Subcomplex Iλ Specifically Controls Integrated Mitochondrial Functions in Caenorhabditis elegans

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Abstract

Complex I dysfunction is a common, heterogeneous cause of human mitochondrial disease having poorly understood pathogenesis. The extensive conservation of complex I composition between humans and Caenorhabditis elegans permits analysis of individual subunit contribution to mitochondrial functions at both the whole animal and mitochondrial levels. We provide the first experimentally-verified compilation of complex I composition in C. elegans, demonstrating 84% conservation with human complex I. Individual subunit contribution to mitochondrial respiratory capacity, holocomplex I assembly, and animal anesthetic behavior was studied in C. elegans by RNA interference-generated knockdown of nuclear genes encoding 28 complex I structural subunits and 2 assembly factors. Not all complex I subunits directly impact respiratory capacity. Subcomplex Iλ, subunits along the electron transfer pathway specifically control whole animal anesthetic sensitivity and complex I upregulation, proportionate to their relative impairment of complex I-dependent oxidative capacity. Translational analysis of complex I dysfunction facilitates mechanistic understanding of individual gene contribution to mitochondrial disease. We demonstrate that functional consequences of complex I deficiency vary with the particular subunit that is defective.

Introduction

The mitochondrial respiratory chain (RC) is crucial to multiple cellular functions including energy generation by oxidative phosphorylation (OXPHOS), reactive oxygen species generation and scavenging, calcium homeostasis, and apoptosis. Mitochondrial RC dysfunction is suspected in the setting of a broad range of findings in high energy-demand tissues, especially those with neuromuscular, cardiac, or gastrointestinal manifestations [1]. Indeed, heterogeneous mitochondrial diseases are now recognized to constitute the most common group of inborn metabolic errors, with a minimal estimated 1 in 5,000 lifetime prevalence [2,3]. Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) is the largest and most commonly implicated RC component in human mitochondrial disease.

Complex I of bovine heart mitochondria has 45 structural subunits, 38 of which are nuclear-encoded, and 7 of which are encoded by mitochondrial DNA (mtDNA) [4]. Fourteen of the subunits have bacterial homologues and represent the catalytic “core” of the enzyme, with presumed roles in redox and proton translocation [5]. Although the three-dimensional structure of its hydrophilic domain in Thermus thermophilus and Escherichia coli has recently been solved to allow for better insight of complex I structure-function relationships [6,7], the functions of many of the 31 supernumerary (accessory) subunits remain unknown [8]. Pathogenic mutations have now been identified in 10 of the 38 nuclear DNA (nDNA) subunits of human complex I [9], including all 7 nDNA-encoded core subunits and 3 supernumerary subunits, as well as in 3 complex I assembly factors (Table 1). The subunits of complex I have been grouped into three major subcomplexes based on differential centrifugation fractions in bovine mitochondria, which generally correspond to structural sublocalization: Iα comprises the matrix arm (Iα) plus several additional membrane bound subunits, Iβ indicates most of the membrane-bound arm, and Iγ refers to subunits having undefined location identified in “breakthrough centrifugation fraction” [4, 14].

We exploited the extensive evolutionary conservation of mitochondrial proteins to undertake a global analysis of subunit contribution to complex I functions. Specifically, polarographic analysis permits assessment of mitochondrial integrated respiratory capacity from electron entry into the respiratory chain through final acceptance by oxygen, in addition to coupling between oxidation and phosphorylation, as well as inner mitochondrial membrane permeability and transporter function. Whereas null alleles of RC subunits are unlikely to produce viable animals to study, large quantities of isogenic hypomorphic Caenorhabditis elegans nematodes can be obtained using feeding RNA interference (RNAi) [10–12]. This approach permits convenient phenotypic...
Table 1. Complex I composition and *C. elegans* homology.

