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Comparison of proteomic and metabolomic profiles of mutants of the mitochondrial respiratory chain in *Caenorhabditis elegans*

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Abstract

Single-gene mutations that disrupt mitochondrial respiratory chain function in *C. elegans* change patterns of protein expression and metabolites. Our goal was to develop useful molecular fingerprints employing adaptable techniques to recognize mitochondrial defects in the electron transport chain. We analyzed mutations affecting complex I, complex II, or ubiquinone synthesis and discovered overarching patterns in the response of *C. elegans* to mitochondrial dysfunction across all of the mutations studied. These patterns are in KEGG pathways conserved from *C. elegans* to mammals, verifying that the nematode can serve as a model for mammalian disease. In addition, specific differences exist between mutants that may be useful in diagnosing specific mitochondrial diseases in patients.

Keywords

Mitochondria; genetics; *C. elegans*; proteomics; metabolomics; gene expression

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Introduction

Since the mitochondrion is crucial to many functions of the cell, consequences of mitochondrial dysfunction can be far-ranging, varied, and severe. In addition, this critical organelle is under dual genetic control, so that patterns of inheritance are very difficult to discern. Heteroplasmy, threshold effects, and compensatory metabolic changes only add to the difficulty of diagnosis. Model systems offer potentially useful methods to search for transcriptional and metabolic patterns that reveal specific mitochondrial defects [1]. Our goal is to identify proteomic and metabolomic fingerprints that characterize specific types of dysfunction in a model organism with well-characterized mitochondrial defects.

Although many other valuable approaches are being used to understand how mitochondria function, the use of molecular genetics in *C. elegans* possesses powerful and unique advantages. The excellent genetics, catalogue of known mitochondrial mutations, well-defined behaviors, and conservation of mitochondrial biology across the animal kingdom make the worm an excellent translational model for study of mitochondria. Using the nematode as a model, we aimed to find novel approaches to characterize mitochondrial defects that may also be useful in patients. *C. elegans* provides the ability to generate a virtually inexhaustible supply of isogenic nematodes that possess well-defined single defects of the mitochondrial respiratory chain (MRC), for which we know the exact nuclear- or mitochondrial-encoded mutation. These mutations affect complexes I, II, III, and coenzyme Q synthesis [2-4]. Each has been extensively characterized biochemically and at the whole-animal level. We have compared nematode mitochondrial mutants by gene set enrichment analysis [1]. Clear patterns of functional variation, *i.e.* mitochondrial fingerprints, emerged that are characteristic for specific mitochondrial defects. Previous studies from others have also documented a high degree of conservation in proteomes between mitochondria of *C. elegans* and mammals [5-7]. This similarity increases the likelihood that compensatory changes in response to genetic mutations will be similar between the nematode and mammals. In addition, a recent focused metabolomic study also found varied patterns in the metabolomes of different mitochondrial mutants [8].

In this report we expand upon prior knowledge of expression profiles caused by mutations in complex I (*gas-1*), complex II (*mev-1*), and a mutation affecting coenzyme Q synthesis (*clk-1*) by applying proteomics and metabolomics. We take on the challenges of a systems biology approach by comparing data sets at different functional levels (*i.e.* proteomics and metabolomics) and compare those to gene expression data previously reported. Our goal was to find easily identifiable (*i.e.* not requiring elaborate isolations procedures) differences between wildtype and mutant mitochondria and comparisons between mutants in different components of the mitochondrial respiratory chain. We find overarching similarities, as well as some potential predictive differences between the mutants in the molecular fingerprints caused by their specific mitochondrial defect. Perhaps more importantly, we find patterns maintained across the proteomic, metabolomic, and expression platforms. These patterns may represent hallmarks for diagnosis and understanding of mitochondrial disease in complex patient presentations.

