (172,173). Blocking mtDNA replication with a loss-of-function mutation in the *C. elegans* DNA polymerase gamma, *polg-1(ok1548)* causes, in homozygous mutants with heterozygous parents, unchanged levels of mtDNA up to the third larval stage but increasingly lower mtDNA levels during adulthood, with concurrent changes to mitochondrial morphology. These adults are sterile, producing only a few arrested embryos, and are short-lived (170). This mutant may be used as a model to further understand mtDNA depletion syndrome and, theoretically, to search for therapeutic targets.

C. elegans is also a good system to screen for novel factors involved in mtDNA copy number. This can be accomplished by low-dose ethidium bromide that sensitizes the animals to changes in mtDNA copy number. Nine genes known to interact with mtDNA directly or indirectly in humans have *C. elegans* homologues whose knockdown by RNAi increases larval arrest and decreases mtDNA copy number in the sensitized animals. These genes of interest are *polg-1* (described above), *mtss-1*, *hmg-5*, Y105E8A.23, *dnj-10*, C47E12.2., *ant-1.4.*, *atad-3*, and *phi-37* (174). Several of the human homologues of these are implicated in disease. It will be interesting to employ this strategy for an unbiased genome-wide screen to identify novel factors that regulate mtDNA copy number, which may also be novel human disease genes. mtDNA copy number may also be regulated in response to insult, for example when *C. elegans* are exposed to a dose of ultraviolet C radiation where mtDNA mutations accumulate but nuclear DNA mutations are repaired. These animals have decreased ATP levels and respiration rates, likely as a direct consequence of reduced mtDNA-encoded MRC components. In order to compensate, *polg-1* expression is induced, and mtDNA copy number rises (175). This system may potentially be employed to evaluate therapeutic treatments that could similarly increase mtDNA copy number.

Another way that MRC activity can indirectly be influenced is through damage by ROS. Although, to our knowledge, no patient has been identified with a defect in ROS scavenging as the primary cause of mitochondrial dysfunction, a simultaneous defect in the MRC and in ROS scavenging has been found to correlate with worsened disease (107,176). One class of enzymes that are involved in ROS scavenging is the superoxide dismutases (SODs), which convert toxic superoxide to less reactive hydrogen peroxide. *C. elegans* have 5 different genes encoding SODs, of which two are mitochondrial: SOD-2 and SOD-3. Although *sod-2(gk257)* and *sod-3(tm760)* have no change in lifespan under standard conditions (94), and neither does the RNAi (177), or the double mutant, they can influence the lifespan of sensitized animals with MRC defects. *sod-2(gk257)* extends the lifespan of *gas-1(fc21)* (94) and *sod-2* RNAi and *sod-2(ok1030)* extend the lifespan of *clk-1(qm30)* but not *isp-1(qm150)* (177,178). *sod-2(gk257)* and *sod-2(ok1030)* have a decrease in fecundity, *sod-2(ok1030)* has slow development and slow defecation, *sod-2(gk257)* and *sod-2* RNAi has an increase in oxidative damage to proteins, *sod-2* RNAi has paraquat hypersensitivity, and *sod-2(ok1030)* has no change to osmotic stress or thermotolerance (94,177,178).

While *sod-3(tm760)* has no significant change to respiration or electron transport, *sod-2(gk257)* has a decrease in complex I-dependent respiration, complex I NADH-decylubiquinone oxidoreductase activity, and NADH-ferricyanide reductase (NFR) activity, and no significant change to complex II-dependent respiration or complex II redox activity. The MRC dysfunction in *sod-2(gk257)* is likely also due to a 30% loss of supercomplex

I:III:IV and I:III stability. In fact, SOD-2 directly binds to the I:III:IV supercomplex, possibly serving a stabilizing function, as evidenced by native immunoblot (94). This novel function of a SOD was discovered in *C. elegans*, and may open new avenues for understanding MRC function, disease, and therapy.

Additional interesting stories not covered here include *C. elegans* homologues of mitochondrial disease genes OPA1 and DRP1 in mitochondrial fission-fusion dynamics (179,180,181,182,183), frataxin and ABCB7 in iron-sulphur cluster biology (184,185,186,187,188,189), mitofilin in mitochondrial christae morphology (190), LRPPRC in mitochondrial RNA metabolism (191), mtSSB in mtDNA maintenance (192), GRO-1 iso-pentenylpyrophosphate:tRNA transferase (193), carnitine-acylcarnitine translocase (194), ANT in ADP/ATP exchange (195), TPL in thiamine pyrophosphate synthesis (196), and uncoupling proteins (197).