| #   | E. coli | B. taurus | H. sapiens | C. elegans | Subcomplex C. elegans | Protein Similarity (H. sapiens - C. elegans) | Predicted Homology in Wormbase | Predicted Homology in KEGG | Identified in N2 worm Mitochondria by BNG/Mass Spectrometry |
|-----|---------|-----------|------------|------------|---------------------|---------------------------------------------|--------------------------------|--------------------------------|--------------------------------------------------|
| 1   | nuoH    | ND1       | ND1        | MTCE.11    | lγ                  | 78.4%                                       | +                              | +                                |                                   |
| 2   | nuoN    | ND2       | ND2        | MTCE.16    | lγ                  | 47.9%                                       | +                              | +                                |                                   |
| 3   | nuoA    | ND3       | ND3        | MTCE.34    | lγ                  | 92.8%                                       | +                              | +                                |                                   |
| 4   | nuoM    | ND4       | ND4        | MTCE.25    | lβ                  | 88.5%                                       | +                              | +                                |                                   |
| 5   | nuoK    | ND4L      | ND4L       | MTCE.4     | lγ                  | 40.0%                                       | +                              | +                                |                                   |
| 6   | nuoL    | ND5       | ND5        | MTCE.35    | lβ                  | 64.9%                                       | +                              | +                                |                                   |
| 7   | nuoJ    | ND6       | ND6        | MTCE.3     | lγ                  | 31.0%                                       | +                              | +                                |                                   |
| 8   | nuoG    | 75 kDa    | NDUFS1**   | Y45G12B.1  | lγ, lδ              | 97.8%                                       | +                              | +                                |                                   |
| 9   | nuoD    | 49 kDa    | NDUFS2**   | K0849.5    | lγ, lδ              | 83.4%                                       | +                              | +                                |                                   |
| 9*  |        |           | gas-1(FC21)|           | lγ, lδ              | 83.4%                                       | +                              | +                                |                                   |
| 10  | nuoC    | 30 kDa    | NDUFS3**   | T10E9.7    | lγ, lδ              | 45.4%                                       | +                              | +                                |                                   |
| 11  |        |           |           |            | lγ, lδ              | 72.7%                                       | +                              | +                                |                                   |
| 12  |        |           |           |            | lγ, lδ              | 57.9%                                       | +                              | +                                |                                   |
| 13  |        |           |           |            | lγ, lδ              | 61.4%                                       | +                              | +                                |                                   |
| 14  | nuoB    | PSST      | NDUFS7**   | Y54H10A.5  | lγ                  | 95.0%                                       | +                              | +                                |                                   |
| 15  | nuoI    | TYKY      | NDUFS8**   | T20H4.5    | lγ                  | 95.8%                                       | +                              | +                                |                                   |
| 16  | nuoF    | 51 kDa    | NDUVF1**   | C09H10.3   | lγ                  | 96.7%                                       | +                              | +                                |                                   |
| 17  | nuoE    | 24 kDa    | NDUVF2**   | F53F4.10   | lγ                  | 93.7%                                       | +                              | +                                |                                   |
| 18* |        |           |           |            | lγ, lδ              | 30.4%                                       | +                              | +                                |                                   |
| 19* |        |           |           |            | lγ, lδ              | 45.4%                                       | +                              | +                                |                                   |
| 20* |        |           |           |            | lγ, lδ              | 93.5%                                       | +                              | +                                |                                   |
| 21* |        |           |           |            | lγ, lδ              | 93.5%                                       | +                              | +                                |                                   |
| 22* |        |           |           |            | lγ, lδ              | [53% by blast]                               | No                             | +                                |                                   |
| 23  | B13     | NDUFA5    | C03A12.1   | lγ, lδ      | 73.3%                                       | +                              | +                                |                                   |
| 24  | B14     | NDUFA6    | Y57G11C.12 | lγ          | 96.2%                                       | +                              | +                                |                                   |
| 25  | B14.5a  | NDUFA7    | F45H10.3   | lγ          | 42.3%                                       | +                              | +                                |                                   |
| 26  |        |           |           |            | lγ, lδ              | 72.7%                                       | +                              | +                                |                                   |
| 27  |        |           |           |            | lγ, lδ              | 64.3%                                       | +                              | +                                |                                   |
| 28  |        |           |           |            | lγ, lδ              | 73.9%                                       | +                              | +                                |                                   |
| 29* | B14.7   | NDUFA11   |           | lγ, lδ      | 90.4%                                       | +                              | +                                |                                   |
| 30  | B17.2   | NDUFA12   | Y9H6A.8    | lγ, lδ      | 90.4%                                       | +                              | +                                |                                   |
| Subunit | Gene Name | Subcomplex | C. elegans Protein Similarity |
|---------|-----------|------------|-----------------------------|
| Location within Bovine Complex | E. coli | B. taurus | H. sapiens | C. elegans | E. coli | B. taurus | H. sapiens | C. elegans | Predicted Homology in Wormbase | Predicted Homology in KEGG | Identified in N2 worm Mitochondria by BNG/Mass Spectrometry |
| 31 | SDAP | NDUFA8-1 | Y56A3A.19 | Iα, Iβ | 97.7% | + | + | + |
| 32* | MNLL | NDUFB1 | | Iβ | | | | |
| 33 | AGGG | NDUFB2 | F44G4.2 | Iβ | 53.8% | + | + | + |
| 34 | B12 | NDUFB3 | C18E9.4 | Iβ | 87.4% | + | + | + |
| 35 | B15 | NDUFB4 | W01A8.4 | Iα, Iβ (Iγ) | 62.8% | + | + | + |
| 36 | SGDH | NDUFB5 | C25H3.9 | Iβ | 71.7% | + | + | + |
| 37 | B17 | NDUFB6 | ZK909.3 | Iβ | 53.3% | + | + | + |
| 38 | B18 | NDUFB7 | D2030.4 | Iβ | 99.2% | + | + | + |
| 39 | ASHI | NDUFB8 | Y51H1A.3 | Iβ | 73.5% | + | + | + |
| 40 | B22 | NDUFB9 | C16A3.5 | Iβ | 39.2% | + | + | + |
| 41 | PDSW | NDUFB10 | F59C6.5 | Iβ | 54.6% | + | + | + |
| 42 | E55S | NDUFB11 | F42G8.10 | Iβ | [39.2% by blast] | No | + | + |
| 43* | KFY1 | NDUFC1 | | Iγ | | | | |
| 44 | B14.5b | NDUFC2 | Y71H2AM.4 | Iβ (Iγ) | 61.2% | + | + | + |
| 45 | B16.6 | GRIM19 | C3482.8 | Iα, Iβ | 84.2% | + | |
| Assembly Factor | NDUFAF1 | NDUFAF1** | C50B8.3 | - | 57.6% | + | |
| Assembly Factor | B17.2L | NDUFAF2** | Y116A8C.30 | - | [24% by blast; 73.9% to bovine] | No | |
| Assembly Factor | C13H20ORF7 | C20orf7** | K09E4.3 | - | 93.0% | + | |
| Assembly Factor | ECSIT | ECSIT | Y17G8.5 | - | 90.9% | + | |

82% (31/38) of mammalian, nuclear DNA-encoded, complex I subunits demonstrate extensive homology across evolution, ranging from 41.5% to 99.2% similarity between *C. elegans* and human proteins. Mitochondrial DNA-encoded complex I subunits, although highly conserved, were not included in this study.

*indicates human subunits not conserved in *C. elegans*.

*indicates subunits in which pathogenic mutations have been identified in human patients.

*gas-1(fc21)* missense mutant allelic to *K09A9.5* RNAi knockdown.

**two RNAi clones identified in silico as *NDUFA2* homologues.
assessments of the biological consequences of specific respiratory chain genetic defects at both the whole animal and isolated mitochondria levels to assess the effects of subunit knockdown on integrated complex I respiratory capacity as well as complex assembly [13]. We report here the relative impact of RNAi-generated gene knockdown on integrated respiratory capacity and whole animal behavior for 28 nuclear-encoded structural subunits and 2 assembly factors of complex I conserved between mammals and C. elegans. We show that individual subunits vary significantly in the extent to which they impact these aspects of complex I function. Deficiency of only a subset of complex I subunits is likely to result in primary mitochondrial disease, as narrowly defined by impairment of integrated respiratory capacity. We extend the correlation between complex I-dependent respiratory capacity impairment and anesthetic sensitivity [13] to specify that subunits comprising subcomplex B. [4,14] in the hydrophilic, matrix arm of complex I most directly influence whole worm anesthetic behavior. Complex II-dependent respiratory capacity correlates inversely with subcomplex B. subunit dysfunction [15], but directly with dysfunction of subunits in membrane-bound subcomplex 1β [4,14]. This translational approach prioritizes a relevant gene subset to investigate in human patients with impaired complex I respiration, and facilitates the study of mechanisms by which individual genes contribute to human mitochondrial RC disease.