Methods

Strains

Methods for growth of strains and mitochondrial function studies have been previously published. *C. elegans* wild type (N2) and mutants *mev-1(kn1)*, *icl-1(ok531)* and *clk-1(qm30)* were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). *gas-1(fc21)* was isolated in our laboratory [9, 10]. Double mutations were generated in our laboratory by standard genetic techniques [11]. All data are from synchronized cultures of young adults, generated by allowing hermaphrodite parents to lay eggs for 4 hours on plates spread with the bacteria OP50.

gas-1(fc21) is a missense mutation in the gene encoding an orthologue of NDUFS2 (4), the 49 kDa subunit of complex I of the MRC. It is thought to be part of the binding site of complex I to coenzyme Q (6). *mev-1(kn1)* is a missense mutation in a gene that encodes the orthologue of SDHC, a subunit of complex II (9). *clk-1(qm30)* is a null (deletion) allele in a gene whose product, COQ7, catalyzes the penultimate step of coenzyme Q synthesis (11). *icl-1(ok531)* (formerly known as *gei-7*) is a null (deletion) allele which encodes the predicted enzyme isocitrate lyase/maltate synthase, an enzyme known to function in the glyoxylate cycle.

Mitochondrial Isolation

The methods for isolating mitochondria from *C. elegans* have been previously published [3, 12]. Four independent wildtype (N2) and *gas-1* samples, and three each from *mev-1* and *clk-1* were used to generate mitochondrial preparations for proteomic studies. Mitochondria were isolated twice from *icl-1* to measure rates of oxidative phosphorylation.

Metabolomic Characterization

All mutants were evaluated by metabolic profiling of the whole animal, using methods similar to those previously described for mammalian tissue extracts [13, 14]. All cultures were synchronized by growth from eggs layed in a four-hour period at 20°C and grown on 100mm agar plates seeded with OP50 *E. coli*. Each culture was harvested on the first day of adulthood when eggs were first identified. This corresponded to day 3 for N2 *clk-1*, and *mev-1*; day 4 for *gas-1*. Worms were quickly washed in cold M9 buffer on ice and flash frozen after 3 washes. Each sample consisted of 100-300 mcL sedimented worms.

Freshly prepared extracts were used for metabolic profiling. Proteins were first removed by precipitation with methanol (1200 µl) or acidified acetonitrile (750 µl ACN + 450 µl 1% formic acid). Samples were mixed well by vortex, spun down, and supernatants were removed immediately for amino acid (AA), organic acid (OA), and acylcarnitine (AC) analysis. Measurement of AA and AC was done by direct-injection electrospray tandem mass spectrometry, using a Quattro Micro LC-MS system (Waters-Micromass) equipped with a model HTS-PAL autosampler (Leap Technologies), a model 1100 HPLC solvent delivery system (Agilent Technologies) and a data system running MassLynx software. For OA, analytes were dried and then converted to trimethyl silyl esters by *N,O*-bis(trimethylsilyl) trifluoroacetamide, with protection of α-keto groups by oximation with

ethoxyamine hydrochloride. OA were analyzed by capillary gas chromatography/mass spectrometry (GC/MS) using a TRACE DSQ instrument (Thermo Electron Corporation; Austin, TX). All MS analyses employed stable-isotope dilution with internal standards from Isotec (St. Louis, MO), Cambridge Isotope Laboratories (Andover, MA), and CDN Isotopes (Pointe-Claire, Quebec, Canada) as detailed previously. All samples were derived from 6-8 independent isolations from each strain.

Proteomic Characterization

Strains were grown as described for the metabolomic studies. Mitochondria were isolated as previously described [3, 12, 15]. Proteins were then isolated as previously published [7]. Prior to analysis by tandem mass spectrometry, samples were divided into 1-4 technical replicates, subjected to bead beating, and digested with trypsin (Promega, Madison WI) in the presence of .1% sodium deoxycholate (Sigma) or .1% PPS silent surfactant (Protein Discovery, Knoxville, TN). Following removal of undigested material by acetonitrile precipitation and brief exposure to a nitrogen dryer, samples were analyzed on an LTQ-Velos mass spectrometer (Thermo Fisher, San Jose CA) coupled to an Eksigent nanoLC 1D + (Eksigent, Dublin CA). Digested peptides were separated on a HALO C¹⁸ nanocolumn by reverse phase chromatography just prior to reaching the nanospray source. The standard experimental protocol has been detailed previously [13].