4. MITOCHONDRIAL DEFECTS IN DEVELOPMENT AND LIFESPAN

As noted in the previous sections, development in both humans and *C. elegans* is often affected by an MRC deficit. Blocking mtDNA replication in *C. elegans* with ethidium bromide, blocking mitochondrial translation with antibiotics, or inducing mtDNA damage using ultraviolet C radiation causes arrest at the third larval stage (110,169,183). Intriguingly, the ethidium bromide-arrested animals were long-lived and the arrest was reversed upon removal of ethidium bromide. The developmental arrest was also similar to that observed in *nuo-1(ua1), atp-2(ua2),* and *clk-1* (on a diet lacking CoQ); in the case of *atp-2(ua2)*, this relies predominantly on presence of ATP-2 in the muscle. These arrests occur before the third and fourth larval stages, when oxygen consumption has been measured to increase in wild-type animals, with a concurrent decrease in glyoxylate cycle activity (69,198). This is followed by increased energy demand during reproduction, and increased mitochondrial proliferation to allow sufficient mitochondrial content in each oocyte. The link between gonad development and mitochondrial function is also consistent with observations in *C. elegans* regarding the synthesis of cardiolipin, a phospholipid of the inner mitochondrial membrane, which is important to several mitochondrial functions. Deletion mutants *crls-1(tm2542)* and *pgs-1(tm2211)*, which are defective in cardiolipin synthesis, are sterile. Although somatic cells appear unaffected as is locomotion, the mutants have impaired cell proliferation, abnormal mitochondrial morphology, and lower mitochondrial membrane potential in the germ cells of the gonad (67).

Many of the genes involved in the mitochondrion are also involved in determining the lifespan of the animal. Repeatedly, under a variety of experimental designs, large-scale screens for lifespan alteration have strikingly yielded MRC subunits and other mitochondrial components, and often with their loss-of-function correlating with lifespan extension (115,160). By one such measurement, genes with mitochondrial function account for 15% of longevity genes, which is higher than any other group (152). It should be noted that *C. elegans* are sensitive to their environment, and therefore the exact lifespan measurements can depend upon the temperature, food, and other experimental factors, which are reviewed elsewhere (199). Of particular note is the use of 5-fluoro-2'-deoxyuridine (FUdR) to sterilize the animals during the lifespan experiment, which can alter the lifespan of certain mutants (200). A general consensus in the field is that *gas-1(fc21), nuo-1(ua1* + A352V/T434M/A443F*), mev-1(kn1)*, *sdhb-1(gk165* + P211L/F/H*)*, and *polg-1(ok1548)* are short-lived, while *nuo-1(ua1)*, *nuo-6(qm200), clk-1(e2519), isp-1(qm150), atp-2(ua2)*, and *lrs-2(mg312)* are long-lived, with caveats detailed in the previous sections on each mutant.

Interestingly, 5-fluoro-2'-deoxyuridine (FUdR), which has no effect on lifespan in wild-type animals, extends the lifespan of *gas-1* mutant animals (200), while dramatically impairing egg production and larval development (201). This may be, at least in part, because the drug-treated animals have less demand for mitochondrial proliferation during gametogenesis. A similar effect may be at least part of the mechanism behind lifespan extension seen for mutants with decreased fecundity, such as *nuo-6(qm200)*, *clk-1(e2519)*, and *isp-1(qm150)*, although it does not explain short-lived mutants with similarly decreased fecundity, such as *gas-1(fc21)* and *sdhb-1(gk165* + P211L/F/H*)* Taken together, it is possible that late-larval development may be regulated by an energy-sensing mechanism, perhaps in the muscle and/or gonad, that halts entry into adulthood and gonad development if MRC activity does not meet the increased demand. Another potential effect of this checkpoint is that ATP production rates later in life may be based on the rates early in life, as suggested by an experiment in which *cco-1* RNAi was ceased

at entry into adulthood, but ATP production never recovered (115). One provocative hypothesis is that this developmental checkpoint could be controlled by the kinase mTOR, in which the loss-of-function mutation *let-363(h111)* causes developmental arrest at the third larval stage and early death in *C. elegans* (59,202).

