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Expression of Ndi1p, an alternative NADH:ubiquinone oxidoreductase, increases mitochondrial membrane potential in a *C. elegans* model of mitochondrial disease

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Abstract

The NADH:ubiquinone oxidoreductase or complex I of the mitochondrial respiratory chain is an intricate enzyme with a vital role in energy metabolism. Mutations affecting complex I can affect at least three processes; they can impair the oxidation of NADH, reduce the enzyme's ability to pump protons for the generation of a mitochondrial membrane potential and increase the production of damaging reactive oxygen species. We have previously developed a nematode model of complex I-associated mitochondrial dysfunction that features hallmark characteristics of mitochondrial disease, such as lactic acidosis and decreased respiration. We have expressed the *Saccharomyces cerevisiae NDI1* gene, which encodes a single subunit NADH dehydrogenase, in a strain of *Caenorhabditis elegans* with an impaired complex I. Expression of Ndi1p produces marked improvements in animal fitness and reproduction, increases respiration rates and restores mitochondrial membrane potential to wild type levels. Ndi1p functionally integrates into the nematode respiratory chain and mitigates the deleterious effects of a complex I deficit. However, we have also shown that Ndi1p cannot substitute for the absence of complex I. Nevertheless, the yeast Ndi1p should be considered as a candidate for gene therapy in human diseases involving complex I.

Keywords: Mitochondria; Membrane potential; Nematode; NADH:ubiquinone oxidoreductase; DiS-C_3(3)

1. Introduction

Structural and functional deficiencies of the mammalian NADH:ubiquinone oxidoreductase (Complex I) are causative of a number of human mitochondrial diseases, including cardiomyopathies, encephalomyopathies and neurodegenerative disorders [1–4]. Complex I, located in the inner mitochondrial membrane, is composed of 45 subunits, one flavin mononucleotide (FMN) cofactor and eight iron–sulfur clusters [5]. Defects in complex I can impair the oxidation of NADH and proton pumping, leading to metabolic imbalances and a reduced proton electrochemical gradient across the inner membrane. In addition, complex I defects are often associated with an elevated production of reactive oxygen species (ROS) [6].

The free-living soil nematode *Caenorhabditis elegans* has the advantages of a short life cycle, a simple anatomy and a mitochondrial respiratory chain (MRC) composed of subunits with high sequence identities with their human homologs [7–10]. We have developed a *C. elegans* model of complex I deficiency to further understand the bioenergetic and biochemical consequences of mitochondrial disease [11]. These mitochondrial mutants contain missense mutations in the *nuo-1* gene, the nematode ortholog of the human *NDUFV1* gene, which encodes the 51-kDa FMN-containing, active site subunit of complex I [11]. In this study, we used nematode strains with A352V and A443F amino acid substitutions in the NUO-1 protein to investigate the utility of gene therapy in treating complex I dysfunction.

In contrast to mammals, the mitochondria of *Saccharomyces cerevisiae* lack complex I and instead contain single subunit rotenone-insensitive NADH:ubiquinone oxidoreductases that do not translocate protons [12–14]. The *S. cerevisiae* Ndi1p, with its

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active site facing the matrix, is a single polypeptide chain that catalyzes the oxidation of NADH and the reduction of ubiquinone [12]. It contains noncovalently bound flavin adenine dinucleotide (FAD) and lacks iron sulfur clusters. Ndi1p is synthesized as a 513-amino acid precursor protein containing a 26-residue amino-terminal signal sequence responsible for its import into mitochondria [15]. Ndi1p catalyzes a two-electron transfer reaction that is believed to avoid the formation of an ubisemiquinone intermediate and thus minimize electron leakage and the formation of ROS [16]. Based on its catalytic mechanism and on its simple structure, it has been suggested that Ndi1p may offer a practical gene therapy approach for treating complex I deficiency. Ndi1p expression should restore NADH oxidation without stimulating ROS production [15]. Gene therapy with Ndi1p will be applicable to deficiencies arising from mutations in either nuclear or mitochondrial DNA encoded complex I subunits. Heterologous expression of Ndi1p in bacteria, plants and fungi does not affect the endogenous complex I [15].