Data Analysis

Protein identification and quantitation was carried out using the SPIRE (Systematic Protein Identification and Relative Expression) analysis platform for high-throughput proteomics data analysis as described previously [16] and was used to search *Wormpep* (version 207) the *C. elegans* sequence database (www.wormbase.org) [21-23]. SPIRE uses the X!tandem search engine combined with a decoy database approach for estimating the error rates of both peptide spectrum matches and protein identifications. The database search was conducted using the following search parameters: fixed modification for carbamidomethylation of cystine, variable modification for oxidation of methionine, fully tryptic cleavage with no more than 2 missed cleavages, and a precursor monoisotopic mass error tolerance of 3.1 daltons. The error threshold for peptide spectrum matches was set to a 1% False Discovery Rate (FDR). Protein expression was measured by the total of identified spectral counts normalized by the total number of spectral counts in each MS run in order to adjust for the variation in signal across runs.

Proteins were mapped to KEGG pathways and mitochondrial respiratory complexes [16] and pathways were compared using a protein set analysis. For this evaluation, we used a cutoff of three members of a pathway as confidently identified (False Discovery Rate [FDR] < 0.01) before including the pathway in the analysis [17]. To decide if a particular pathway is differentially expressed, we employed two approaches: total expression, as measured by normalized spectral counts to explore pathway specific differential expression, and the max-mean statistics, which takes the maximum of the average of over-expressed and under-expressed proteins to compare differential expression relative to other pathways (<http://statweb.stanford.edu/~tibs/GSA/>). A randomization test of the biological replicate labels is used to calculate statistical significance while maintaining the correlation structure

between proteins. Pathways were ranked based on each method and sorted with respect of the combined ranking. For the total expression method an expression ratio (ER) is also calculated between to strains by taking a ratio of the total expression as measured by normalized spectral counts across all proteins in the set. Proteomics data will be available through MOPED, Model Organism Protein Expression Database (<https://www.proteinspire.org/MOPED/>) [18-20].

Results

Proteomic Characterization

At a false discovery rate (FDR) of 1%, a total of 630 different mitochondrial proteins were identified in N2, 525 in *clk-1*, 487 in *gas-1* and 423 in *mev-1* (Figure 1, Figure S1). 283 proteins were present in all four strains, while 296 proteins were only found in one strain (109 in N2, 147 in *clk-1*, 26 in *gas-1*, 14 in *mev-1*). The remaining proteins were found in 2 or 3 of the strains. 305 proteins mapped to 135 KEGG metabolic pathways or specific cellular functions. The complete list of proteins identified is given in Supplemental Table 1.

Individual Protein Expression—Several proteins were differentially expressed in the mutants compared to the wildtype N2. The most significant of these individual proteins are listed in the Supplemental Tables 2-4. Table 1 shows those proteins differentially expressed in 2-3 of the mutant strains. Of particular note were the increased expressions of ICL-1 (isocitrate lyase) and PYC-1 (pyruvate carboxylase) in all three mutants, compared to N2, consistent with compensatory changes to maintain energy production.

Pathway Expression—We evaluated the proteins expressed in the mutants by grouping them in functional protein sets as described previously. For this evaluation, we limited pathways to KEGG pathways/groups in which at least 3 members were identified. We identified 177 proteins that were mapped to 17 pathways or groups of proteins across the four strains. Several significant differences were seen in the spectral counts of subunits of complexes of the MRC between the different strains (Table 2). Complex I subunits were under-expressed in both *gas-1* and *mev-1* compared to N2. Proteins involved in the synthesis of ubiquinone were overexpressed both in *mev-1* and *gas-1* compared to N2. Complex III subunits were overexpressed in *gas-1* compared to N2.