It may be counterintuitive that a loss-of-function in something as important as the MRC could extend life. This conundrum has resulted in the debate of several models to explain the observed results. One explanation, termed the "rate of living" hypothesis, argues that the MRC mutants with delayed development and decreased physical activity deplete their life force slower than others, accounting for their longer life (203). Along these lines, it could be argued that any ATP-requiring processes that damage the cells would be slowed along with other cellular functions. This is consistent with "slow" long-lived mutants, such as *nuo-1(ua1)*, *nuo-6(qm200)*, *clk-1(e2519), isp-1(qm150), atp-2(ua2),* and *lrs-2(mg312)*. However, it does not explain "slow" short-lived mutants, such as *gas-1(fc21)*, *nuo-1* point mutants, *mev-1(kn1)*, *coq-3(qm188)*, nor the "slow" *isp-1(qm150);ctb-1(qm189)* and *sod-2(gk257)* with normal lifespan.

Along those same lines, the free radical theory of aging hypothesizes that decreased ROS production, and therefore less oxidative damage, allows a healthier, longer life (204). Given that the MRC is the main source of ROS, it follows that MRC mutants with decreased respiration could have decreased ROS and increased lifespan. It could also be argued that MRC mutants could also decrease respiration while increasing ROS, and this would decrease lifespan, and together these arguments are called the mitochondrial oxidative stress theory of aging. This is consistent with certain mutants such as *clk-1(e2519)*, where ROS is decreased and life is extended, and *gas-1(fc21)*, *mev-1(kn1)*, and *sdhb-1(gk165* + P211L/F/H*)*, where ROS is increased and life is shortened. However, it does not explain long-lived mutants with no change to ROS, such as *nuo-6(qm200)*, *isp-1(qm150)*, and *lrs-2(mg312)*.

Mitochondrial threshold effect theory, or "window of hope," is analogous to the concept of hormesis in pharmacology, and argues that for any single necessary function, while a complete disruption will be detrimental, a small decreased in function could be life-extending. Such small decreases in function can be achieved by mutating an accessory subunit of a complex, making a small point mutation, knocking down the protein levels only mildly via an inefficient RNAi, or by looking at the first generation where maternal effects mask the full disruption. Taking these theories one step further, the double threshold hypothesis states that different tissues may be able to tolerate a different level of MRC dysfunction, since different tissues have different energy supply and demand. This may explain why phenotypes of different tissues, such as fertility versus locomotion, can be uncoupled in some experiments. It is also consistent with the variety of tissue-specific symptoms seen in patients with MRC dysfunction (205). Mitochondrial threshold effect theory was investigated in an elegant study by targeting MRC components by RNAi in dilution series (73). A three-stage threshold effect is observed for RNAi of *atp-3*, *isp-1*, and *cco-1* in different dilutions and for RNAi of *frh-1* over different generations, and was confirmed in an independent study of RNAi of *isp-1* and *nuo-6* in different dilutions (72). However, lifespan kept on increasing for higher concentrations of *nuo-2* RNAi and kept on decreasing for higher concentrations of *mev-1* RNAi (73), and while *nuo-1(ua1)* is long-lived, less severe point mutants are short-lived. For the four *sdhb-1* point mutants, no one measure of MRC activity correlates with lifespan: P211L has no effect on live-animal oxygen consumption but has a similar lifespan shortening as the two that have decreased oxygen consumption (P211F and P211H). Also, the one that is not short-lived (P211R) has a similar decrease in reductase activity as for the short-lived point mutants (125).

In instances displaying threshold effect, induction of genes in compensatory pathways may alleviate a mild deficit but be insufficient to rescue a major deficit. There is substantial evidence for altered transcriptional profiles in MRC mutants, and the lifespan and "slow" phenotypes may depend on a subset of the activated transcription factors (206,207,208). In this manner, metabolic reprograming may alleviate MRC defects by activating alternate sources of energy metabolism such as the glyoxylate pathway in *clk-1(qm30)*, *isp-1(qm150)*, and *cyc-1* RNAi (135,136). Alternate metabolic pathways may be better than respiration at promoting health and longevity in *C. elegans*, but in some cases metabolic reprogramming can be uncoupled from longevity (207). Other survival pathways may be activated that protect the animal from stress, via the retrograde response model, which is consistent with long-lived mutants that display stress resistance. For example, long-lived

clk-1(e2519) and *nuo-6(qm200)* are also UV- and paraquat-resistant, respectively, and short-lived *nuo-1* point mutants are hyperoxia and paraquat hypersensitive. However, it does not explain long-lived mutants that are stress-sensitive, such as *lrs-2(mg312)*. As an offshoot to this, it has also been hypothesized that a slightly increased level of ROS, and in particular superoxide, could be the messenger that activates the cytoprotective pathways (115). This is consistent with long-lived mutants that have increased superoxide, but no change to overall ROS, such as *nuo-6(qm200)* and *isp-1(qm150)*.