In this study, we demonstrate that Ndi1p expression results in significantly improved reproductive success, better survival under conditions of oxidative stress, fewer signs of premature aging, increased respiration rates and a complete restoration of the in vivo mitochondrial membrane potential in *C. elegans nuo-1* mutants. However, Ndi1p is not able to fully replace complex I and cannot support larval development when complex I is missing. We suggest that gene therapy approaches involving Ndi1p may offer substantial clinical benefits in cases of complex I deficiency.

2. Materials and methods

2.1. Worm strains and maintenance

C. elegans strains were cultured on Nematode Growth Medium supplemented with 1 mg/ml G418 antibiotic to select for the retention of the extrachromosomal arrays [8]. We used the following *C. elegans* strains: N2 (Bristol) wild type; LB25 (also referred to as A352V), *nuo-1(ua1)* II, *unc-119(ed3)* III, *uaEx25* [p016bA352V]; and LB27 (also referred to as A443F) *nuo-1(ua1)* II, *unc-119(ed3)* III, *uaEx27*[p016bA443F]; LB52, *nuo-1(ua-1)* II, *unc-119(ed3)* III, *uaEx25* [p016bA352V], *uaEx30*[Plet-858::NDI1, pDP#SU006, pTG96, pPD118.25NEO]; LB53, *nuo-1(ua-1)* II, *unc-119(ed3)* III, *uaEx25*[p016bA352V], *uaEx31*[Pnuo-1::NDI1, pDP#SU006, pTG96, pPD118.25NEO]; LB54, *nuo-1(ua-1)* II, *unc-119 (ed3)* III, *uaEx25*[p016bA352V], *uaEx32*[pDP#SU006, pTG96, pPD118.25NEO]. *uaEx25* and *uaEx27* are extrachromosomal arrays carrying the *nuo-1(A352V)* and the *nuo-1(A443F)* point mutations, respectively.

2.2. Plasmid constructs and the generation of transgenic lines

The *Plet-858::NDI1* expression plasmid was constructed by inserting the entire *NDI1* gene downstream of a 3.5-kb fragment encoding the *let-858* promoter using Gateway Cloning technology [17]. The *Pnuo-1::NDI1* expression plasmid was constructed by inserting the *NDI1* gene downstream of a 0.6-kb fragment encoding the *nuo-1* promoter region [17]. *NDI1* expression constructs were injected independently into the syncytial gonads of young adult LB25 hermaphrodites along with the reporter plasmids, pDP#SU006, pTG96 and pPD118.25NEO. pDP#SU006 expresses GFP under the control of the F25B3.3 promoter, which is active in the nervous system [17,18]. pTG96 expresses a *sur-5*::GFP fusion that localizes to nuclei [19]. pPD118.25NEO contains the neomycin resistance gene under control of the ubiquitous *let-858* promoter. Six independent transgenic lines were isolated for each *NDI1* construct and two were chosen for further study. A control line, LB54, contains the reporter plasmids but does not contain either of the *NDI1* expression plasmids. In all cases except in Fig. 3, data from independent lines were combined.

2.3. Phenotypic analyses

Brood size measurements were conducted as described [11]. Morphological analysis was performed by mounting worms onto 2% agarose pads for observation using a Zeiss Axioskop-2 research microscope equipped with fluorescence optics, Nomarski imaging and a SPOT-2 digital camera (Carl Zeiss Canada Ltd., Calgary, Canada).

2.4. Western blot analyses

Mitochondria isolated from synchronized L4 larvae were isolated and used for Western blot analysis (100 μg protein per lane) as previously described [11]. The yeast strain, YNN214 was used as a positive control for the presence of Ndi1p, while a knockout strain, *NDI1*Δ was used as a negative control.

2.5. Polarographic analyses

Oxygen consumption rates were measured using a Strathkelvin 1302 oxygen electrode with a MT200 Mitocell respiration chamber (Strathkelvin Instruments, Glasgow, UK). Whole-animal respiration rates were obtained using extensively-washed, bacteria-free synchronized L4 larvae [17]. A minimum of ten replicates per strain was performed. Whole mitochondria for respiration analyses were isolated from bacteria-free worms grown in liquid culture. Mitochondria were lysed by hypotonic shock and ultrasonication to allow access of NADH to the matrix-facing active site of complex I and respiration rates were measured as described [11]. Complex I-specific respiration was measured as the NADH-dependent, rotenone-sensitive rate of oxygen consumption.

