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Mitochondrial DNA Variant in COX1 Subunit Significantly Alters Energy Metabolism of Geographically Divergent Wild Isolates in *Caenorhabditis elegans*

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Abstract

Mitochondrial DNA (mtDNA) sequence variation can influence the penetrance of complex diseases and climatic adaptation. While studies in geographically defined human populations suggest that mtDNA mutations become fixed when they have conferred metabolic capabilities optimally suited for a specific environment, it has been challenging to definitively assign adaptive functions to specific mtDNA sequence variants in mammals. We investigated whether mtDNA genome variation functionally influences *Caenorhabditis elegans* wild isolates of distinct mtDNA lineages and geographic origins. We found that, relative to N2 (England) wild-type nematodes, CB4856 wild isolates from a warmer native climate (Hawaii) had a unique p.A12S amino acid substitution in the mtDNA-encoded COX1 core catalytic subunit of mitochondrial complex IV (CIV). Relative to N2, CB4856 worms grown at 20 °C had significantly increased CIV enzyme activity, mitochondrial matrix oxidant burden, and sensitivity to oxidative stress but had significantly reduced lifespan and mitochondrial membrane potential. Interestingly, mitochondrial membrane potential was significantly increased in CB4856 grown at its native temperature of 25 °C. A transmitochondrial cybrid worm strain, *chpIR* (*M*, CB4856 > N2), was bred as homoplasmic for the CB4856 mtDNA genome in the N2 nuclear background. The cybrid strain also displayed significantly increased CIV activity, demonstrating that this difference results from the mtDNA-encoded p.A12S variant. However, *chpIR* (*M*, CB4856 > N2) worms had significantly reduced median and maximal lifespan relative to CB4856, which may relate to their nuclear–mtDNA genome mismatch. Overall, these data suggest that *C. elegans* wild isolates of varying geographic origins may adapt to environmental challenges through mtDNA variation to modulate critical aspects of mitochondrial energy metabolism.

Keywords

N2; CB4856; mitochondria; adaptation; bioenergetics

Introduction

Mitochondrial DNA (mtDNA) harbors extensive sequence variation whose patterns trace the evolutionary migration of human populations [1]. Specific mtDNA sequence lineages, or haplogroups, have increasingly been associated with altered risk of complex human diseases ranging from optic neuropathy to cancer [2]. Indeed, correlation of mtDNA variation with geographically defined human populations suggests that mtDNA mutations have become fixed when they conferred metabolic capabilities optimally suited for a specific environment [3]. It has remained a challenge to definitively assign adaptive function to specific mtDNA sequence variants in mammals [4,5], although recent evidence has demonstrated a clear effect of mtDNA sequence variants in *ND1* on human adaptation to altitude [6] and in *ND3* and *COX3* on cardiac function of different inbred mouse lines [7].

Invertebrate model animals, including the non-parasitic soil nematode *Caenorhabditis elegans*, show similar mtDNA-based evolutionary haplogroup clustering by geographic origin [8]. While the N2 Bristol (N2) strain originating in Bristol, England, is the original and most commonly studied wild isolate, other *C. elegans* isolates that originate in diverse locations across the globe from Hawaii (CB4856) to Australia (AB4) have also been

characterized [9]. Indeed, wild isolates are increasingly recognized to differ in basic phenotypic characteristics such as lifespan, social behavior, and brood size [10]. Recent advances have further demonstrated the utility of the nematode to study a host of *in vitro* and *in vivo* mitochondrial phenotypes [11]. Thus, this robust model can permit detailed investigations of functional effects of naturally occurring mtDNA genome variation on inherent metabolic capacity in living animals.

We specifically investigated whether mtDNA genome variation has discernible functional effects in *C. elegans* wild isolates of distinct mtDNA lineages and geographic origins. We resequenced and compared the mtDNA genome of two *C. elegans* wild isolates, N2 from England and CB4856 from Hawaii, that significantly differ in originating continent, latitude, and ambient temperature. Remarkably, we found that the mitochondrial genomes of these two geographically divergent isolates differed by only a single non-synonymous amino acid change, which replaces an alanine with a serine in the N-terminal region of the COX1 subunit of mitochondrial complex IV (CIV). Multidimensional investigations of *in vitro* and *in vivo* mitochondrial functions in these *C. elegans* wild isolates were performed to assess the potential functional effects of this sole mtDNA non-synonymous sequence variant [11,12]. Significant differences in functional mitochondrial parameters were identified between these two isolates and found to generally correlate with predicted effects of the non-synonymous amino acid change in the COX1 subunit that lies within the matrix side of the CIV catalytic core. Attribution of varying functional effects to the specific mtDNA variant were confirmed by analyses in a transmitochondrial cybrid worm strain, *chp1R* (*M*, *CB4856* > *N2*), that was bred to be homoplasmic for the CB4856 mtDNA genome in the N2 nuclear background. This work elucidates specific functional effects of mtDNA sequence variation in an invertebrate model animal, thereby suggesting that *C. elegans* may adapt to natural environmental challenges through mtDNA-based modulation of mitochondrial energy metabolism.

Results

mtDNA genomes of N2 and CB4856 strains differ by a single non-synonymous alanine-to-serine replacement in COX1

In silico comparison of publicly accessible mtDNA genome sequences from *C. elegans* wild isolates [8] was initially performed to reveal that 5 non-synonymous and 35 synonymous single nucleotide variants (SNVs) existed between N2 and CB4856 (Table 1). To validate these homoplasmic SNVs, we performed manual Sanger-based analyses with CB4856 and N2 mtDNAs. This resequencing of 93.5% (12,912 of 13,813 base pairs) of the CB4856 mtDNA genome and 73.6% of the N2 mtDNA genome provided coverage for 97.8% and 86.7% of the 12 protein-coding mtDNA genes in CB4856 and N2, respectively (Fig. 1a). Resequencing also confirmed the previously reported 28 synonymous SNVs and an A-to-G tRNA^{leu} mutation at the 27th nucleotide position of the tRNA, while 3 previously reported synonymous SNVs were confirmed to be absent and 4 sites originally identified to represent synonymous SNVs were not resequenced in CB4856 (although one of these was the 8540 variant that our N2 resequencing showed was not present) (Table 1). In addition, 6 novel synonymous SNVs were identified, 5 of which were located in an area of the mtDNA

genome that was not originally reported in the public National Center for Biotechnology Information (NCBI) sequence. Most importantly, resequencing validated only a single non-synonymous SNV between the wild isolates: a G-to-T transversion at base pair 7878 (m. 7878G > T) occurring in CB4856 that falls within the CIV subunit I gene, *coxI*, and results in the replacement of alanine with serine (p.A12S) (Table 1).

Subsequent massively parallel sequencing in CB4856 verified the Sanger sequencing results and provided additional coverage of the complete mtDNA genome, including all protein-coding genes, tRNAs, and both (16S and 12S) ribosomal RNAs. However, it did not identify any additional non-synonymous variants relative to N2 (data not shown). Therefore, the other 4 non-synonymous variants originally suggested by analysis of public NCBI sequence data [8] must represent errors in the public CB4856 sequence.