Results and Discussion

C. elegans complex I subunit homologue knockdown studied by RNA interference

All 7 mtDNA subunits, at least 31 nDNA subunits, and 4 known complex I assembly factors demonstrate extensive evolutionary conservation between humans and C. elegans (Table 1). At the individual subunit level, the extent of protein similarity by length between C. elegans and humans ranges between 25% and 99.2% (http://ucsc.genome.edu; www.wormbase.org; www.genome.jp/kegg). This similarity extends across all structural subcomplexes and known assembly factors (Table 2). 26 subunits were experimentally confirmed via mass spectrometry analysis of complex I isolated by blue native gel electrophoresis from wildtype (N2) C. elegans mitochondria; interestingly, no mtDNA-encoded subunits were identified by this analysis [16].

| Table 2. General overview of human-worm complex I subunit and subcomplex homology. |
|---------------------------------|-------|-------|-------|
| H. sapiens C. elegans % conserved Studied by RNAi |
| Total Cl subunits | 45 | 38 | 84% | 28 |
| mtDNA-encoded | 7 | 7 | 100% | 0 |
| nDNA-encoded | 38 | 31 predicted in silico | 82% | 28 |
| (26 confirmed by BNG) |
| Subcomplex of nDNA subunits |
| α alone | 8 | 5 | 63% | 4 |
| α, β | 2 | 2 | 100% | 2 |
| β | 11 | 10 | 91% | 9 |
| γ** | 1 | 0 | 0% | 0 |
| undefined*** | 1 | 0 | 0% | 0 |
| CI Assembly Factors | 4 | 4 | 100% | 2 |

Notes: * Two strains were studied for homologues of two α subunits, NDUFS2 and NDUFA2 - see Table 1 notes for details.
** γ consists of “breakthrough fractions” in the purification of subcomplexes α and β from bovine heart mitochondria, but only KFY1.
*** Extensive conservation is apparent between human and C. elegans complex I composition, as broken down by either genome of origin or subcomplex. Percent conserved indicates the number of human complex I subunits in each group for which a C. elegans homologue is identifiable, rather than the extent of similarity between specific subunits. * two strains each were studied for homologues of two α subunits, NDUFS2 and NDUFA2, per details in Table 1 legend. ** only KFY1 is found in no other fraction except γ. *** MLRQ is not localized to any subcomplex (4).

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Table 3. RNAi-generated hypomorphic *C. elegans* strains for 28 nDNA-encoded complex I subunits and 2 complex I assembly factors were studied by a gene knockdown approach in strains exposed for 3 generations to RNAI.

| SUBUNIT | GENE NAME | SUBCOMPLEX | RNA INTERFERENCE | RESPIRATORY CAPACITY | COMPLEX QUANTITY | ANESTHESIA |
|---------|-----------|------------|------------------|----------------------|------------------|------------|
|         | E. coli | B. taurus | H. sapiens | C. elegans | Localization within Bovine Complex I* | RNAI Clone Source | Relative transcript knockdown in F2 worms* | % CI-dependent OXPHOS in Isolated Mitochondria* | CI OXPHOS p-value* | % CI-dependent OXPHOS in Isolated Mitochondria* | CI OXPHOS p-value* | % N2 complex I on BNG | % N2 complex V on BNG | Halothane EC50 in Whole Worms** |
| #       | # | # | # | # | # | # | # | # | # | # | # | # | # | # | # | # | # |
| 8       | nuoG    | 75 kDa     | NDUF51 | Y5G12.1 | 1s, 0, GS | 59% | 76% | 0.130 | 142% | 0.022 | 76% | 40% | 2.9% |
| 9       | nuoD    | 49 kDa     | NDUF52 | K09A9.5 | 1s, 0, GS | 51% | 57% | 0.120 | 148% | 0.051 | - | - | 1.8% |
| 10      | nuoC    | 30 kDa     | NDUF53 | T10E9.7 | 1s, 0, GS | 27% | 1.088 | 143% | 0.088 | 87% | 114% | 2.6% |
| 11      | 12 kDa  | NDUF54 | ZK973.10 | 0.230 | 127% | 0.170 | 10% | 185% | 2.5% |
| 12      | 15 kDa  | NDUF55 | Y5E10B5.5 | 0.011 | 141% | 0.022 | 87% | 94% | 2.3% |
| 13      | 13 kDa  | NDUF56 | F22D6.4 | 0.006 | 169% | 0.011 | - | - | 1.8% |
| 14      | nuoB    | 50 kDa     | NDUF57 | W10D5.2 | 1s, 0, OB | Ind | 74% | 0.150 | 100% | 0.920 | - | - | 2.9% |
| 15      | nuol    | 51 kDa     | NDUF58 | T2OHN.5 | 0.022 | 134% | 0.023 | - | - | 2.1% |
| 16      | nuoF    | 51 kDa     | NDUF59 | C09H10.3 | 0.022 | 133% | 0.130 | - | - | 1.8% |
| 17      | nuoE    | 24 kDa     | NDUF60 | F53F4.10 | 0.130 | 147% | 0.022 | - | - | 2.8% |
| 20      | B8      | NDUF62 | Y5D3A3.7 | Ind | 127% | 0.170 | 3.0% | 32% | 3.2% |
| 22      | B13     | NDUF63 | C3A12.1 | Ind | 84% | 0.230 | 94% | 0.920 | 3.0% |
| 24      | B14     | NDUF64 | Y5G711C12 | Ind | 59% | 0.010 | 121% | 0.340 | 85% | 144% | 2.8% |
| 25      | B14.5a  | NDUF65 | F45H10.3 | Ind | 0.230 | 97% | 0.920 | 106% | 134% | 3.1% |
| 27      | 39 kDa  | NDUF66 | Y5G88.2 | Ind | 0.026 | 119% | 0.410 | - | - | 1.9% |
| 28      | 42 kDa  | NDUF67 | K04G7.4 | GS | 59% | 0.022 | 186% | 0.006 | - | - | 3.8% |
| 31      | SDAF    | NDUF68 | Y5F3A3.19 | 0.088 | 131% | 0.130 | 79% | 98% | 2.9% |
| 33      | AGGG    | NDUF69 | F44G4.2 | Ind | 56% | 0.117 | 0.170 | 107% | 27% | 2.1% |
| 34      | B12     | NDUF70 | C16E9.4 | Ind | 90% | 0.230 | 42% | 0.220 | 88% | 39% | 3.2% |
| 35      | B15     | NDUF71 | M01A8.4 | 0.022 | 128% | 0.230 | 61% | 58% | 3.1% |
| 36      | SQDH    | NDUF72 | C25F9.3 | Ind | 145% | 0.111 | 110% | 46% | 3.1% |
| 37      | B17     | NDUF73 | ZK809.3 | Ind | 27% | 0.022 | 111% | 0.660 | 57% | 59% | 4.8% |
| 38      | B18     | NDUF74 | D2030.4 | Ind | 77% | 0.088 | 154% | 0.130 | 58% | 27% | 3.0% |
| 39      | ASHI    | NDUF75 | Y5H1A3 | Ind | 0.170 | 136% | 0.039 | 117% | 57% | 2.9% |
| 40      | B22     | NDUF76 | C16A3.5 | Ind | 90% | 0.170 | 148% | 0.022 | 78% | 290% | 3.0% |
| 41      | PDSW    | NDUF77 | F59G6.5 | Ind | 64% | 0.039 | 112% | 0.390 | 90% | 13% | 3.2% |
| 42      | ESSS    | NDUF78 | F42G8.10 | Ind | 69% | 0.060 | 125% | 0.100 | 90% | 110% | 2.8% |
| 45      | B16.6   | GRIM19 | C34B2.8 | Ind | 23% | 0.006 | 131% | 0.290 | 117% | 31% | 2.0% |
Individual subunits significantly vary in their relative impairment of complex I-dependent OXPHOS capacity. Table 3 presents the RNAi knockdown of each subunit, as well as the effect on complex I respiratory capacity and whole worm anesthetic behavior. Subunit knockdowns were performed in C. elegans, B. taurus, H. sapiens, and C. elegans. NDUFS2 knockdown significantly decreased complex I-dependent respiration compared to wildtype (N2) (p<0.05), with an additional 3 subunits and 1 assembly factor causing marginal impairment of complex I-dependent respiration (p<0.10) (Table 3). Curiously, knockdown of the NDUFB5 homologue (C25H3.9) results in significantly increased complex I-dependent respiratory capacity. The degree of complex I dysfunction does not strictly correlate with extent of gene knockdown (Figure S1) or subcomplex localization (Figure 2a). Among the two in silico-predicted homologues to human subunit NDUFA2, neither significantly alters complex I-dependent respiratory capacity. It is possible that these two paralogues are able to substitute for each other, or that knockdown of either gene is insufficient to cause measurable changes in its function. Uncoupled rates in isolated mitochondria do not substantially differ from state 3 rates for a given strain (Figure S2). Knockdown of 13 complex I subunits results in significantly (p<0.05) upregulated complex II-dependent respiration; one additional subunit increases complex II-dependent respiration but not significantly (p<0.10) (Table 3 and Figure 1b), irrespective of subcomplex (Figure 2b). While there is no significant overall correlation between complex I- and II-dependent OXPHOS capacity (r = −0.19, p = 0.29), reanalysis by subcomplex localization reveals stronger and opposite correlations for subunits in the matrix versus membrane-bound subcomplexes (Figure 3). Dysfunction of subunits in subcomplex Iₐ along the electron transfer pathway in the hydrophilic, matrix arm of complex I demonstrates a marginal, inverse correlation with complex II-dependent respiratory capacity (Pearson r = −0.53, p = 0.042; Spearman r = −0.46, p = 0.081). This presumably represents a compensatory response, as has been observed in a knockout mouse model of a subcomplex Iₐ subunit, NDUFS4 (15). In contrast, dysfunction in subunits comprising the hydrophobic, membrane-bound, subcomplex 1B demonstrates a marginal, positive correlation with complex II-dependent respiratory capacity (Pearson r = 0.57, p = 0.067; Spearman r = 0.73, p = 0.012). Further subdivision into core versus accessory subunits does not significantly alter these results. Future studies are needed to elucidate the mechanism underlying this finding.