2.6. Oxidative stress assays

The survival of transgenic strains was measured at room temperature by placing L1 larvae on seeded NGM plates in a chamber continuously flushed with 100% oxygen [17]. Survival was measured after 5 days as the fraction of L1 larvae that developed into adults. A minimum of ten trials were performed per strain.

2.7. Membrane potential determination

Mitochondrial membrane potential was determined using the cationic fluorescent probe, diS-C_3(3) (3,3′-dipropylthiocarbocyanine iodide; Sigma-Aldrich Chemie GmbH (Buchs, Switzerland)). Healthy, well-fed, mixed staged worms were harvested from plates by washing with M9 [20] and centrifuging at 275×*g*. Worms were incubated in M9 for 30 min with gentle shaking to allow digestion of bacteria, washed and stained for 1 h in 4×10^{-6} M diS-C_3(3). The worms were washed four times in M9 and fluorescence measurements were

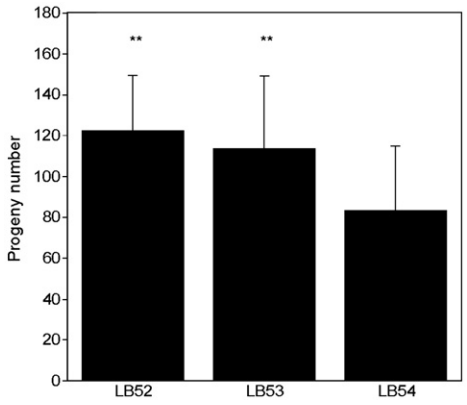

Fig. 1. Brood size analysis of transgenic strains. Hermaphrodites were incubated at 20 °C and the number of progeny produced was counted. Values are the average of a minimum of 34 broods counted. $^{**}P<0.01$ when compared with the LB54 control strain using a two-sample *t*-test.

Table 1
Ndi1p expression increases respiration rates in living animals

Strain	Respiration rate [a]
LB52	6.4±2.1 **
LB53	5.6±0.7 **
LB54	3.3±1.4

[a] Values are expressed as nmol O_2 consumed min^{-1} mg $protein^{-1}$ and are the means±SD of a minimum of ten trials.
** $P<0.01$ when compared to the control line LB54 using a two-sample *t*-test.

performed as described [21] using a PTI spectrofluorophotometer (PTI Technologies Inc., London, Ontario, Canada).

3. Results

We expressed the *NDI1* gene, which encodes the single subunit *S. cerevisiae* NADH dehydrogenase, under the control of the *let-858* and *nuo-1* promoters. Both promoters will drive constitutive expression [17,22]. LB25, also referred to as A352V, is the parent strain for our studies; it is homozygous for the lethal *nuo-1(ua1)* allele and carries an extrachromosomal array with a Ala352Val substituted *nuo-1* gene [17]. LB52 is derived from LB25 by transformation with the *Plet858::NDI1* construct; LB53 is derived from LB25 by transformation with *Pnuo-1::NDI1*. LB54, the control strain, was transformed with the reporter plasmids only.

NDI1 expression improves the overall health and fertility of the A352V mitochondrial mutants [17]. Significant increases of 47% and 36% in the average brood sizes of LB52 and LB53, respectively were observed in comparison with LB54 (Fig. 1).

We have previously shown that the A352V mutant consumes oxygen at approximately half the wild-type rate [11]. We measured the oxygen consumption rates of the *NDI1* expressing strains. LB52 and LB53 showed 94% and 70% higher respiration rates than LB54, respectively (Table 1).

Unlike complex I, Ndi1p catalyzes the oxidation of NADH in a rotenone-insensitive reaction. We predicted that the rotenone-insensitive NADH oxidase activity should increase in *NDI1* expressing strains. Isolated mitochondria of LB52 and LB53 show an increase in the total NADH-dependent rates of oxygen consumption (Table 2), consistent with the increased respiration rates seen in live animals (Table 1). The rotenone-insensitive respiration rates, which have increased 2–3 fold in the Ndi1p expressing strains, account for the entire increases. The rotenone-sensitive rates, attributable to complex I are unchanged in LB52 and LB53 compared to LB54 (Table 2).