To assess potential structural consequences of the confirmed *coxI* non-synonymous SNV, we performed *In silico* modeling of the *C. elegans* COX1 protein structure based on that reported for *Bos taurus* COX1 (Fig. 1b). The non-synonymous amino acid substitution falls near the N-terminus of the protein in an eight-amino-acid string and alters the hydrogen bonding of this looped structure. This string is not highly evolutionarily conserved across disparate taxa and is not present in mammalian COX1. The *coxI* SNV also is not present in two other geographically isolated *C. elegans* wild strains (CB4855 from Palo Alto, California, and AB4 from Adelaide, Australia) for which mtDNA genome sequence was publicly available in NCBI and which we confirmed by Sanger sequencing. COX1 analysis in the NCBI UniProt Database suggests that COX1 is most likely located on the matrix side as opposed to being embedded in the inner mitochondrial membrane. Human COX1 starts in the matrix and then is followed by multiple transmembrane domains[†]. Although only the trans-membrane domains of COX1 are annotated in *C. elegans*, its first predicted transmembrane domain begins distal to the p.A12S variant and N-terminal loop structure, providing further suggestion that the p.A12S variant region of *C. elegans* COX1 is located in the mitochondrial matrix[‡].

Generation of a transmitochondrial cybrid worm strain that is homoplasmic for the CB4856 mtDNA genome in the N2 nuclear background

To determine the functional consequences of the sole mtDNA mutation in *coxI*, we mated N2 and CB4856 strains to obtain a transmitochondrial cybrid strain that contained the CB4856 mtDNA genome (harboring the *coxI* m.7878G > T mutation) in the N2 wild-type nuclear background. Verification that vertical transmission of mtDNA occurs in *C. elegans* through the hermaphrodite and not through males was first performed by sequencing the individual offspring of matings between N2 hermaphrodites and CB4856 males for the mtDNA *coxI* variant and a nuclear variant in *npr-1* [13], as well as in offspring of the reciprocal mating experiment. These analyses confirmed that mtDNA genome transmission occurs exclusively through the hermaphrodite in *C. elegans*.

[†]http://www.uniprot.org/uniprot/P00395#section_interactions

[‡]http://www.uniprot.org/uniprot/P24893#section_attribute

We then backcrossed CB4856 hermaphrodites and N2 males for at least eight generations with sequence verification of offspring. A novel strain that was found to be homoplasmic (by Sanger sequencing) for the CB4856 mtDNA genome in the N2 nuclear genome background was named per *C. elegans* nomenclature as *chp1R* (*M*, *CB4856* > *N2*). All subsequent functional analyses were performed comparing *chp1R* (*M*, *CB4856* > *N2*) to both parent strains (N2 and CB4856).

CIV enzyme activity was increased in isolated mitochondria of CB4856 and *chp1R* (*M*, *CB4856* > *N2*) relative to N2

Reagents commonly used to analyze CIV activity such as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and dodecyl- β -maltoside, respectively, alter COX kinetics and can monomerize the enzyme [14]. Therefore, we determined COX specific activity in the absence of TMPD and dodecyl- β -maltoside by titrating increasing amounts of bovine-heart-derived cytochrome *c*, which can also reveal possible enzyme differences such as sigmoidal *versus* hyperbolic kinetics [15,16]. Interestingly, isolated mitochondria from all three strains produced hyperbolic kinetics with similar K_m values of COX for cytochrome *c* [8.3 μ M, N2; 10.6 μ M, CB4856; 9.4 μ M, *chp1R* (*M*, *CB4856* > *N2*)] (Fig. 2a). N2 showed a maximal catalytic rate of 73 nmol O₂/min/mg protein. In comparison, COX specific activity at maximal turnover was increased by 47% in CB4856 and by 110% in *chp1R* (*M*, *CB4856* > *N2*). Thus, the CB4856 and cybrid strains harboring the p.A12S mutation had significantly increased CIV enzyme activity relative to N2.

Interestingly, no differences in isolated mitochondria of wild isolates were seen on blue native polyacrylamide gel electrophoresis (PAGE) analyses of CIV activity within respiratory chain supercomplexes (Fig. 2b) or of overall supercomplex assembly (Fig. 2c). COX1 protein level quantitation by Western blot analysis of isolated mitochondria was also unchanged between N2 and CB4856 (Fig. 2d). Collectively, these data suggest that the p.A12S substitution in CB4856 increases its specific CIV enzyme activity without increasing COX1 or CIV protein content in mitochondria.

Since enzyme activity rates can be regulated by post-translational modifications, such as phosphorylation, we investigated whether the replacement of an alanine with a potentially phosphorylatable serine in CB4856 altered a phosphorylation site in COX1. Using a phosphorylation prediction algorithm, Scansite [17], *In silico* analysis suggested that COX1 in CB4856 has an additional binding target of the ERK2 kinase that is not present in N2. Under medium stringency selection conditions, ERK2 binding sites were gained in CB4856 at L11 (YKKYQGGLSVWLESS) and with increased likelihood at V13 (KYQGGLSVWLESSNH). Under high stringency conditions, only V13 remained a binding site for ERK2 in CB4856. ERK2 is known to localize to mitochondria [18], where it has been previously shown to function as a key kinase that modulates mitochondrial respiration by affecting complex V (CV; ATP synthase) function [19] and membrane potential [20]. ERK2 also shares 82% protein similarity by length to the *C. elegans* kinase, MAPK-1[§].

[§]www.wormbase.org

Collectively, these data suggested that the A12S non-synonymous substitution in COX1 might increase mitochondrial CIV activity through the predicted gain of a MAPK-1 binding site and subsequent phosphorylation of the new serine in the COX1 subunit of CIV. *in vitro* confirmation of the predicted phosphorylation site by mass spectrometry (MS) represents a challenge due to low sequence coverage for this highly hydrophobic subunit [15,21]. Indeed, phosphoproteomics analysis performed at the level of unfractionated mitochondria isolated from CB4856 [22] failed to either confirm or exclude the presence of a phospho-site at p.A12S (data not shown).

Integrated respiratory capacity was similar in isolated mitochondria of the wild isolates, except for relative uncoupling in the transmitochondrial cybrid strain

Polarographic analysis of freshly isolated mitochondria from the two wild isolates and the cybrid strain showed no differences between the wild isolates but a consistent trend toward lower state 3 (near-maximal respiratory capacity) rates with substrates that donate electrons specifically at complex I (CI; malate), complex II (CII; succinate), or cytochrome *c* and CIV (TMPD/ascorbate) in the cybrid strain, *chp1R* (*M*, *CB4856* > *N2*) relative to either wild isolate (Fig. 3a). State 4 rates also showed a decreased trend in mitochondria isolated from the cybrid strain (Fig. 3b). These changes translated to a significant decrease in the respiratory control ratio (RCR = state 3/state 4 rates) of CB4856 relative to the cybrid strain and a trend toward decrease relative to *N2*, when the integrated OXPHOS (oxidative phosphorylation) capacity of CI through CIV were tested with malate ($p < 0.05$) (Fig. 3c). These findings suggested that the *N2* nuclear background supports more tightly coupled OXPHOS capacity than does the CB4856 nuclear background when worms are grown at 20 °C. No significant differences were seen in mitochondrial coupling efficiency (ADP/O ratio) between any of the three strains (Fig. 3d), suggesting that the moles of ATP synthesized per atom oxygen consumed are not affected by the p.A12S variant.

Relative *in vivo* mitochondrial membrane potential in CB4856 was reduced when grown at 20 °C and increased when grown at 25 °C

Respiratory chain CI, CIII, and CIV generate the proton gradient across the inner mitochondrial membrane that is then dissipated by CV (ATP synthase) to generate ATP. The relative uncoupling of oxidation to phosphorylation that was seen in CB4856 relative to *N2* by integrated respiratory capacity analysis of isolated mitochondria (Fig. 3c) was suggestive that CB4856 may have a lower mitochondrial membrane potential (Ψ_m). Therefore, *in vivo* analysis of relative Ψ_m in both wild *C. elegans* isolates was performed by quantitation of tetramethylrhodamine ethyl ester (TMRE)-based mean fluorescence within their mitochondria-dense terminal pharyngeal bulb (PB) [11]. Indeed, CB4856 had a decreased *in vivo* membrane potential as conveyed by a 39.4% decrease relative to *N2* in terminal PB mean TMRE fluorescence at 20 °C ($p < 0.001$) (Fig. 4a).