Given the predominant role of complex I in regulating respiratory control (23), we analyzed the relative impact on activity [16]. Therefore, the lack of such a correlation between complex I subunits indicates they differ in their biological contributions to complex I respiratory capacity and anesthetic behavior.

Polarographic analysis of subunit impact on mitochondrial respiratory capacity

Complex I subunit knockdown variably impact integrated respiratory function, as referred to the polarographically-assessed combined efficacy of metabolic substrate uptake and transport across the inner mitochondrial membrane, the transfer of electrons through RC complexes to oxygen, the generation of a proton gradient, and ultimately, the generation of ATP. Indeed, deficiency of only a subset of complex I subunits results in primary mitochondrial dysfunction, as characterized by significant respiratory capacity impairment (Table 3). Substantial variability is seen between state 3 (near-maximal) rates of biological replicates with a given substrate (Figures 1a and 1b), as is consistent with broad normal ranges for freshly isolated human mitochondria [21,22]. Both the gas-1 (gc21) missense allele and RNAi-generated hypomorph for the NDUFS2 homologue (K9949.5) significantly impair complex I capacity, demonstrating the validity of the knockdown technique. 15 of 26 genes when knocked down (when counting the missense and knockdown, NDUFS2 animals only once) significantly decrease complex I-dependent respiration compared to wildtype (N2) (p<0.05), with an additional 3 subunits and 1 assembly factor causing marginal impairment of complex I-dependent respiration (p<0.10) (Table 3). Curiously, knockdown of the NDUFB5 homologue (C25H3.9) results in significantly increased complex I-dependent respiratory capacity. The degree of complex I dysfunction does not strictly correlate with extent of gene knockdown (Figure S1) or subcomplex localization (Figure 2a). Among the two in silico-predicted homologues to human subunit NDUFA2, neither significantly alters complex I-dependent respiratory capacity. It is possible that these two paralogues are able to substitute for each other, or that knockdown of either gene is insufficient to cause measurable changes in its function. Uncoupled rates in isolated mitochondria do not substantially differ from state 3 rates for a given strain (Figure S2). Knockdown of 13 complex I subunits results in significantly (p<0.05) upregulated complex II-dependent respiration; one additional subunit increases complex II-dependent respiration but not significantly (p<0.10) (Table 3 and Figure 1b), irrespective of subcomplex (Figure 2b). While there is no significant overall correlation between complex I- and II-dependent OXPHOS capacity (r = −0.19, p = 0.29), reanalysis by subcomplex localization reveals stronger and opposite correlations for subunits in the matrix versus membrane-bound subcomplexes (Figure 3). Dysfunction of subunits in subcomplex Iₐ along the electron transfer pathway in the hydrophilic, matrix arm of complex I demonstrates a marginal, inverse correlation with complex II-dependent respiratory capacity (Pearson r = −0.53, p = 0.042; Spearman r = −0.46, p = 0.081). This presumably represents a compensatory response, as has been observed in a knockout mouse model of a subcomplex Iₐ subunit, NDUFS4 (15). In contrast, dysfunction in subunits comprising the hydrophobic, membrane-bound, subcomplex 1B demonstrates a marginal, positive correlation with complex II-dependent respiratory capacity (Pearson r = 0.57, p = 0.067; Spearman r = 0.73, p = 0.012). Further subdivision into core versus accessory subunits does not significantly alter these results. Future studies are needed to elucidate the mechanism underlying this finding.