When we ranked relative protein abundance for *gas-1* compared to N2 for KEGG pathways overrepresented in *gas-1*, we obtained the results presented in Table 3. Similar, though not identical, results were obtained by comparing protein abundance for either *mev-1* or *clk-1* to N2 (Tables 4 and 5). In all three comparisons, the glyoxylate pathway (In *C. elegans*, all functions of the glyoxylate pathway are accomplished by one protein, ICL-1, and thus the pathway is not listed in the tables) was increased in the mutants. Those pathways that were up-regulated in samples from each of the three mutants are highlighted in yellow as are those upregulated in our previously published GSEA studies [1]. Pathways upregulated in two mutants are highlighted in grey (*clk-1* and *mev-1*) or blue (*gas-1* and *mev-1*). No pathways were upregulated only in *clk-1* and *gas-1* but not in *mev-1*.

There were differences between the MRC mutants. Glycine, serine and threonine metabolism, butanoate metabolism, tryptophan metabolism and ketone metabolism were up-regulated in our *gas-1* GSEA results [1] and in the *gas-1* and *clk-1* PSEA results. In contrast, these pathways were not up-regulated in either the GSEA or protein sets analyzed from the complex II mutant *mev-1* compared to N2 [1].

Metabolomics Characterization

We measured the organic acids for N2, *gas-1*, *mev-1*, and *clk-1*. The most striking change was in *gas-1*, the complex I defect, which had no measurable succinate (Figure 2). In addition, there was a decrease in lactate in *gas-1* compared to N2 and a moderate decrease in malate in *mev-1* compared to N2.

We also measured the amino acids for the three mutants compared to N2 (Figure 3). The levels of several amino acids were decreased in the mutants. Glycine, proline, methionine, histidine, phenylalanine, tyrosine and glutamate/glutamine were significantly decreased in *gas-1* compared to N2. In *mev-1* valine, histidine and tyrosine were decreased relative to N2, and no significant differences were seen in *clk-1* compared to N2. We also measured acylcarnitine levels in the mutants but noted no significant differences between mutant and wildtype (Figure S2).

Genetics

Our results suggested that *gas-1* might require increased succinate production for survival. To test this we constructed the double mutant *icl-1;gas-1*. The gene *icl-1* encodes the single member of the glyoxylate pathway that can generate malate and succinate from isocitrate (and acetyl CoA) without producing NADH. As a single mutation, the mutant *icl-1* has no obvious phenotype and no alteration in mitochondrial oxidative phosphorylation (Data not shown). Loss of the glyoxylate pathway (which is upregulated in both the transcription [1] and protein analysis in *gas-1*), was lethal in a *gas-1* background. Deleting *icl-1* in either *mev-1* or *clk-1* did not produce any synthetic phenotypes compared to *mev-1* or *clk-1* alone. The effect of *pyc-1* differed from that of *icl-1*. Knockdown of *pyc-1* by RNAi was lethal to *clk-1* animals but had no discernable effect on the phenotypes of N2, *mev-1* or *gas-1*.

Gene products required for synthesis of ubiquinone were upregulated in both *gas-1* and *mev-1*. Inhibition of ubiquinone metabolism by *clk-1*, which by itself does not cause a strong phenotype, caused sterility (no viable eggs/offspring) when combined with *gas-1* or *mev-1*, confirming the importance of this compensation.

Discussion

The majority of the proteins identified from our samples were homologous to proteins in the mouse Mitocarta [20]. Approximately 35% of the mouse Mitocarta had clear orthologues in the *C. elegans* sample, though more have been found in previous extensive surveys in wildtype (N2) samples [5-7]. However, our approach was not intended to provide an exhaustive list of mitochondrial proteins in *C. elegans*, since that has been previously accomplished [7]. In this study we focused on a comparison between mutant and wildtype mitochondrial proteins to identify consistent changes between them that do not require

“deep” metabolomic and proteomic characterization. Such an approach may be useful in diagnosis of patients. The changes we have identified represent potential compensatory changes that may be necessary for the survival of the mitochondrial mutants. Two of these changes (increased expression of proteins responsible for ubiquinone synthesis and in the glyoxylate pathway) were suggested as essential for the survival of *gas-1*. The fact that decreases in two of these pathways (increased expression of proteins responsible for ubiquinone synthesis and in the glyoxylate pathway by construction of *clk-1;gas-1* and *icl-1;gas-1*, respectively) are synthetic lethals is consistent with the model that their upregulations are essential for the survival of *gas-1*. However, it is necessary to block their upregulation, rather than removing the protein, to unequivocally prove this model. In addition, the lethal effect of knocking down *pyc-1* in *clk-1* may also indicate a crucial compensatory response when both complex I and complex II-dependent respiration are theoretically at risk. PYC-1 is involved in gluconeogenesis under conditions of starvation and may play such a role in the mitochondrial mutants as well.