To investigate whether the observed decrease in mean TMRE fluorescence might be attributable to diminished mitochondrial content, we performed *in vivo* terminal PB fluorescence quantitation analysis in nematodes fed MitoTracker Green FM [11]. This fluorescent dye is a monovalent cation that accumulates in mitochondria largely independent of membrane potential and covalently binds its chloromethyl group with sulfhydryls of inner

mitochondrial membrane proteins (Invitrogen, Carlsbad, CA). No difference was seen between the two wild isolates by analysis of relative mitochondrial content based on MitoTracker Green FM (Fig. 4b). Therefore, the relatively decreased membrane potential of CB4856 was not attributable to an alteration of mitochondrial amount.

As another indicator of mitochondrial mass, we also assessed the relative mtDNA genome content of the two wild isolates by quantitative polymerase chain reaction (qPCR) analysis of the mtDNA-encoded CI subunit gene, *nd4*, relative to the nuclear-encoded *drs-1* housekeeping gene [23] in both wild isolates at the young adult stage. Interestingly, CB4856 young adults had a 30% reduction in mtDNA genome content relative to N2 (Fig. 5). Since relative mtDNA depletion could plausibly be attributed to strain differences in egg numbers within young adult worms (Bernard Lemire, personal communication), relative mtDNA content was also quantified in L1 larval stage animals that do not have eggs. CB4856 L1 stage larvae showed a 50% reduction in mtDNA content relative to N2 (Fig. 5). Therefore, mtDNA depletion was consistently seen in CB4856 relative to N2 worms in both early larval and young adult stages. Altered mtDNA content likely represents the influence of nuclear gene variation between these two isolates [9], as has been well-characterized regarding mtDNA content in mammalian mitochondria [24].

To assess whether environmental temperature quantifiably influenced mitochondrial metabolism in the wild isolates from different climatic origins, we assessed *in vivo* TMRE-based Ψ_m in both isolates maintained for at least three consecutive generations at either 15 °C or 25 °C (Fig. 4a). Maximal Ψ_m in both strains was seen at 15 °C relative to either 20 °C or 25 °C. However, the relative Ψ_m of N2 dropped as environmental temperature increased. Furthermore, the membrane potential of CB4856 was significantly lower at 20 °C relative to either 15 °C or its more native environmental temperature of 25 °C. In fact, whereas Ψ_m was significantly greater in N2 than CB4856 at both 15 °C and 20 °C ($p < 0.001$), the reverse was seen at 25 °C ($p < 0.001$). These data indicated that environmental temperature significantly influences mitochondrial membrane potential in a genetic-background-dependent fashion.

chp1R (*M*, CB4856 > N2) worms grown at 20 °C also had a 9.6% decreased mitochondrial membrane potential relative to N2 ($p < 0.05$) (Fig. 4c), although it did not reach the same degree as seen in CB4856 (Fig. 4a). However, *chp1R* (*M*, CB4856 > N2) also showed a significant small increase by 7.3% in their mitochondrial content relative to N2 ($p < 0.001$) (Fig. 4c). Given this relatively increased mitochondrial content, *chp1R* (*M*, CB4856 > N2) membrane potential per mitochondrion may possibly be further decreased than is conveyed by isolated interpretation of TMRE-based membrane potential. Thus, *chp1R* (*M*, CB4856 > N2) displays a similar reduction in mitochondrial membrane potential as occurs in CB4856 relative to N2. The mtDNA-encoded p.A12S variant of COX1 further appears to directly influence mitochondrial membrane potential in living worms.

***in vivo* matrix oxidant burden and *in vitro* oxidative stress sensitivity were increased in CB4856 relative to N2**

To investigate the potential impact of altered mitochondrial coupling and membrane potential seen in the wild isolates on oxidative stress, we assessed the relative *in vivo* matrix

oxidant burden (i.e., the balance of oxidant production and oxidant scavenging) in the mitochondria-dense terminal PB of living worms by MitoSOX mean fluorescence quantitation [11]. This dye localizes via its triphenylphosphonium cation to mitochondria where it accumulates 100- to 1000-fold, reacts first with superoxide and then any other mitochondrial oxidant, and ultimately fluoresces upon binding to mtDNA. CB4856 showed a 31.3% increase in mean MitoSOX terminal PB fluorescence relative to N2 ($p < 0.001$) (Fig. 6a). These data suggested that the steady-state mitochondrial matrix oxidant burden is increased in CB4856 relative to N2 nematodes. Interestingly, no differences were seen between the *chpIR* (*M*, CB4856 > N2) and N2 in levels of matrix oxidants (data not shown). However, since the mitochondrial content of *chpIR* (*M*, CB4856 > N2) was increased, this may account to actually a slight decrease in oxidant burden per mitochondrion.

Relative *in vitro* oxidant production was also quantified by Amplex red assay of isolated mitochondria from both wild isolates at baseline and under conditions of oxidative stress. No difference in basal concentrations of hydrogen peroxide were seen when mitochondria were maximally stimulated with NADH, malate, and succinate (Fig. 6b). However, upon addition of an oxidative stressor (antimycin A, which binds to the Q_i site of complex III (CIII) [25] to increase electron leak and superoxide production [26]), CB4856 mitochondria showed a 62.3% increase in mean hydrogen peroxide concentration relative to N2 ($p < 0.05$). These data suggest that the increased *in vivo* mitochondrial superoxide burden in CB4856 (Fig. 6a) may relate to their being under relatively increased oxidative stress when studied at 20 °C.

CB4856 and *chpIR* (*M*, CB4856 > N2) worms were short-lived relative to N2

Lifespan analyses were performed to assess the cumulative impact on animal health of the diverse variations in mitochondrial physiology observed in the wild isolates [27]. Significant reduction was observed in both median and maximal lifespan of CB4856 worms grown at 20 °C relative to N2 ($p < 0.0001$) (Fig. 7a). Mean lifespan of nematodes grown at 20 °C was 17 days for N2 compared to 11 days for CB4856 (Fig. 7a). These results were confirmed by replicate analysis (Fig. 7b) and were highly consistent with previously published lifespan analyses of these wild isolates [10,28]. It is possible that the shorter lifespan of CB4856 worms at 20 °C may be influenced by their relatively increased mitochondrial oxidant burden and oxidative stress sensitivity. Lifespan analysis of the cybrid strain, *chpIR* (*M*, CB4856 > N2), revealed that it had a median lifespan of 11 days whereas CB4856 had a median of 12 days. This further decrease in median and maximal lifespan relative to CB4856 ($p < 0.001$) (Fig. 7b) may be indicative that an adverse impact on animal physiology occurs in the setting of nuclear–mitochondrial genome mismatch.

Discussion

We used the model organism *C. elegans* to investigate whether mtDNA genome sequence variation has functional significance that underlies geographic adaptation. *C. elegans* wild isolates of distinct geographic origins and native environmental temperatures were studied to determine whether non-synonymous mutations in mtDNA-encoded respiratory chain complex subunit proteins directly influence mitochondrial energy capacity. Remarkably, we

showed that the mitochondrial genomes of the N2 wild isolate from England and the CB4856 wild isolate from Hawaii have no functionally significant protein-coding sequence variants except for a non-synonymous amino acid change that replaces an alanine with a serine (A12S) in the CIV COX1 subunit (Fig. 1a).