In summary, no single phenotype is predictive of lifespan, and no single theory is consistent with all lifespan alterations observed. As with many debates in biology, it is possible that any number of these hypotheses are true, and which one is most relevant will depend on the particular gene, allele, and genetic background, as well as the conditions of the experiment. There are many theories and postulated pathways thought to regulate aging, such as in caloric restriction, insulin signaling, and mTOR signaling, which are reviewed elsewhere (209). This is further complicated by the fact that some components have more than one function: cytochrome c also functions in apoptosis, CoQ has redox functions outside of the MRC, and SOD-2 is not only a superoxide dismutase but also a direct stabilizer of the I:III:IV supercomplex. The complexity of the system underscores the utility of more simple model organisms such as *C. elegans*, and necessitates careful, direct measurements of the biochemical activities involved. This is an active area of exciting current research, and further layers of complexity are sure to be uncovered over the coming decade.

5. CONCLUSION

In this fascinating field of mitochondrial dysfunction, many unanswered questions remain. Experience with *isp-1(qm150);ctb-1(qm189)* demonstrates that identifying the factors that control supercomplex levels, such as SOD-2, will be an important piece of the puzzle. Understanding the molecular players that either help assemble or stabilize complexes could aid in developing future therapies that act by promoting supercomplex availability or decreasing ROS production in patients with mitochondrial defects. Even small improvements in ETC efficiency could translate into noticeable increases in patient health.

To help understand how ROS can simultaneously be damaging and beneficial, precise measurements of superoxide anion and other oxygen species, and not simply ROS in general, might prove enlightening. Perhaps some ROS are purely damaging, while others (potentially superoxide) have cytoprotective signaling roles. If we knew which species is primarily responsible for which function, then we could attempt to modulate them independently. The big black box in this hypothetical pathway is figuring out how ROS mediates the response: is it via a post-translational modification such as the HNE-fluorophore? Is it by a conformational change induced when an oxygen species binds a receptor? Our current understanding of other complex biological pathways, including insulin signaling and apoptosis, has greatly benefitted from research in *C. elegans*, and so this organism may play a role in mapping out the emerging pathways downstream of oxygen species signaling.

Insights from *C. elegans* mitochondrial mutants are rapidly building a more complete picture of the biology of aging. No single molecule, mechanism, phenotype, or theory seems to be able to account for all the observed results; this is evidence that there is a complex balance of a combination of factors, such as energy production versus consumption, ROS-mediated signaling versus ROS-mediated damage, and harmful stress versus inducible cytoprotective stress-response pathways, Translating life extension research to the clinic will require assessing the quality of life in the long-lived but stressed animals, such as by measurements of resistance to pathogens. It remains to be seen whether this work will be capable of benefitting children who are diagnosed with primary mitochondrial disorders with a short life expectancy, as the cause of death in patients, often by organ failure, may not necessarily be the type of death that the *C. elegans* long-lived mutants are able to avoid. Results from MRC mutant model organism have also yet to yield practical applications for treating premature aging diseases such as Hutchinson Gilford Progeria Syndrome and Werner Syndrome or diseases associated with aging such as Parkinson's disease and Alzheimer's disease.

Experiments with MRC mutant *C. elegans* have allowed us to evaluate the therapeutic potential of several dietary supplements and drug treatments. These include antioxidants such as vitamin C, vitamin E, and N-acetyl-L-cysteine, potential coenzyme Q

substitutes, and riboflavin. As this area of research unfolds, we are beginning to expand a palette of exciting new potential therapeutic strategies for improving the life expectancy and quality of life for patients with mitochondrial disorders. There is still room for *C. elegans* MRC mutant secondary screens for phenotype reversal. Genetic suppressor screens may help identify therapeutic targets for which inhibitor compounds or blocking antibodies could alleviate symptoms in patients with mitochondrial disorders. Pharmacologic suppressor screens, or chemical genetics, may help identify promising drug classes as well as targets. Some methods exist for high-throughput drug screening in *C. elegans*, but these have yet to be applied to primary mitochondrial disorders. It is clear that *C. elegans* models of disease will undoubtedly continue to provide a useful system for identifying and evaluating novel therapies in mitochondrial biomedicine.

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Abbreviations: CoQ: coenzyme Q; DMQ_9: demethoxy-Q_9; LOF: loss-of-function; MRC: mitochondrial respiratory chain; mtDNA: mitochondrial DNA; NADH: reduced nicotinamide adenine dinucleotide; NFR: NADH-ferricyanide reductase; RNAi: RNA interference; ROS: reactive oxygen species; SOD: superoxide dismutase

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