We then combined metabolic data with proteomic data to determine consistent patterns characterizing the compensatory changes in the mutants. In a general sense, our metabolic data agrees with the earlier studies of Falk *et al.* [1] though the differences between mutant and wildtype did not reach significance in as many instances in our studies as in the previous study. The differences between this study and the recent publication from the Falk laboratory are more striking and difficult to reconcile at this time [8].

The majority of our results are most consistent with metabolic alterations to rescue energy production in the face of defects in specific parts of the respiratory chain. The most striking differences in profiles were observed for *gas-1*, the complex I mutation. The absence of measurable succinate in these animals likely results from increased use of succinate to drive complex II dependent respiration, which we have shown is increased in *gas-1* [3]. This is also consistent with the previous finding that the double mutation, *mev-1;gas-1* is lethal [21]. Pfeiffer *et al.* showed that *ucp-4* acts as a succinate transporter into mitochondria [22]. The importance of succinate availability for survival of *gas-1* was further implicated by the fact that the double mutant *ucp-4;gas-1* is a synthetic lethal [22]. The increased representation of ICL-1 (formerly known as GEI-7) may indicate that the animals are also generating succinate by use of the glyoxylate pathway, which generates fewer NADH molecules than use of the TCA cycle (which, without complex I activity, are difficult to remove). The glyoxylate pathway allows production of succinate from isocitrate without NADH production. The lethality of the *icl-1;gas-1* double mutant is consistent with this model.

In *gas-1*, decreases in the levels of lactate and several amino acids may reflect their increased use to generate glucose (gluconeogenesis has previously been described in *C. elegans* [23, 24]) to enable increased glycolysis in subsets of tissues to help compensate for the loss of complex I activity in other tissues. The use of lactate or amino acids to drive the TCA cycle directly would likely be counterproductive since the NADH produced could not be removed effectively by the defective complex I. However, lactate may be used to generate acetyl-CoA, which is necessary for the glyoxylate pathway to generate malate and succinate.

It is interesting that gluconeogenesis/glycolysis and glyoxylate pathways were both upregulated in several long-lived *C. elegans* mutants [23, 24]; the present results show that such changes are not sufficient for increased lifespan. The increased presence of PYC1 in all three mutants may indicate that it is generating oxaloacetate indirectly for the glyoxylate pathway and, more directly, for gluconeogenesis (via PEPCK). Components of both pathways (gluconeogenesis and glyoxylate synthesis) are increased in all three mutants. Both pathways allow a mechanism for generating ATP without involvement of complex I and a mechanism for reducing the levels of lactate by metabolism of pyruvate.

Our amino acid results need to be interpreted with caution. A recent report from the Falk laboratory focused on metabolomics and found different results of the effects of *gas-1* and *mev-1* on amino acid levels [18]. In particular, they reported increased levels of alanine and decreased levels of glutamate in *gas-1* compared to N2. However, there was a large scatter in the values for these amino acid resulting in overlap of the values. Our results may reflect that overlap. In addition, the phenotype of *gas-1* is exquisitely dependent on the growing temperature around 20°C; slight differences in temperature may also explain the differences in amino acids between the two studies.