A host of investigations into mitochondrial biochemical parameters at both *in vitro* and *in vivo* levels was performed to determine if this mtDNA-encoded sequence variant directly modulates critical aspects of mitochondrial energy metabolism (Table 2). Relative to N2 worms grown at 20 °C, CB4856 showed significantly increased CIV activity in isolated mitochondria (Fig. 2a); significantly decreased *in vivo* mitochondrial membrane potential (Fig. 4a); no difference in integrated OXPHOS near-maximal capacity, coupling, or efficiency (Fig. 3); increased *in vivo* mitochondrial matrix oxidant burden (Fig. 6a); increased *in vitro* oxidant production with oxidative stress (Fig. 6b); and decreased median and maximal lifespan (Fig. 7). No significant differences were observed between the two wild isolates in terms of their relative *in vivo* mitochondrial content (Fig. 4b), COX1 protein level (Fig. 2d), or supercomplex assembly (Fig. 2c).

To definitively assign which of these functional differences in mitochondrial physiology were attributable to the sole non-synonymous mutation, we generated a transmitochondrial cybrid *C. elegans* strain with the mtDNA genome of CB4856 that has the *cox1* A12S mutation in the N2 nuclear background. This cybrid strain, named *chpIR* (*M*, *CB4856* > *N2*), was generated by backcrosses after experimentally verifying that mtDNA inheritance occurs only through hermaphrodites in *C. elegans*. Functional analyses in this cybrid nematode strain demonstrated that it had significantly increased CIV activity (Fig. 2a) and mitochondrial content (Fig. 4c), along with significantly decreased membrane potential (Fig. 4c) and lifespan relative to N2 (Fig. 7b). The cybrid worms also showed increased OXPHOS coupling (Fig. 3c) relative to CB4856. No significant differences were seen in levels of *in vivo* matrix oxidant burden (data not shown) or integrated respiratory capacity in isolated mitochondria of the cybrid strain, although a consistently decreased trend was apparent for CI, CII, and CIV-based respiration (Fig. 3a and b). All together, these data suggest that mitochondrial metabolism in *C. elegans* wild isolates of varying geographic origins is directly influenced by mtDNA genome variation.

Although the *cox1* mutation clearly affects CIV activity, nuclear genome factors appear to regulate other aspects of their mitochondrial function since not all differences found to occur between CB4856 and N2 were seen when comparing *chpIR* (*M*, *CB4856* > *N2*) with N2 (Table 2). While CB4856 had increased matrix oxidant burden, similar levels of *in vivo* oxidant burden were seen in *chpIR* (*M*, *CB4856* > *N2*) as in N2. This could potentially be attributable to the N2 and *chpIR* (*M*, *CB4856* > *N2*) strains' similar nuclear background that encodes the mitochondrial enzymes to scavenge reactive oxygen species, such as superoxide dismutase and glutathione peroxidase. However, the increased level of matrix oxidant burden seen in CB4856 could also be influenced by a known nuclear mutation in *npr-1* that leads to altered feeding behavior in these worms, such that they tend to clump in groups and feed at the edge of bacterial lawns. Such behavior creates local hypoxic environments that could, in turn, potentially influence oxidative stress generation.

Several of the observed differences in multidimensional aspects of mitochondrial physiology may potentially relate to structural and genomic effects of the specific non-synonymous amino acid change present in the COX1 protein, which is the most evolutionarily conserved subunit of CIV that lies at its catalytic core within the inner mitochondrial membrane [29]. The A12S substitution alters local hydrogen bonding to cause a conformational change of the COX1 protein's loop structure. *In silico* modeling suggested that the *cox1* mutation in CB4856 increased affinity at one, and possibly a second, ERK2 (MAPK-1) kinase binding residue that is adjacent to the phosphorylatable serine variant. Indeed, phosphorylation has emerged as an important mechanism to modulate RC activity, including direct effects at CIV [30]. ERK2, in particular, has been shown to alter relevant mitochondrial properties [31]. Thus, increased COX1 binding affinity of the *C. elegans* ERK2 homolog, MAPK-1, in CB4856 may be partially responsible for the increased CIV activity relative to N2.

Preliminary studies at the level of isolated mitochondria did not provide sufficient insight to either confirm or refute the possibility that phosphorylation is altered in the CB4856 COX1 protein. Given the predicted matrix localization of the p.A12S variant in COX1, it is not clear whether cytosolic kinases such as ERK are able to function on matrix-localized proteins. However, it remains theoretically possible that the p.A12S changes a phosphorylation site by other kinases. For example, PKA (protein kinase A) has recently been suggested to regulate COX function via matrix-side phosphorylation events [32], although whether PKA is active within the mitochondrial matrix remains an intense area of research, with evidence supporting both possibilities [33,34]. Overall, matrix-side phosphorylation remains a reasonable potential mechanism by which a sequence variant can alter protein function, albeit a complex one which will require highly sensitive additional analyses to decipher.

Further, our investigation of whether the non-synonymous substitution in the N-terminal string of COX1 might directly reduce CIV affinity to form structural RC supercomplexes that collectively generate the proton gradient across the inner mitochondrial membrane revealed no alterations either in CIV activity within the supercomplexes (Fig. 2b) or in the relative amounts of supercomplexes between the two wild isolates (Fig. 2c). Thus, the reduced membrane potential and increased CIV activity observed in CB4856 did not result from an obvious alteration of RC supercomplex assembly. However, the assays performed may lack sensitivity to detect changes of the magnitude observed by *in vitro* CIV enzyme activity.

The shorter lifespan of CB4856 worms at 20 °C relative to N2 is consistent with previously published analyses [28]. Although reduced longevity was associated in our study with increased *in vivo* mitochondrial matrix oxidant burden and *in vitro* mitochondrial oxidant production [11], the degree of lifespan reduction may not be evolutionarily unfavorable since adult CB4856 worms still live well beyond the egg-laying period. The further decreased lifespan in *chp1R* (*M*, CB4856 > N2) worms is intriguing and could be related to detrimental effects from the mismatch of nuclear and mitochondrial genomes in the cybrid strain, as has recently been observed in copepods [35], drosophila [36,37], and mammals [7,38,39].

It is notable that, while both “wild” *C. elegans* isolates harbor an extensive range of structural and single nucleotide variations that affect their nuclear genes [40,41], only a single non-synonymous amino acid substitution was identified in their mtDNA genomes despite these hermaphroditic animals having passed through up to 1000 generations since their original isolation from the wild [42]. This observation suggests not only that there remains substantial constraint on the acquisition of new non-synonymous mtDNA mutations in this nematode species but also that the unique native environmental niche of CB4856 may have contributed to fixation of the A12S mtDNA non-synonymous COX1 mutation.

mtDNA genome mutations have long been hypothesized to enable populations to adapt to energetic demands of novel climates because of the mitochondria's metabolic role as an environment sensor and its high rate of mutagenesis [1,3,5]. Mitochondrial generation of ATP and heat are inversely linked through RC coupling efficiency, as determined by the proton-pumping efficiency of CI, CIII, and CIV and the physiologic proton gradient dissipation caused by the ATP synthase activity of CV. Whereas “tightly coupled” mitochondria efficiently transform consumed calories into ATP, “loosely coupled” mitochondria require additional calories to generate equivalent amounts of ATP and dissipate some of the energy potential created from electron flow down the RC as heat. While mtDNA variation is a reasonable mechanism for adaptation to nutrient and environmental challenges, proof of the hypothesis ultimately requires more than correlative studies among human populations.