Given the predominant role of complex I in regulating respiratory control [23], we analyzed the relative impact on
Figure 1. Integrated respiratory capacity of intact mitochondria from all complex I knockdown strains studied. Boxplots indicate cumulative state 3 (near-maximal) rate data from all replicates of each *C. elegans* strain interrogated with (a) a complex I-dependent substrate (malate) or (b) a complex II-dependent substrate (succinate). The length of the box represents 25th to 75th percentile inter-quartile range, interior cross represents mean, interior horizontal line represents median, and vertical lines issuing from the box extend to minimum and maximum values of the analysis variable. Figure insets depict specific aspects of integrated respiratory capacity interrogated with each substrate. Significance of individual strain mean differences from wildtype (N2) is detailed in Table 3.

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Figure 2. Analyses of subunit functions in *C. elegans* by known structural role. (a) Relative mean complex-I dependent respiratory capacity of each knockdown strain by subcomplex localization. (b) Relative mean complex-II dependent respiratory capacity of each knockdown strain by subcomplex localization. (c) Anesthetic behavior of each knockdown strain by subcomplex localization. Dotted lines indicate wildtype function. To discriminate subunits present in both subcomplexes 1α and 1α from those present only in 1α, the former are labelled "1α," and the latter "1α". "Accessory subunits" include all structural complex I subunits studied except for 1α core subunits.

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respiratory control of each complex I subunit knockdown strain (Figure S3). Mean respiratory control ratio (RCR) is significantly decreased compared to wildtype for 11 strains located across the structural subcomplexes, corresponding to human subunits NDUFS2, NDUFS3, NDUFS6, NDUFS8, NDUFV1, NDUFA6, NDUFA10, NDUFA11, NDUFB4, NDUFB9, NDUFB10, and marginally decreased for NDUFB7. Analysis of subunit knockdown strains having relatively impaired respiratory control showed no significant correlation of RCR and mean state 3 rate (Pearson $r = 0.30$, $p = 0.32$).

RC Complex Quantitation. Complex I subunit knockdown variably decreases the amount of complex I (Table 3 and Figure 4). Observed decreases in complex I-dependent OXPHOS capacity are not predicted entirely by decreased

| SUBCOMPLEX                | n  | PEARSON | SPEARMAN |
|---------------------------|----|---------|----------|
|                           |    | r      | r²       | p   | r    | r²   | p    |
| ALL SUBUNITS              | 33 | -0.19  | 0.036    | 0.290 | -0.073 | 0.005 | 0.6900 |
| 1α - all                  | 15 | -0.53  | 0.28     | 0.042 | -0.46  | 0.22  | 0.0810 |
| 1α - core                 | 8  | -0.50  | 0.25     | 0.2000 | -0.38  | 0.15  | 0.3500 |
| 1α - accessory            | 7  | -0.49  | 0.24     | 0.2600 | -0.18  | 0.03  | 0.7000 |
| 1α + 1β                   | 21*| -0.43  | 0.18     | 0.0520 | -0.28  | 0.08  | 0.2200 |
| IB                         | 11*| 0.57   | 0.33     | 0.0670 | 0.73   | 0.53  | 0.0120 |
| All accessory subunits    | 17 | -0.06  | 0.00     | 0.7900 | 0.11   | 0.013 | 0.5900 |

Figure 3. Mean complex I- and complex II-dependent respiratory capacities in C. elegans complex I knockdown strains. No overall significant correlation is observed between complex I dysfunction and complex II-dependent respiration. However, subcomplex analysis highlights that reduced complex I function in in membrane-bound subcomplex I$\beta$ subunits directly correlates with reduced complex II-dependent OXPHOS capacity. In contrast, depletion of matrix arm subcomplex 1$\alpha$ subunits evokes a modest compensatory increase in complex II-dependent OXPHOS capacity. See Table 1 legend for subcomplex descriptions. * The two subunits which localize both to 1$\alpha$ and 1$\beta$ are likely at the interface of both subcomplexes, and were therefore included in the statistical analysis for each of these subcomplexes. Results of both parametric (Pearson) and non-parametric (Spearman) analyses are shown to allow interpretation of effects of outliers and differences in subcomplex size. doi:10.1371/journal.pone.0006607.g003
complex I content. In other words, complex I function may be impaired without corresponding changes in the amount of assembled complex I (e.g., knockdown of the GRIM-19 homologue (C34B2.8) results in 53% complex I-dependent OXPHOS capacity, but 117% complex I content when compared to the wild-type, N2). Among most subcomplex Iα and/or Iβ subunits, however, complex I content approximates polarographically-determined percent wildtype function (Table 3). Further analysis by organization into core versus accessory subunits permits additional insight, although interpretation is limited by sample size of core subunits in which complex I content was quantified (Figure S4). The two subunits localized to both bovine subcomplexes Iα and Iβ (homologues of NDUFA1 (Y56A3A.19) and NDUFB4 (W01A8.4), plausibly localized at the interface of these two subcomplexes, directly impact both complex I-dependent (and complex II-dependent) respiratory capacity and assembly. In contrast, the only significant respiratory capacity change observed upon knockdown of two complex I assembly homologues was a marginally significant decrease in complex I-dependent OXPHOS capacity in NDUAF1. However, both assembly knockdown strains have marginally increased complex I content and significantly decreased complex V content (Table 3). These data raise the possibility these genes are not functioning as complex I assembly factors in C. elegans. Indeed, complex V content appears significantly altered in many of the complex I subunit knockdown strains, although no consistent pattern is evident (Figure 4).

Anesthetic sensitivity of complex I subunit knockdown strains

The degree of complex I-dependent OXPHOS impairment at the level of isolated mitochondria highly correlates with anesthetic sensitivity at the level of whole worms for the corresponding genetic knockdown strains (Pearson $r = 0.45$, $p = 0.0079$ and Spearman $r = 0.60$, $p = 0.0002$) (Figure 5). As seen in the figure, however, there are both outliers (decreased complex I rates with no decrease in EC50) and an apparent ceiling effect when complex I rates are increased. Thus, maximal complex I rates do not entirely predict anesthetic sensitivity, despite the overall strong correlation between these two parameters.