It remains curious, however, that these changes lead to the decreases in lactate and succinate in *gas-1* but not in *clk-1* or *mev-1*. This may indicate a unique role of complex I in these responses. In mammals, defects in the MRC are generally associated with increases in lactate, likely secondary to limitations in oxidation of pyruvate in the citric acid cycle. In contrast, in *C. elegans* the presence of the glyoxylate pathway offers an alternative method to remove pyruvate in addition to lactate dehydrogenase. While this may seem to be a curiosity of nematodes, creating an escape mechanism to generate succinate for complex II function without increasing NADH levels may represent a potential therapy for rescue of mitochondrial function when complex I is defective.

It is important to note that the recent metabolic study of similar mitochondrial mutants (*gas-1*, *mev-1* and *clk-1*) did not find a decrease in lactate levels in *gas-1* [18]. It is not clear what underlies the differences between the two studies, though isolation procedures were somewhat different. Schrier-Vergano *et al.* did a more detailed study of organic acids and amino acids studying flux as well as absolute levels. However, the differences in results for succinate and lactate are not easily reconciled. As discussed in the previous paragraph, subtle differences in growing temperature may also affect these results. In either case, there do appear to be differences in metabolic patterns between these mutants and wildtype, though the nature of the patterns remains to be unequivocally determined. There are also multiple other excellent metabolomic studies in *C. elegans* of the roles of mitochondrial mutants in aging [32, 34-36]. However, these are difficult to compare to the present study due to different groups of mutants and differing phenotypes from those in our study.

The specific changes in organic acids and amino acids may aid in diagnosis of specific mitochondrial diseases of the MRC in humans. Our data indicate that defects in different parts of the MRC induce distinct responses in metabolic pathways that serve as a coordinated compensatory response to the particular lesion. However, the changes in pathways that may translate to mitochondria of higher organisms will need to be confirmed.

In general, the changes seen in our studies for all three MRC mutants are consistent with metabolic adjustments to maintain energy production in spite of a defective MRC. In addition, the upregulation of ubiquinone synthesis in both *gas-1* and *mev-1* may indicate that ubiquinone plays an important role in transducing these changes. The ability to create genetic defects in multiple aspects of mitochondrial function in *C. elegans* represents a powerful mechanism to use different data platforms to compare compensatory mechanisms resulting from varied mitochondrial defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Metabolic compensation in MRC mutants shows adjustments to maintain energy production.
- Differences in patterns of compensation allows comparison of the effects of different MRC changes.
- Upregulation of ubiquinone synthesis plays an important role in MRC compensation.
- Limiting NADH production may be important in compensation for complex I dysfunction.

Intersection Diagram

Figure 1. Total number of proteins identified in each strain

Indicated in each box is the number of proteins in that strain identified at a false discovery rate of 1%. The top row represents the proteins found only in single mutants (*clk-1*, *gas-1*, *mev-1*) and the wild type, N2. The second row represents the number of proteins found in two strains (each listed separately), the third row proteins found in three strains (listed separately) and the fourth row the number of proteins found in all four strains. 283 proteins were present in all four strains, while 296 proteins were only found in one strain (109 in N2, 147 in *clk-1*, 26 in *gas-1*, 14 in *mev-1*). The remaining proteins were found in 2 or 3 of the strains.

Figure 2. Organic acid levels in each strain

The mean values of seven organic acids found in each of the strains. Error bars are standard deviations. Lactate is shown separately due to its relatively high levels compared to the other organic acids. Note the extremely low amounts of succinate and lactate in *gas-1*. Error bars are standard deviations. Significance defined as $p < 0.05$ with a Bonferroni correction for $n=4$ ($0.05/3 = 0.017$; we used 3 for the Bonferroni correction since there were 3 degrees of freedom with 4 strains studied.). N2 refers to the wildtype strain. *fc21* refers to *gas-1(fc21)*; *kn-1* refers to *mev-1(kn-1)*; *qm30* refers to *clk-1(qm30)*. All samples were derived from 6-8 independent isolations from each strain.

Figure 3. Amino acid levels in each strain

The mean values of 18 amino acids found in each of the strains. Error bars are standard deviations. Three groups include two amino acids unable to be identified separately. Significance defined as $p < 0.05$ with a Bonferroni correction for $n=4$ ($0.05/3 = 0.017$). Values for alanine were too high to be clearly represented on the same graph as the other amino acids; thus a discontinuous graph is presented with a change in scale. All samples were derived from 6-8 independent isolations from each strain.