Here, we sought to understand the causality of a single non-synonymous mtDNA mutation located in the catalytic subunit COX1 of CIV on altering multiple complex physiologic parameters between two natural *C. elegans* isolates from very different wild environments. Generation of a transmitochondrial cybrid strain *chpIR* (*M*, CB4856 > *N2*) containing the CB4856 mtDNA genome in the *N2* nuclear genome background confirmed that this mtDNA variant directly influences CIV activity and mitochondrial membrane potential. Thus, naturally occurring homoplasmic mtDNA mutations can influence basic metabolic parameters that may allow adaptation to new environments.

Materials and Methods

Strain growth and maintenance

C. elegans wild isolates, *N2* and CB4856, were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN). Nematodes were grown on nematode growth media (NGM) plates spread with OP50 *Escherichia coli*, per standard technique [43]. For the majority of analyses, worms were grown at 20 °C. However, fluorescence-based mitochondrial membrane potential assays were also performed at 15 °C and 25 °C. Synchronous young adult populations were obtained by bleaching adult worms, plating recovered eggs onto unspread NGM plates, and then transferring L1-arrested animals the following day to NGM plates spread with OP50 *E. coli*.

Lifespan assessment

Lifespan studies were conducted on worms maintained at 20 °C as previously described [11]. To prevent larvae of the generation being studied from reaching adulthood and complicating analyses, we added fluorodeoxyuridine to the OP50-spread NGM plates to 100 µg/ml final concentration.

mtDNA genome sequence analysis

DNA was isolated from nematodes using QIAamp DNA Mini Kit (Qiagen). The protocol for DNA purification from tissues was adapted for nematode DNA isolation following manual homogenization by pestle of a freeze-thawed worm pellet. PCR amplifications were performed in 22.5-µl reactions containing 16.5 µl H₂O, 1.25 µl of each 10 pmol/µl forward and reverse primer, 2.5 µl of 10× buffer with MgCl₂ and 1 µl of dNTP mix (Roche), 5 U of Taq DNA polymerase (Sigma), and 1 µl of isolated DNA. Amplification was carried out by an initial 95 °C denaturation stage followed by 40 cycles of denaturation at 95 °C for 45 s, 30 s of annealing at a primer-dependent temperature (Table S1), and extension at 72 °C for 2 min/kb. mtDNA PCR products were separated by gel electrophoresis in a 1% agarose gel. Bands were excised and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen).

DNA was diluted to a concentration of 10 ng/100 bp of product in 9 µl and mixed with 1 µl of 2 µM primer for forward and reverse reactions. Sequence analysis was conducted using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). After 25 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 5 s, and extension at 60 °C for 4 min, sequencing products were analyzed on a 3730 DNA Analyzer (Applied Biosystems) in the DNA and Protein Core Facility at The Children's Hospital of Philadelphia.

Massively parallel sequencing was conducted on CB4856 using a modified Nextera protocol. First, *C. elegans* mitochondria were prepared as previously described [44] followed by DNA purification using the QIAamp DNA Mini Kit (Qiagen). The tissue purification protocol was followed with the exception of the following adaptations: 10–40 µl of mitochondria with protein concentrations in the range of 20– 50 µg/µl were used in replacement of tissue and DNA was eluted from the column with 100 µl of Buffer AE.

DNA was fractionated by transposon insertion. A total of 5 ng of total isolated DNA was incubated at 55 °C in the presence of 1.5 µl of Amplicon Tagment Mix (Illumina) for 2 min. DNA containing the transposon was amplified by PCR using primers that contained the required sequences needed for sequencing using the Illumina's MiSeq technology and discrete sample indices. Reactions were incubated at 94 °C for 2 min to denature the DNA template and were amplified for 14 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s. Unincorporated oligonucleotides were removed both by QIAquick (Qiagen) and AMPure beads (Beckman Coulter).

Amplicon libraries were quantitated by Nanodrop, pooled together, and diluted to 10 nM. Pooled sample libraries were diluted 1:5 in 10 µl of EBT buffer (Illumina). An equal volume of 0.2 N NaOH was added to the samples. Denaturation reactions were mixed, briefly spun, and incubated at room temperature for 5 min. Reactions were quenched with 980 µl of

hybridization buffer (Illumina). Both the sample and the mock community libraries were diluted 1:1 while the PhiX library was diluted 1:4. Libraries were mixed together at equal volume and were added to the sequencing reagent cartridge according to the manufacturer's instructions (Illumina).

We performed 2× 150-bp paired-end sequencing using the Illumina MiSeq sequencer. Base calling, quality control, and de-multiplexing were performed with the onboard MiSeq Control software and MiSeq Reporter (version 2.1.43). A total of 11,528,243 pairs of reads were obtained for CB4856. Mitochondrial reads were first selected by aligning all reads against the complete *C. elegans* mitochondrial genome sequence (NC_001328.1) using BWA (version 0.6.2-r126). A total of 31,409 (0.27%) of all read pairs could be aligned to the mitochondrial genome. These read pairs were then used for assembling the full-length mitochondrial genome via *de novo* assembly using cap3^{||}. Sequences were combined and analyzed using Sequencher (Gene Codes Corp.).

COX1 protein modeling and phosphorylation site prediction in *C. elegans* wild isolates

The structure of *C. elegans* COX1 was constructed based on that of *B. taurus* cytochrome *c* oxidase (PDB ID: 1OCR) COX1. After homology was determined sufficient for modeling by ClustalW2, model construction and visualization were performed in SWISS-MODEL and DeepView. Predicted phosphorylation sites of COX1 in N2 and CB4856 were determined using the Scansite Motif algorithm [17].

Analysis of CB4856 COX1 for the *In silico* predicted phosphorylation site

Mitochondria were isolated from CB4856 adult worms as previously described [49], with two minor modifications that included (1) An additional slow centrifugation spin (300 g, 10 min, 4 °C) was performed to remove remaining intact worms from the mitochondrial fraction prior to the first high-speed (7000 g) centrifugation spin; and (2) proteinase inhibitors and phosphatase inhibitors were added in all buffer solutions used throughout the mitochondrial isolation procedure beginning immediately after degradation of the outer nematode cuticle. Isolated mitochondrial samples were analyzed for mitochondrial protein phosphorylation as described previously [22]. Briefly, pelleted CB4856 mitochondria (1 mg protein) were re-solubilized in 500 µl Resuspension Buffer [8 M urea, 40 mM Tris (pH 8.0), 30 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂, 1× Roche Complete mini ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor tablet, and 1× Roche PhosSTOP phosphatase inhibitor tablet]. DTT was added to a final concentration of 2 mM and the sample was warmed at 37 °C for 1 h, with intermittent incubation in a sonicating water bath in three 10-min intervals. After cooling to room temperature, iodoacetamide was added to a final concentration of 7 mM, and the sample was vortexed and incubated at room temperature in the dark for 30 min. After DTT was added to a final concentration of 7 mM, 10 µg LysC was added, and the samples were vortexed and incubated at 37 °C for 4 h. The urea concentration was subsequently diluted to 1.5 M by the addition of 40 mM Tris (pH 8.0), 1 mM CaCl₂, and 2 mM MgCl₂. After the addition of 10 µg trypsin, the samples were vortexed and incubated overnight at 37 °C.

^{||}<http://seq.cs.iastate.edu/cap3.html>

After acidifying with 10% trifluoroacetic acid (TFA) (final concentration, ~1%), the peptides were desalted by solid phase extraction using 50 mg tC18 Sep-Pak cartridges (Waters) and dried in a speed vacuum. The sample was resuspended in 1 ml of 80% acetonitrile/0.1% TFA and 5% (50 μ l) was removed, dried in a speed vacuum, and saved at -80°C for analysis of unmodified peptides. The remaining 95% (950 μ l) was subjected to immobilized metal affinity chromatography with magnetic beads (Qiagen) to enrich for phosphopeptides.