These data extend our previously reported correlation between complex I-dependent respiratory capacity and anesthetic sensitivity [13]. Specifically, subunit analysis by subcomplex localization reveals that the respiratory capacity of subunits in subcomplex Iα along the electron transfer pathway in the hydrophilic, matrix arm of complex I most directly influence whole worm anesthetic behavior (Pearson $r = 0.89$, $p<0.0001$). The magnitude and significance of this finding remains unchanged when accounting for possible outliers and differences in number of subunits studied within each subcomplex (Spearman $r = 0.89$, $p<0.0001$). While subcomplex Iα includes all subcomplex Iα subunits plus several subunits located in the membrane-bound arm (Table 2), the correlation falls considerably when including Iβ and Iγ subunits together (Pearson $r = 0.60$, $p = 0.0037$; Spearman $r = 0.64$,
Further breakdown of subunits into “core” versus “accessory” highlights that it is the core subunits (all part of subcomplex Ia) that demonstrate the greatest correlation between complex I-dependent OXPHOS capacity and anesthetic behavior (Pearson \( r = 0.97 \), \( p < 0.0001 \); Spearman \( r = 0.91 \), \( p = 0.0017 \)). In contrast, a substantially diminished correlation (Spearman \( r = 0.46 \), \( p = 0.0250 \)) is observed among the combined accessory subunits (located in Ib, Ia, and I\( \lambda \) subcomplexes), which appears driven by a clear absence of correlation between these parameters among membrane-bound subcomplex Ib subunits (Spearman \( r = 0.18 \), \( p = 0.60 \)) (Figure 5).

The magnitude of anesthetic sensitivity is not predicted solely by complex I rate nor by subcomplex localization, as strains with similarly increased degrees of anesthetic sensitivity occur in multiple subunits of subcomplexes Ia and I\( \lambda \), and in a single subcomplex Ib subunit homologue \( \langle \text{NDUF}B2 \rangle \) (Figure 2c). In contrast, subcomplex analysis highlights three anesthetic resistant knockdown strains of homologues to an assembly factor \( \langle \text{NDUFAF1} \rangle \), a subcomplex Ib subunit \( \langle \text{NDUFA110} \rangle \), and a subcomplex Ib subunit \( \langle \text{NDUF}B6 \rangle \). Notably, no I\( \lambda \) subunits cause anesthetic resistance. Future analysis of these exceptions may elucidate mechanisms underlying the role of complex I in anesthetic behavior.

This work confirms that complex I subunits variably impact both mitochondrial respiration and whole worm anesthetic sensitivity. Indeed, the extent of volatile anesthetic hypersensitivity directly correlates with the degree of complex I dysfunction in \( C. \) elegans, rather than with dysfunction of any other respiratory chain.
Biological Significance

Significant genetic heterogeneity across dual genomes and high morbidity associated with mitochondrial RC dysfunction limits efforts to discern the pathogenic mechanisms underlying widespread phenotypic variability in human mitochondrial diseases [25,26]. Currently available diagnostic techniques are unable to establish the genetic cause in at least 60% of suspected mitochondrial disease patients who have demonstrable biochemical abnormalities [26]. The pathogenesis of human complex I deficiency to date has involved homozygous or complex heterozygous mutations resulting in near total loss of function in 10 human complex I subunits and 3 assembly factors [27]. However, synergistic or partially recessive mutations in two different complex I subunit genes has also been described [28]. Whereas null alleles of respiratory chain subunits are unlikely to produce viable animals to study, RNAi permits an opportunity to assess the physiologic importance of individual complex I subunits in multicellular animals [12]. Here, we exploited the extensive evolutionary conservation of mitochondrial composition and function between mammalian and C. elegans mitochondria to identify a biologically-relevant cadre of complex I subunits to investigate in human patients with impaired complex I respiratory capacity.

Since we were interested in physiologic consequences of complex I subunit dysfunction, we investigated integrated respiratory capacity of intact mitochondria from each complex I subunit knockdown strain by polarographic analysis of oxygen consumption, rather than spectrophotometry of in vitro enzyme activity. In so doing, we learned that deficiency of only a subset of complex I subunits likely results in primary mitochondrial RC dysfunction. We observed that complex II-dependent respiratory capacity is upregulated in matrix arm, subcomplex 1α, subunit dysfunction, as is consistent with a mouse knockout model of the subcomplex 1α, NDUFS4 subunit [15]. Conversely, complex II-dependent respiratory capacity is significantly impaired upon dysfunction of membrane-bound subcomplex 1β subunits. Based upon our data, the lowest complex I-dependent maximal respiratory capacity (state 3 rate) measured in isolated mitochondria from C. elegans (specifically in the gas-1(fc21) missense allele of the NDUFS2 homologue) is on the order of 30 nA/O/min/milligram protein. However, given that complex II-dependent respiratory capacity is upregulated in these animals, we postulate the minimum complex I capacity necessary for survival in the setting of an otherwise normal functioning respiratory chain to be slightly lower than the measured rate in these viable nematodes. The gas-1(fc21);mev-1(kn1) double mutant (harboring missense mutations in the NDUFS2 complex I subunit and SDH-C complex II subunit homologues) is non-viable [29]. Thus, these animals cannot survive alone at this degree of complex I impairment without intact complex II respiration. While difficult to extrapolate these values to mammalian cells given the nematode ability to ferment [30], anaerobic respiration is insufficient to rescue the gas-1(fc21);mev-1(kn-1) double mutant in complexes I and II. The minimal respiratory activity for survival in mammalian tissue is known to depend on tissue-specific thresholds [31], which in human fibroblasts correlates with a 40% reduction in complex I activity [32].

We also applied this translational genetic approach to explore the mechanism underlying the long-recognized, but poorly understood, role of complex I in whole animal anesthetic behavior. Our results strongly suggest that subunits comprising the subcomplex 1α matrix arm involved in electron transfer through complex I most directly influence volatile anesthetic hypersensitivity. Future work will focus on discerning if this association is attributable to anesthetic exacerbation of membrane potential dissipation caused by selective impairment of complex I subunits that contribute to proton gradient flux.

In summary, the results presented here demonstrate that not all subunits of complex I contribute equally to efficient oxidative phosphorylation or anesthetic sensitivity. While impaired complex I respiration is the implicated mechanism in a large proportion of human mitochondrial diseases, the heterogeneity of clinical disease manifestations likely results from a host of secondary cellular consequences. Prior work in C. elegans indicates that complex I mutations exert their pathogenic effects in multiple ways, such as by impeding NADH metabolism, significantly altering expression of multiple intermediary metabolic pathways, increasing reactive species production, dissipating mitochondrial membrane potential, initiating apoptosis, and interfering with the function or assembly of other RC components [29,33–37]. The functional importance of a variety of post-translational modifications of complex I subunits also remains an area of active investigation [27]. C. elegans presents the opportunity to discern relative contributions of complex I subunits to these plethora of mitochondrial roles in a tractable model animal.