Table 1
Individual proteins differentially expressed in multiple mutants compared to N2

The raw data for these groupings is found in Supplemental Table 1 and is from the proteomic analysis. “+” indicates that expression was increased significantly compared to N2; “-” indicates that expression was decreased compared to N2. No genes were strongly differentially found only in *gas-1* and *clk-1*, but not in *mev-1*. Two different eukaryotic elongation factors (EEF) orthologs were upregulated in *gas-1* and *clk-1* and are listed separately by that function. Two proteins (VIT-2 and VIT-6) not usually considered to be mitochondrial were underrepresented in *gas-1* and *mev-1* compared to N2. These may represent contamination but are listed for completeness.

Increased				
Gene	Annotation	<i>gas-1</i>	<i>mev-1</i>	<i>clk-1</i>
C05E4.9a	ICL-1, isocitrate lyase	+	+	+
D2023.2	PYC-1, pyruvate carboxylase (1)	+	+	+
K03H1.4	TTR-2, transthyretin- like (JC8.8 in <i>clk-1</i>)	+	+	(+)
F40F4.6	EGF-like repeats, Notch-like, unknown function		+	+
F54F11.2a	Zinc-binding metalloprotease (2)		+ up	- down
T25C12.3	EGF-like repeats, nematode specific		+	+
C44B7.10	Acetyl-CoA hydrolase/transferase	+	+	
C17C3.1e	Acyl-coenzyme A thioesterase (3)	+	+	
F18E3.7e	DDO-2, D-amino acid oxidase (4)	+	+	
Y25C1A.13	dienoyl-CoA isomerase, mitochondrial	+	+	
T05H4.12	ATP-4 (subunit of compex V)	+	+	
F56D2.1	UCR-1, processing protease, subunit beta	+	+	
	EEF, eukaryotic elongation factors	Y41E3.10a		F25H5.4
Decreased				
C42D8.2e	VIT-2, VITellogenin protein 2	-	-	
K07H8.6a	VIT-6, VITellogenin protein 6	-	-	

Table 2
Mitochondrial respiratory chain protein sets expression in mitochondrial mutants

Each mutant is compared to the wildtype, N2 first by expression ratio (ER), then by p value. ER and p values calculated as described in the Methods. Significance was defined as p value<0.05 with a Bonferroni correction for n=3. Significance was therefore corrected to be (0.05/3)=0.017; significant p values listed in red.

Pathway	ER <i>clk-1</i> /N2	p value <i>clk-1</i> vs. N2	ER <i>gas-1</i> /N2	p value <i>gas-1</i> vs. N2	ER <i>mev-1</i> /N2	p value <i>mev-1</i> vs. N2
complex I	.976	.240	.816	.000	.921	.016
complex II	.962	.214	1.016	.353	.992	.426
complex III	.888	.026	1.108	.017	1.07	.107
complex IV	1.051	.233	1.008	.450	1.019	.396
complex V	1.034	.261	1.009	.430	1.005	.469
Q synthesis	1.225	.367	2.461	.0007	2.537	.012

Table 3
Protein pathways ranked by differential expression in *gas-1* compared to N2

To decide if a particular pathway is differentially expressed, we employed two approaches: total expression, and the max-mean statistics. Pathways were ranked based on each method and sorted with respect of the combined ranking by using the sum of the ranks of minmax and total expression. P values for differences in expression are given next to the ranking. Those pathways highlighted in yellow were upregulated in all three mutants compared to N2; those in grey were up-regulated in *gas-1* and *clk-1*, but not *mev-1*; those in blue were up-regulated in *gas-1* and *mev-1*, but not *clk-1*.