Table 2
Ndi1p expression increases rotenone-insensitive respiration rates in isolated mitochondria

Strain [a]	Total respiration [b]	Rotenone-insensitive [b]	Rotenone-sensitive [b, c]
LB52	48±24 (100%)	40±16 (83%) **	8±23 (17%)
LB53	29±10 (100%)	22±4 (76%) **	7±9 (24%)
LB54	23±12 (100%)	12±8 (52%)	11±18 (48%)

[a] The number of replicates performed are 10, 16 and 14 for LB52, LB53 and LB54, respectively.
[b] Values are expressed as nmol O_2 min^{-1} mg $protein^{-1}$ and are the means±SD.
[c] Rotenone-sensitive rates are calculated as the differences between the total and the rotenone-insensitive rates.
** $P<0.01$ when compared with the LB54 control strain using a two-sample *t*-test.

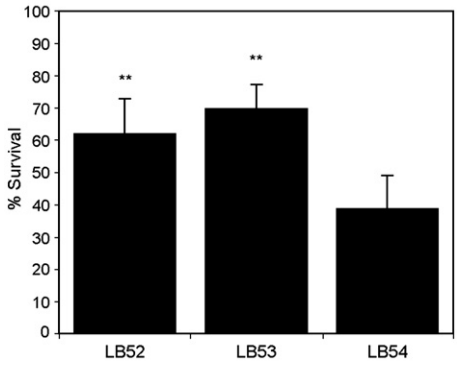

Fig. 2. Survival of transgenic strains under hyperoxia. L1 larvae were incubated at room temperature in a tank continuously flushed with 100% oxygen for 5 days. Animals not responding to touch were scored as dead. Values are the means of a minimum of ten trials with at least 75 animals per trial. **$P<0.01$ when compared with the LB54 control strain using a two-sample *t*-test.

Mitochondria generate ROS as a normal but toxic by-product of respiration [23]. Mitochondrial dysfunction can result in the elevated production of ROS, leading to increased damage of cellular proteins, lipids and DNA [24]. We showed previously that the imposition of an exogenous oxidative stress, such as a 100% oxygen atmosphere, on the *nuo-1(A352V)* mutant overwhelms its already challenged defense mechanisms and results in decreased survival [25]. Ndi1p is believed to catalyze ubiquinone reduction using a two-electron transfer mechanism, thus eliminating the formation of an ubisemiquinone intermediate that might contribute to reactive oxygen species formation. We measured the survival of the Ndi1p expressing strains under hyperoxia; 62% of LB52 and 70% of LB53 animals survived after 5 days in 100% oxygen, while only 39% of LB54 animals survived (Fig. 2). These results indicate that Ndi1p expression renders LB52 and LB53 more resistant to oxidative stress, suggesting that Ndi1p diminishes ROS generation under hyperoxic conditions and probably also under normoxic conditions.

Expression of Ndi1p has marked effects on *C. elegans* fertility and resistance to oxidative stress. It stimulates respiration in live animals and in isolated mitochondria. With an antibody directed against Ndi1p, we used Western blot analysis to investigate whether Ndi1p could be detected in worms (Fig. 3). We detected a strong band migrating at ~53 kDa in mitochondria from the wild type yeast strain YNN214 that was absent in the *ΔNDI1* mitochondria. We detected markedly variable amounts of a 53-kDa band in the LB52 and LB53 mitochondria isolated from

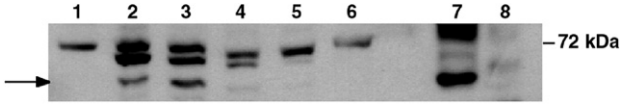

Fig. 3. Ndi1p is expressed in transgenic *C. elegans* strains. Mitochondrial protein from two independent lines of LB52 (lanes 1, 2), LB53 (lanes 3, 4) and LB54 (lanes 5, 6) was loaded (100 μg per lane). Yeast mitochondrial protein from the wild type strain, YNN214 (lane 7) and from the *NDI1* deletion strain (*ΔNDI1*, lane 8) was also loaded as controls.