Following three washes with water, the beads were incubated in 40 mM EDTA, pH 8.0 for 30 min while shaking, and subsequently washed with water again three times. The beads were then incubated with 100 mM FeCl_3 for 30 min while shaking and were washed three times with 80% acetonitrile/0.1% TFA. The sample was added to the beads and was incubated for 30 min while shaking and subsequently washed four times with 1 ml of 80% acetonitrile/0.1% TFA and eluted for 1 min by vortexing in 100 μ l of 1:1 acetonitrile:0.7% NH_4OH in water. Eluted phosphopeptides were acidified immediately with 5% formic acid and dried in a speed vacuum.

The samples were resuspended in 20 μ l of 0.2% formic acid and subjected to high mass accuracy tandem MS. Briefly, peptides were separated by reversed phase (C18) liquid chromatography on a nanoACQUITY UPLC system and subjected to online infusion into an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., Rockford, IL). For all samples, MS [1] survey scans of peptide cations were performed in the Orbitrap. The phosphopeptide fraction was subjected to MS/MS interrogation (Orbitrap MS [2]) using both (in separate runs) data-dependent selection prior to fragmentation with HCD and data-independent selection targeting the monoisotopic masses of the predicted phosphopeptide—UniProt entry “G4XR22” phosphorylated on serine 12, including charge states +2, +3, and +4 and allowing for 0 to 1 missed site of trypsin cleavage. The unmodified peptides were subjected to data-dependent selection prior to fragmentation with CAD (ion trap MS [2]).

The Open Mass Spectrometry Search Algorithm was used to search peak lists against a concatenated target-decoy database consisting of *C. elegans* proteins (including the protein of interest, “G4XR22”) that are nonsense reverse complements. The *C. elegans* complete proteome set, consisting of reviewed (UniProtKB/Swiss-Prot) and unreviewed (UniProtKB/TrEMBL) protein sequences from UniProt, was utilized as the target protein database (downloaded 10/14/13). In-house software (COMPASS) was used to filter the search results to 1% FDR at the peptide and protein level. For the targeted phosphopeptide runs, the data were also inspected prior to FDR filtering.

CIV enzyme activity assay

Specific COX activity was determined using isolated, solubilized mitochondria in the absence of TMPD and dodecyl- β -maltoside using a setup as previously described [16,45]. Briefly, experiments were performed at 25°C in the presence of 20 mM ascorbate and increasing amounts of cow heart cytochrome c (Sigma) from 0 to 80 μ M. COX activity measurements were performed in 10 mM K⁺ Hepes (pH 7.4), 40 mM KCl, 1% Tween 20, and 1 mM PMSF after sonication using a closed 200- μ l chamber equipped with a micro Clark-type oxygen electrode (Oxygraph system; Hansatech).

Polarographic analysis of integrated respiratory chain capacity in freshly isolated mitochondria

Integrated respiratory capacity analysis was performed using a Clark-type electrode on freshly isolated mitochondria from *C. elegans* grown in liquid culture, as previously described [44]. Within each run, the following oxygen consumption rates were determined and normalized to milligrams of mitochondrial protein: no substrate background, state 3 (near maximal), state 4 (ADP depleted), high ADP (maximal), DNP (uncoupled), and TMPD/ascorbate (CIV specific). Statistical comparison of all oxygen consumption rates for the biological replicates of each strain substrate were performed by random effect ANOVA.

Western blot analysis

Mitochondrial proteins (25 mg/each lane) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 2% nonfat milk in Tris-buffered saline/Tween 20 (0.3%) overnight and then incubated with the monoclonal anti-CIV subunit I (Invitrogen) overnight. SuperSignal kit (Thermo Fisher Scientific) was used for detection.

Blue native gel electrophoresis and in-gel activity staining of CIV enzyme activity in isolated *C. elegans* mitochondria

For the assessment of absolute levels of and CIV, blue native PAGE was performed using isolated nematode mitochondria, as previously described [46,47]. In brief, 600 µg of mitochondrial protein was resuspended in 60 µl of 50 mM NaCl, 2 mM 6-aminocaproic acid, 1 mM EDTA, 0.2 mg/ml DNase, and 2% (w/v) dodecyl-β-maltoside in 50 mM imidazole-HCl (pH 7.0). Following incubation and centrifugation, 5 µl of each samples was loaded on a 5–13% separating gel with a 3.5% stacking gel. For the quantification of supercomplexes, blue native gel electrophoresis was performed as previously described [48]. Isolated mitochondria were solubilized as described above, with the exception that 20% digitonin was used instead of dodecyl-β-maltoside, with a detergent-to-protein mass ratio of 6:1. A total sample volume of 10 µl was loaded on a 3–8% gel.

Following electrophoresis performed in the cold room, the gels were washed twice in 2 mM Tris-HCl (pH 7.5) and subjected to 3,3'-diaminobenzidine-cytochrome c staining (1 mg/ml 3,3'-diaminobenzidine, 24 U/ml catalase, 1 mg/ml cytochrome c, and 75 mg/ml sucrose in 100 mM phosphate buffer, pH 7.6) to visualize CIV. The amount of super-complexes was normalized to the amount of protein by comparing the CV band following staining with Coomassie Brilliant Blue G-250 (Invitrogen).

Relative quantitation of mitochondrial matrix superoxide burden, mitochondrial membrane potential, and mitochondria content by fluorescence microscopy

Fluorescence studies were performed at 15 °C, 20 °C, or 25 °C, as previously described [11]. All worms were grown at the experimental temperature for a minimum of three generations before treatment with a fluorescent dye. Synchronous populations of young adults were moved to 10-cm NGM plates spread with OP50 *E. coli* and either 10 µM MitoSOX Red (matrix oxidant burden), 100 nM TMRE (mitochondrial membrane

potential), or 10 μ M MitoTracker Green FM (mitochondria content) for 24 h. On the following day, the worms were transferred by washing in S. basal onto 10-cm plates spread with OP50 *E. coli* without dye for 1 h to allow clearing of residual dye from the gut. Worms were then paralyzed *in situ* with 10 mg/ml levamisole.

Photographs were taken in a darkened room at a magnification of 160 $\times$ with a Cool Snap cf2 camera (Nikon, Melville, NY). A CY3 fluorescence cube set (MZFLIII; Leica, Bannockburn, IL) was used for MitoSOX and TMRE. A GFP2 filter set was used for MitoTracker Green FM. Respective exposure times were 2 s, 320 ms, and 300 ms for MitoSOX, TMRE, and MitoTracker Green FM. The nematode terminal PB was manually circled and mean intensity of the region was quantified using NIS Elements BR imaging software (Nikon). A minimum of three independent experiments of approximately 50 animals per replicate were studied per strain. Statistical comparison between groups was performed by student's *t*-test (Excel). All of the analyses were performed by a single individual (S.D.D.).

Mitochondrial hydrogen peroxide quantitation

Isolated mitochondria were prepared by differential centrifugation from synchronous young adult nematode populations grown in liquid culture, as previously described [11], and frozen in -80°C . Hydrogen peroxide production from maximally stimulated thawed mitochondria at room temperature was fluorometrically quantified in 96-well microtiter plates using Amplex Red Hydrogen Peroxide Assay Kit (Catalog#: A22188; Invitrogen, Molecular Probes, Carlsbad, CA). Amplex Red reagent in the presence of horseradish peroxidase reacts with hydrogen peroxide to produce a red-fluorescent oxidation product, resorufin (530 nm excitation/590 nm emission). A total of 40 μ g isolated mitochondria from N2 and CB4856 were incubated in a 100- μ l reaction mixture per assay containing 50 μ M Amplex Red reagent, 0.1 U/ml horseradish peroxidase in 50 mM sodium phosphate buffer (pH 7.4), 10 mM malate, 10 mM succinate, and 500 μ M NADH in the presence or absence of 10 μ M antimycin A or catalase (5 U/well in early experiments and 1790 U/well in later experiments). While 5 U/well of catalase did not completely scavenge all hydrogen peroxide, the use in later experiments of higher catalase concentrations (1800 U/well) did completely scavenge hydrogen peroxide levels. These results affirmed that the increased Amplex red concentration reliably reflects hydrogen peroxide levels.