Pathway	Rank (minmax)	P value (minmax)	Rank (total expression)	P value (total expression)	N in Pathway
Glycolysis / Gluconeogenesis	2	.086	2	.002	40
Lysine degradation	1	.035	4	.007	37
Valine, leucine and isoleucine degradation	9	.155	1	.001	47
Pyruvate metabolism	3	.106	11	.090	28
Calcium signaling pathway	7	.144	9	.055	43
Butanoate metabolism	12	.213	5	.028	29
Tryptophan metabolism	11	.212	6	.034	39
Fatty acid metabolism	15	.238	3	.004	57
Oxidative phosphorylation	14	.228	7	.037	113
Peroxisome	4	.108	17	.285	65
Citrate cycle (TCA cycle)	8	.154	16	.283	33
Protein processing in endoplasmic reticulum	6	.12	19	.29	126
Phagosome	13	.221	13	.152	52
Spliceosome	10	.177	18	.292	107
Purine metabolism	5	.113	25	.469	110
Fatty acid elongation in mitochondria	24	.805	8	.055	13
Glycine, serine and threonine metabolism	20	.401	14	.160	21
Glyoxylate and dicarboxylate metabolism	25	.840	10	.056	13
Synthesis and degradation of ketone bodies	23	.803	12	.095	6
Alanine, aspartate and glutamate metabolism	18	.270	21	.355	32

Table 4
Protein pathways ranked by differential expression in *mev-1* compared to N2

Statistics and ranking were as described in Table 3. Those pathways highlighted in yellow were up-regulated in all three mutants compared to N2; those in blue were up-regulated in *gas-1* and *mev-1*, but not *clk-1*.

Pathway	Rank (minmax)	P value (minmax)	Rank (total expression)	P value (total expression)	N in Pathway
Valine, leucine and isoleucine degradation	1	.011	1	.005	47
Fatty acid metabolism	3	.055	5	.121	57
Fatty acid elongation in mitochondria	11	.157	3	.052	13
Pyruvate metabolism	4	.063	13	.207	28
Arginine and proline metabolism	9	.130	9	.174	36
Glyoxylate and dicarboxylate metabolism	10	.136	8	.172	13
Phagosome	5	.066	15	.283	52
Alanine, aspartate and glutamate metabolism	19	.450	2	.047	32
Oxidative phosphorylation	7	.073	14	.273	113
Peroxisome	18	.439	7	.167	65
beta-Alanine metabolism	6	.071	20	.358	23
Glycolysis / Gluconeogenesis	20	.464	6	.159	40
Propanoate metabolism	2	.045	24	.443	32
Butanoate metabolism	22	.55	10	.19	29
Protein processing in endoplasmic reticulum	17	.39	16	.30	126
Lysine degradation	24	.69	12	.20	37
Citric cycle (TCA cycle)	12	.22	27	.48	33

Table 5

Protein pathways ranked by differential expression in *clk-1* compared to N2

Statistics and ranking were as described in Table 3. Those pathways highlighted in yellow were up-regulated in all three mutants compared to N2; those in grey were up-regulated in *gas-1* and *clk-1*, but not *mev-1*.

Pathway	Rank (minmax)	P value (minmax)	Rank (total expression)	P value (total expression)	N in Pathway
Fatty acid metabolism	1	.01	6	.08	57
Glycine, serine and threonine metabolism	8	.15	2	.01	21
Lysine degradation	2	.01	8	.11	37
Phagosome	7	.08	3	.02	52
Synthesis and degradation of ketone bodies	5	.05	5	.06	6
Alanine, aspartate and glutamate metabolism	12	.26	1	.01	32
Peroxisome	10	.21	9	.12	65
Butanoate metabolism	16	.33	4	.03	29
Valine, leucine and isoleucine degradation	9	.20	11	.19	47
Tryptophan metabolism	4	.03	17	.28	39
Calcium signaling pathway	3	.02	20	.40	43
Glycolysis / Gluconeogenesis	11	.25	15	.23	40
Propanoate metabolism	24	.62	7	.10	32
Fatty acid elongation	18	.42	16	.23	13
Glyoxylate/dicarboxylate metabolism	19	.44	18	.31	13
Oxidative Phosphorylation	26	.66	13	.23	113
Citrate cycle (TCA cycle)	21	.49	25	.43	33