Materials and Methods

Identification of complex I subunit C. elegans homologues

As complex I has no complete crystal structure elucidated in any species, the identity of the estimated 45 nDNA-encoded subunits of complex I in bovine mitochondria as determined by mass spectrometry was used as the initial reference list [4]. Each of the 45 protein sequences corresponding to the human complex I subunits was blasted in several browsers (www.homologene.com, www.wormbase.org, http://genome.ucsc.edu) against the C. elegans proteome to identify the closest C. elegans homologue (Table 1). Homology extent was determined in www.wormbase.org by blast based on percentage similarity along the length of the C. elegans protein corresponding to the longest human protein. Protein similarity of the nuclear-encoded complex I subunits and assembly factors as compiled by KEGG pathways (cel 100190) was cross-referenced against the final list (www.genome.jp/kegg). 31 complex I subunits and 2 complex I assembly factors were identified for C. elegans in this manner (Table 1). Two additional, recently-identified, human complex I assembly factors, ECSIT and C20orf7, were found to have C. elegans homologues by protein sequence blast (www.wormbase.org) [38,39]. Mass spectrometry analysis performed on blue native gel electrophoresis of wildtype (N2 Bristol) C. elegans mitochondria (see below) independently confirmed the presence of 26 complex I subunits (Table 1). No C. elegans homologue was identifiable in silico for 6 complex I subunits (NDUFAC1, NDUFAC3, NDUFA4, NDUFAC11, NDUFB1, NDUFCL1). Although a potential NDUFS3 homologue (CO9D1.1) was identified by early blast search, this was not confirmed in the most recent version of the genome browser and was therefore omitted from final analysis. C. elegans homologues for two human mtDNA subunits, ND4L and ND6, were not identifiable by protein sequence blast in either Wormbase or the UCSC genome browser; however, protein homology of these subunits is predicted by manual alignment to be 42% and 31% similarity by length, respectively (MacVector v10.0, Symantec, Cary, North Carolina) [40]. Subcomplex localization utilized for each subunit was that
determined in complex I of bovine heart mitochondria [4,14]. Of note, subcomplex 1β includes all subcomplex 1λ subunits comprising the matrix arm, in addition to other subunits that are present in the membrane arm (Table 1).

**Generation of RNAi-induced single gene C. elegans knockdown strains**

N2 Bristol (wildtype) *C. elegans* were obtained from the Caenorhabditis Genetics center (St. Paul, Minnesota) and grown and maintained by standard culture techniques [41]. RNAi bacterial clones corresponding to 28 subunits and 2 complex I assembly genes identified by the *in silico* approach, as discussed above, were obtained from one of two publicly accessible libraries (Geneservice, Cambridge, United Kingdom or OpenBiosystems, Huntsville, Alabama). Knockdown of the NDUF2 homologue, K09A9.3, was generated by RNAi using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and demonstrated similarity to the well-studied gas-1 (fe21) missense [G(X:15,58),0(73)] allele of this gene, which results in a non-conservative amino acid substitution (R290K) [18]. A second NDUF2 homologue that is expressed in *C. elegans*, T26A5.3, has 95 percent identity to K09A9.3 but is of unknown function and was not analyzed [42]. Two potential NDUF2 homologues (Y63D3A.7 and C2A11.13) were identified, both of which were studied by RNAi. Bacterial RNAi feeding clone identity was verified by direct sequencing using a universal forward promoter specific to its library of origin. Standard RNAi feeding protocols were performed using 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce transcription of double-stranded RNA [11]. Worms were grown in the presence of a given bacterial RNAi feeding clone at 20°C for two generations on agar nematode growth media (NGM) plates, transferred to liquid media for a third generation, washed clear of bacteria when most worms reached adulthood, and isolated by sucrose gradient centrifugation [13,18]. 1–4×10⁶ worms were obtained in each of 2 to 7 (average 6) replicates per gene knockdown.

**Confirmation of RNAi-induced gene knockdown by relative qPCR**

Whole worm total RNA was isolated from F2 generation (meaning 3rd consecutive *C. elegans* generation fed a specific RNAi bacterial clone) *C. elegans* populations and studied by relative quantitation using SYBR green gene-specific primers for eighteen strains, as previously described [36]. For five strains where SYBR green qPCR results were ambiguous, as well as the last nine complex I subunit knockouts generated, relative quantitation was instead performed using Taqman *C. elegans* gene expression assays (Applied Biosystems), on an Applied Biosystems 7500 real time PCR system using Sequence Detection Software v.1.2.3 or v.1.2.4 (Foster City, CA). For Taqman assays, *T04C12.8* was used as the endogenous control, with consistent ΔCt of 20 to 30 in all samples analyzed. qPCR analyses on all RNA isolated from knockdown animals generated by OpenBiosystems RNAi open reading frame (ORF) clones failed due to positive RT(-) controls; cDNA from these RNAi clones initially fed to the worms could not be completely eliminated from worm total RNA, despite repeated worm washing prior to RNA isolation and aggressive DNAse treatment. Genomic DNA-based Geneservice RNAi clones presented no similar problem.

**Polarographic analysis of integrated respiratory chain capacity in freshly isolated mitochondria**

Freshly washed, living, adult *C. elegans* populations were immediately subjected to an isolation procedure performed on ice which involved homogenization, protease degradation of their outer cuticle, and differential centrifugation to collect the mitochondrial fraction, as previously described [13]. Polarographic measurement of intact mitochondrial integrated OXPHOS capacity was immediately performed utilizing a Clark-type electrode (Oxytherm, Hansatech Instruments, United Kingdom), as previously established [33]. Substrates specific to complex I (malate alone is sufficient to stimulate state 3 respiration in *C. elegans* mitochondria without supplemental glutamate, as is required in mammalian mitochondria [13]) or II (succinate) were used to obtain respiratory rate profiles: baseline, state 3 (near-maximal) in the presence of limited ADP, state 4 (ADP-depleted), high ADP (maximal) in the presence of excessive ADP, uncoupled with dinitrophenol (DNP), and cytochrome C stimulated with tetramethyl-p-phenylenediamine (TMPD) and ascorbate (Figure S2). Rates were calculated as nanoatomsO/minute/mg protein and expressed for comparison as percent wildtype rate (Table 3). Respiratory control ratios (state 3/state 4) and ADP/O ratio were calculated to assess mitochondrial coupling and efficiency, respectively.

**Anesthetic sensitivity assessment**

Freshly washed F2 young adult worms from each strain were transferred to NGM plates and exposed to varying concentrations of the volatile anesthetic, halothane, to determine each strain’s EC₅₀ (effective concentration at which 50% of the animals are immobilized), as previously described [13]. A single observer (PGM) performed all anesthetic analyses.