The reaction mixture was incubated for 30 min at room temperature protected from light, after which fluorescence was detected with a microplate reader (Spectramax 250; Molecular Devices, Sunnyvale, CA) at 550 nm excitation and 590 nm emission using a 570-nm cutoff. A hydrogen peroxide standard curve ranging between 0 and 5 μ M was performed on each plate. Data were recorded and analyzed using Softmax PRO 3.1.1 software (Molecular Devices). All of the assays were performed by a single individual (E.P.).

Relative quantitation by qPCR of mtDNA content

Relative levels of mtDNA were measured in synchronous populations of young adult and L1 larval stage worms by comparing levels of the nuclear-encoded housekeeping gene, *drs-1*, to the mtDNA gene, *nd4*. Worms were bleached and recovered eggs were transferred to NGM

plates without bacteria to obtain a synchronous population of L1 stage animals, and the following day, L1-arrested animals were collected. DNA was isolated from either synchronous L1 larval stage or synchronous young adult stage worms grown on NGM plates using QIAamp DNA Mini Kit (Qiagen). DNA concentration was measured spectrophotometrically at 230 nm, 260 nm, and 280 nm wavelengths (Nanodrop ND-100 Spectrophotometer v3.1.2; NanoDrop Technologies, Inc., Wilmington, DE).

A total of 40 ng of DNA was used per qPCR reaction containing Taqman Gene Expression Assays (Applied Biosystems) for the housekeeping gene *drs-1* (Ce02451127_g1) and mtDNA-encoded CI subunit gene *ND4* (Custom Taqman Gene Expression Assay, Applied Biosystems; forward primer sequence: GAGGCTCCTACAACAGCTAGAATAC and reverse primer sequence: TCATACATTGTTGTGTACAAATCTTAAACTACCT). Taqman Universal PCR Master Mix (Applied Biosystems, Roche Inc., Branchburg, NJ) was used with Taqman Gene Expression Assay (Taqman MGB Probes, FAM dye-labeled), per standard Applied Biosystems protocol. Real-time qPCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) and analyzed using the provided software (7500 Software V2.0.1).

Generation of transmitochondrial cybrid worm strain containing CB4856 mtDNA genome in N2 nDNA background

The *chp1R* (*M*, *CB4856* > *N2*) worm strain was obtained by crossing an N2 male with a CB4856 hermaphrodite and then repeatedly crossing the offspring with N2 males. The crossings were conducted for at least eight generations with sequence validation along the way to ensure successful mating events. The offspring obtained were sequence validated as having the N2 nuclear background and the CB4856 *cox1* mutation. The nuclear background was verified by the non-synonymous difference in the gene *npr-1* [13].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

mtDNA	mitochondrial DNA
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV

SNV	single nucleotide variant
NCBI	National Center for Biotechnology Information
CV	complex V
PB	pharyngeal bulb
TMRE	tetramethylrhodamine ethyl ester
TMPD	N,N,N',N'-tetramethyl- <i>p</i> -phenylenediamine
NGM	nematode growth media
EDTA	ethylenediamine tetraacetic acid
TFA	trifluoroacetic acid
MS	mass spectrometry
qPCR	quantitative PCR

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Fig. 1.

C. elegans mtDNA genomes of N2 and CB4856 isolates differ by only a single non-synonymous coding variant, which alters COX1 protein conformation. (a) Mitochondrial genomes of N2 and CB4856 animals were manually resequenced and compared. Red and blue lines represent extent of CB4856 mtDNA genome sequence determined in this study and previously deposited in NCBI [8], respectively. Synonymous and non-synonymous SNVs in CB4856 relative to N2 are indicated by black dashes and black stars, respectively. Primer pair names and locations are indicated within the circle. (b) The SNV in CB4856

(right panel) results in the replacement of a hydrophobic alanine with a hydrophilic serine, which appears by protein modeling to alter the N-terminal loop structure conformation through hydrogen bonding with the COX1 protein core.

Fig. 2. *in vitro* analyses of isolated mitochondrial CIV activity and protein levels, in N2 and CB4856 isolates. (a) COX specific activity was determined in the absence of TMPD and dodecyl- β -maltoside by increasing the concentration of substrate cytochrome *c* (note that higher concentrations of exogenously added cow heart cytochrome *c* are required to reach maximal turnover due to the imperfect fit with *C. elegans* COX). K_m values of COX for cytochrome *c* are 9.4 μ M [*chp1R* (M, CB4856 > N2)], 10.6 μ M (CB4856), and 8.3 μ M (N2). Maximal catalytic rates were 73, 154, and 108 nmol O₂/min/mg total mitochondrial protein for N2, *chp1R* (M, CB4856 > N2), and CB4856, respectively. Experiments were performed in triplicates and each sample was measured three times (error bars indicate standard deviation). (b) The activity of CIV in the respiratory chain supercomplexes was similar between N2 and CB4856. S1–S4 represents the amount of CIV associated with CI and CIII, V₂ represents the amount of CIV associated with CII, and V represents the amount of CIV associated with CIV.

where S1: CI, CIII, CIV; S2: CI, CIII, CIV₂; and so on. (c) The total amount of mitochondrial protein associated with each supercomplex was also similar between these two wild isolates. Molecular mass is shown on the y-axis in kilodaltons and V2 represents the CV dimer. (d) Western blot analysis showed similar COX1 content in isolated mitochondria from both wild isolates (molecular mass is indicated in kilodaltons).

Fig. 3.

Integrated respiratory capacity in isolated mitochondria is similar between N2 and CB4856 isolates but showed a reduced trend in *chpIR (M, CB4856 > N2)* cybrid worms. Polarographic analysis of integrated respiratory capacity was performed with substrates that donate electrons at CI (malate), CII (succinate), or cytochrome *c*/CIV (TMPD/ascorbate). (a) Similar state 3 (near-maximal) and (b) state 4 (ADP depleted) respiratory capacity rates were seen with all substrates tested in CB4856 relative to N2. Values represent mean and standard error of raw values from 10 biological replicates of N2, 3 of CB4856, and 5 of *chpIR (M, CB4856 > N2)*. (c) The relative coupling efficiency as assessed by calculating the state 3/state 4 respiratory control ratio (RCR) was statistically similar between N2 and CB4856, although it trended toward decreased for CI- and CII-based respiration. CI-based RCR was significantly increased in *chpIR (M, CB4856 > N2)* relative to CB4856. (d) No significant difference in the ratio of ADP/O was observed between the three *C. elegans* strains.

Fig. 4.

Significant strain differences were observed in *in vivo* assays of relative mitochondrial membrane potential and mitochondria content. (a) Mitochondrial membrane potential (Ψ_m) was studied in both wild strains at three different temperatures, with peak Ψ_m occurring in both strains at 15 °C. Interestingly, while N2 (black bars) had higher Ψ_m than CB4856 (gray bars) at 15 °C and 20 °C, CB4856 had relatively increased Ψ_m at 25 °C. (b) No significant difference was seen in mitochondria content between CB4856 and N2, as assessed at 20 °C by MitoTracker Green FM mean fluorescence intensity. (c) *chpIR (M,*

CB4856 > *N2*) worms had increased mitochondrial content and decreased membrane potential relative to *N2*. Statistical analyses were performed by student's t-test, where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. For the *in vivo* fluorescence experiments, total number of animals studied per strain and condition are indicated within bars. Bars and error bars represent mean and standard error of the mean, respectively.