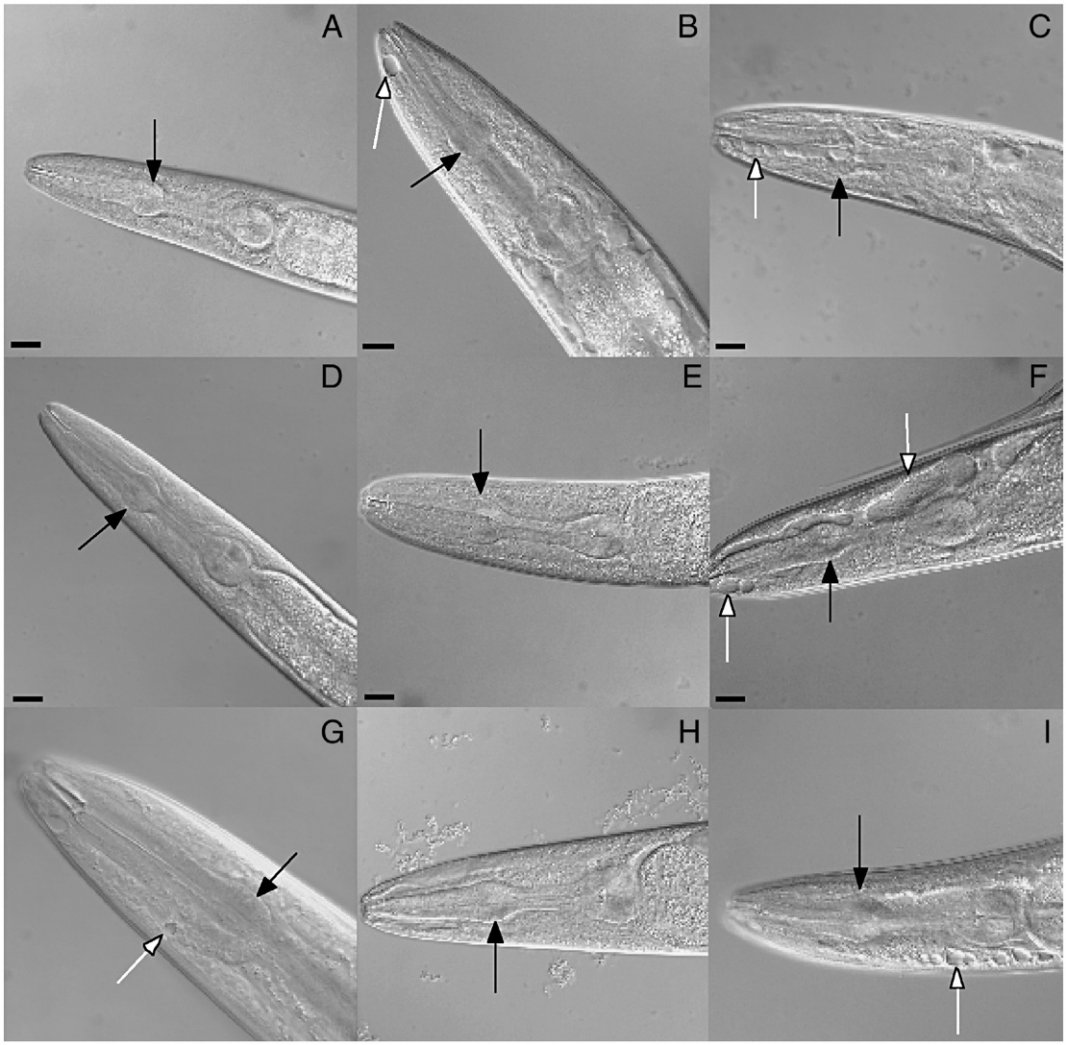

Fig. 4. Vacuole formation is delayed by Ndi1p expression. A classification system was developed to assess the severity of vacuolar structure formation. Representative examples are shown in A–C. (A) No vacuoles; (B) Moderate phenotype (1–3 vacuoles); (C) Severe phenotype (4 or more vacuoles). Representative Nomarski photographs of the head regions of 1-day-old hermaphrodites are shown in D–F. (D) LB52, (E) LB53, (F) LB54. Representative Nomarski photographs of the head regions of 2-day-old hermaphrodites are shown in G-I. (G) LB52, (H) LB53, (I) LB54. Bars = 15 μm. The metacarpus of the pharynx is indicated with a black arrow and examples of vacuolar structures are indicated with white arrows.