**Assessment of mitochondrial complex content by blue native gel (BNG) electrophoresis, optical densitometry, and mass spectrometry**

BNG [43] was performed to isolate mitochondrial respiratory complexes and supercomplexes. Capillary column liquid chromatography/tandem mass spectrometry analysis of complex I-containing bands on BNG electrophoresis of *C. elegans* N2 mitochondria was used to identify individual complex I subunits (Table 1). Complex content of individual BNG bands was quantified by optical densitometry in all complex I subunit knockdown strains for which isolated frozen mitochondria remained following polarographic assessment (Figure 4b). Specifically, 200 ug of mitochondrial protein determined by Lowry assay [44], were subject to BNG electrophoresis by modification of the technique of Wittig et al. [43] and Schagger et al. [46] using Triton X-100 with a 3:1 detergent to protein mass ratio. Mitochondrial solubilization was performed at room temperature for 10 minutes, followed by 21,000 x g centrifugation at 4°C for 20 minutes. Supernatants were collected and Coomassie blue G-250 was added to obtain an 8:1 dye to detergent mass ratio before loading onto a 3.5–11% polyacrylamide gel (Hoefer Inc, Hollister, MA). Individual bands representing complexes I, III, and V were quantified by optical density (Multi Gauge V3.0, Fujifilm Life Science, Tokyo, Japan and ImageJ software, NIH) and normalized to the constant F0 bands of complex V (Table 3).

**Statistical analyses**

Two-sided, non-parametric ANOVA analyses were performed in SAS version 9.1 (SAS Institute Inc, Cary, North Carolina) to compare state 3 polarographic rates for a given substrate of all RNAi-generated complex I subunit strains to N2 wildtype controls grown on HT115 *E. coli* (Table 3). Significance was set at p<0.10 given the small sample size of most strains each having two or three independent biological replicates. Pearson (parametric) correlation coefficients, Spearman (non-parametric) coefficients,
and R-squared values were calculated in SAS version 9.1 using mean state 3 rates and mean Halothane EC50 from all biological replicates of a given gene knockdown (Figures 3 and 5). Parametric analyses provide greater power for small sample sizes, whereas non-parametric analysis is more robust to outliers; thus, results of both statistical analyses are provided, where relevant.

Appendices
See Supporting Figures (S1–S4).

Supporting Information

**Figure S1** Correlation of target gene expression with complex I OXPHOS capacity for all complex I knockdown strains in C. elegans. Mean RNA knockdown of each target gene (assessed in whole worm populations) alone does not predict complex I-dependent respiratory capacity (state 3) of each corresponding mutant strain (assessed in intact mitochondria isolated from separate populations of each worm strain). Each point represents average knockdown and malate-dependent state 3 OXPHOS rate for a particular subunit from 3 replicate experiments. Apparent lack of correlation between relative transcript knockdown and complex I function may relate to limited transcriptional analyses performed due to the experimental model used. However, individual subunits appear to differ in their biologic contribution to complex I respiratory capacity. Red diamond indicates N2. Found at: doi:10.1371/journal.pone.0006607.s001 (0.01 MB PDF)

**Figure S2** Compilation of isolated mitochondria complex I-dependent OXPHOS mean rates for all complex I knockdown strains in C. elegans using malate as a substrate. Uncoupled rates in the presence of dinitrophenol (DNP) are not substantially higher than respective state 3 (near-maximal ADP stimulated) or high ADP (utilizing non-rate limiting ADP concentrations) rates for each mutant. Mitochondrial viability following uncoupling is confirmed by robust TMMP plus ascorbate stimulated cytochrome C-dependent OXPHOS capacity. Found at: doi:10.1371/journal.pone.0006607.s002 (0.02 MB PDF)

**Figure S3** Respiratory control analysis in complex I knockdown strains. Malate-dependent mean respiratory control ratios (RCR), defined as state 3 rate/state 4 oxygen consumption rates, in isolated mitochondria of C. elegans complex I mutants. Among the 12 complex I mutants with impaired respiratory control, no consistent or similar magnitude decrease is observed in mean state 3 rate (r = 0.56). Error bars indicate standard deviation. Asterisks indicate p<0.0015 (to account for multiple hypothesis testing), except for D2030.4 where p = 0.0016. Found at: doi:10.1371/journal.pone.0006607.s003 (0.02 MB PDF)

**Figure S4** Correlation of relative mean complex I-dependent respiratory capacity and complex I content in C. elegans RNAi-generated complex I knockdown strains. An overall modest correlation is present between impaired mitochondrial complex I-dependent respiratory capacity assessed by polarography and impaired complex I content assessed by BNG electrophoresis (Pearson r = 0.30, p = 0.014). While a very strong correlation (Pearson r = 0.95, p = 0.2) is seen between complex I respiratory function and assembly among subcomplex IΔ core subunits, this does not reach significance; this may in part be based on analysis of only three subunits including the missense mutant (gas-1(fc21)), which has the greatest impairment in both respiratory capacity and content (Spearman r = 0.50, p = 0.25). Similarly, subcomplex IΔ accessory subunits do not appear to affect complex I content (Spearman r = −0.30, p = 0.62). This preliminary analysis is suggestive that core subunits may be crucial for holocomplex assembly/stability and activity, whereas accessory subunits are not. However, definitive conclusions are limited by the small number of subunits in which complex content was studied. Of note, two subunits localizing to both subcomplexes 1 and 1B, presumably located at their interface, also appear to have a very high correlation between complex I function and content. Statistical analyses are included, as described in Figure 3. Found at: doi:10.1371/journal.pone.0006607.s004 (0.05 MB PDF)

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Author Contributions
Conceived and designed the experiments: MJF PGM MMS. Performed the experiments: MJF EP PGM MMS. Analyzed the data: MJF EP ZC PGM MMS. Contributed reagents/materials/analysis tools: MJF PGM MMS. Performed the bioinformatics analysis: EP. Contributed reagents/materials/analysis tools: PS. Assisted with mass spectrometry studies: EK. Contributed reagents/materials/analysis tools: MR. Conducted blue native gel quantitation, Matthew Deardorff, M.D., Ph.D. for his assistance with mass spectrometry studies, David Kehres, Ph.D. and Charles Hoppel, Ph.D. for their assistance with blue native gel electrophoresis quantitation, Matthew Deardorff, M.D., Ph.D. for his genomics expertise, as well as Britton Chance, Ph.D. for his critical review of this manuscript.

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