Fig. 5.

mtDNA content was reduced in CB4856 relative to N2 worms at both early larval and young adult stages. qPCR analysis of mtDNA-encoded (*nd4*) and nDNA-encoded (*drs-1*) genes showed that relative mtDNA depletion occurred in CB4856 worms relative to N2. Results were similar in both L1 and young adult stage comparisons, which suggested that the difference in mtDNA content cannot be attributed to differences in oocyte content in adult stage worms (from two replicate experiments).

Fig. 6.

CB4856 demonstrated an increased matrix oxidant burden and increased sensitivity to oxidative stress relative to N2. (a) Significantly increased oxidant burden occurs in CB4856 relative to N2, as indicated by 31.3% increase in terminal PB mean MitoSOX fluorescence intensity. Bars and error bars indicate mean and standard error of mean, respectively. Statistical analyses were performed by student's *t*-test, where **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. (b) Baseline levels of H₂O₂ were approximately the same in isolated mitochondria from the two wild isolates. However, CB4856 mitochondria were more sensitive to oxidative stress, evidenced by a 62% increase in the level of hydrogen peroxide measured in the presence of antimycin A (AA). Bars indicate mean and standard error of four biological replicates per strain. M, malate. S, succinate.

Fig. 7. Lifespan analysis of *C. elegans* wild strains at 20 °C. (a) CB4856 has significantly shorter median and maximal lifespan relative to N2 at 20 °C ($p < 0.0001$). (b) Repeat analysis of CB4856 relative to N2 at 20 °C confirmed the strain's decreased lifespan ($p < 0.0001$) and demonstrated that *chp1R* (*M*, *CB4856* > *N2*) cybrid worms have an even further decreased lifespan relative to N2 ($p < 0.0001$).

Table 1

SNVs in protein-coding and ribosomal RNA genes between N2 and CB4856 mtDNA genomes

Genes	NCBI N2 Bristol vs NCBI CB4856		NCBI N2 Bristol vs Resequenced CB4856	
	Synonymous	Non-Synonymous	Synonymous	Non-Synonymous
<i>nd1</i>	1924 G:A		1924 G:A	
	2038 A:C		2038 A:C	
	2210 T:C	–	2210 T:C	–
	2536 C:T		2536 C:T	
	3444 C:T		3444 C:T	
<i>nd2</i>	3546 C:T		3546 C:T	
	3672 C:T	–	3672 C:T	–
	4227 T:C		4227 T:C	
<i>nd3</i>	–	–	–	–
<i>nd4</i>	6616 A:G		–	
	7255 T:C	6639 A:G	7255 T:C	–
	7390 T:C	Tyr→Cys	7390 T:C	
<i>nd4l</i>	–	–	–	–
<i>nd5</i>			12320 A:G	
			12371 C:T	
	No sequence	–	12719A:T	–
			12998 C:A	
			13069 T:C	
<i>nd6</i>	–	–	–	–
<i>ctb-1</i>	4665 C:T		4665 C:T	
	5079 G:A		5079 G:A	
	5127 C:T	–	5127 C:T	–
	5289 T:C		5289 T:C	
	5479 T:C		5479 T:C	
	5496 A:G		5496 A:G	
	8027 G:A		8027 G:A	
	8093 C:T		8093 C:T	
	8177 G:A		8177 G:A	
	8327 G:A	7878 G:T	8327 G:A	7878 G:T
<i>cox1</i>	8429 A:G	Ala→Ser	8429 A:G	Ala→Ser
	8540 A:G		8540 A:G	
	9194 C:T		9194 C:T	
	9227 C:T		9227 C:T	
	9344 G:A		9344 G:A	
<i>cox2</i>	9900 G:A		9900 G:A	
	10104 C:T	–	10104 C:T	–
	–		10239 T:A	
	5737 C:T	5962 A:C	5737 C:T	

Genes	NCBI N2 Bristol vs NCBI CB4856		NCBI N2 Bristol vs Resequenced CB4856	
	Synonymous	Non-Synonymous	Synonymous	Non-Synonymous
<i>cox3</i>	5782 C:T	Trp→Cys	5782 C:T	
	6283 C:T		6283 C:T	–
	6361 G:A	6380 T:A	6361 G:A	
	6397 T:C	Tyr→Asn	–	
<i>atp6</i>	2843 T:C	2923 T:G	2843 T:C	
	2912 A:G	Leu→Trp	2912 A:G	–
	12S	N/A	1221 A:G	N/A
	10408 T:A		–	
16S	10568 T:C		–	
	10808 T:C		–	
	11045 T:C	N/A	–	N/A
	No sequence		11207 A:C	
	No sequence		11208 C:T	
	No sequence		11211 T:C	

The analysis of sequences for N2 and CB4856 that were publicly deposited in NCBI [8] initially suggested that their mtDNA genomes differed by 35 synonymous and 5 non-synonymous SNVs. To evaluate these results, we manually resequenced the entire mtDNA genome of CB4856 and nearly all sites of the mtDNA genome of N2 in which sequence variants were initially reported relative to CB4856. This work confirmed that CB4856 has 34 synonymous variants across the 12 protein-coding mtDNA genes, including 28 that were previously reported in the NCBI sequence as well as 6 additional variants we identified in regions not previously sequenced per NCBI data. In addition, we did not manually resequence the first 3 variants listed in *ctb-1*, but did confirm absent by resequencing 3 variants that had been reported by NCBI sequence in either N2 (the 8540 variant in *cox1*) or CB4856 (1 variant each in *ND4* and *cox3*). Moreover, a single non-synonymous SNV difference between the N2 and CB4856 mtDNA genomes was confirmed. The validated non-synonymous SNV results in an alanine-to-serine substitution in the N-terminus of the COX1 subunit of respiratory chain CIV.

Table 2

Summary comparison of genetic background and observed physiologic differences in N2, CB4856, and *chp1R* (*M*, *CB4856* > *N2*) strains. N.D., not determined. N.S., no significant difference.

		N2	CB4856	<i>chp1R</i> (<i>M</i> , <i>CB4856</i> > <i>N2</i>)
Genetics	Nuclear background	N2	CB4856	N2
	mtDNA COX1	p.12A	p.12S	p.12S
Whole Animal	Lifespan	Baseline	↓25%	↓31%
Mitochondria		Baseline	Relative to N2	Relative to N2
	Matrix oxidant burden		↑31%	↔
	Mitochondrial content		↔	↑7%
	mtDNA content		↓30%	N.D.
	Oxidative stress - induced H2O2 production		↑62%	N.D.
	State 3 respiratory rate		↔	↓ (N.S. trend)
	State 4 respiratory rate		↔	↓ (N.S. trend)
	Respiratory control ratio		↔	↑ relative to CB4856
	ADP/O		↔	↔
	Membrane potential	Relative to N2 at 20°C	Relative to N2 at same temperature	Relative to N2 at 20°C
		15°C	↑30%	↓16%
		20°C	Baseline	↓39%
		25°C	↓20%	↑22%
				N.D.
Complex IV		Baseline	Relative to N2	Relative to N2
	Activity		↑47%	↑110%
	COX1 protein levels		↔	N.D.
	Complex IV activity in respiratory supercomplexes		No detectable change	N.D.
	Complex IV amount in respiratory supercomplexes		↔	N.D.

N.D. denotes not determined.

N.S. denotes not significant.