independently-derived transgenic lines. This suggests that the expression levels of Ndi1p, the efficiency of Ndi1p targeting to mitochondria or Ndi1p assembly with its flavin cofactor into the *C. elegans* mitochondrial membrane differ between transgenic lines. Alternatively, Ndi1p may be susceptible to degradation in a non-native membrane. The amount of immunologically-detectable Ndi1p does not accurately reflect the amount of functional Ndi1p present. We did not detect any significant amount of staining at 53 kDa in any of our LB54 control lines.

MRC function is closely related to aging and lifespan determination [9]. The A352V mutant shows signs of premature aging as a young adult [11]. The body wall muscle appears to degenerate and large vacuolar structures form beneath the cuticle (Fig. 4). We counted the number of vacuolar structures seen in 1-day- and 2-day-old control or Ndi1p-expressing adults. Worms were classified by the number of vacuolar structures present in the vicinity of the pharynx. Worms with 1–3 vacuolar structures were scored as moderate (Fig. 4B) while worms with 4 or more were scored as severe (Fig. 4C). One day into adulthood, we measured a decrease in the number of LB52 (9%) and LB53 (14%) worms scored as severe compared to LB54 (20%) animals (Fig. 4D–F; Table 3). The number of vacuolar structures

Table 3
Ndi1p expression reduces the formation of vacuolar structures

Strain	% None		% Moderate [a]		% Severe [a]	
	1 day [b]	2 days [c]	1 day [b]	2 days [c]	1 day [b]	2 days [c]
LB52	79.2	59.2	12.3	31.6	8.5	9.2
LB53	55.4	66.5	30.5	27.3	14.1	6.2
LB54	40.6	28.2	39.3	38.5	20.1	33.3

[a] One- and 2-day-old adults were examined and classified as moderate when they contained 1–3 vacuolar structures and as severe when they contained 4 or more structures.

[b] The numbers of LB52, LB53 and LB54 animals scored was 221, 208 and 252, respectively.

[c] The numbers of LB52, LB53 and LB54 animals scored was 219, 253 and 297, respectively.

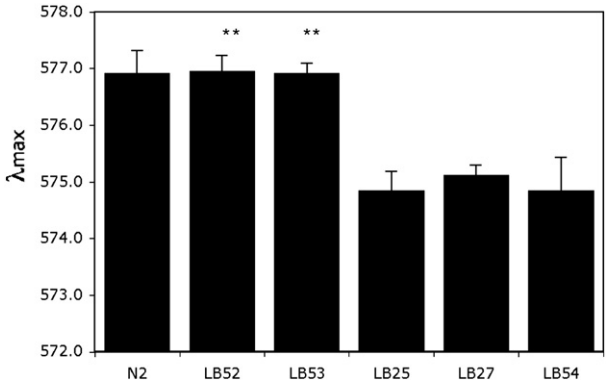

Fig. 5. Worms expressing Ndi1p have an increased membrane potential. The λ_{max} values of worms stained with diS-C_3(3) were determined; the λ_{max} values are proportional to the mitochondrial membrane potential. LB25 is the parent strain for LB52–LB54. LB27 is another *nuo-1* mutant carrying an A443F mutation. Values are means of 13, 18, 10, 9, 6 and 14 replicates for N2, LB52, LB53, LB25, LB27 and LB54, respectively. $^{**}P<0.01$ when compared with the LB54 control strain using a two-sample *t*-test.

increased significantly in 2-day-old LB54 adults but not in 2-day-old LB52 or LB53 adults (Fig. 4G–I; Table 3). The percentage of 2-day-old LB54 animals that remain vacuole free is only 28%, while 59% and 67% of LB52 and LB53 animals remain vacuole-free, respectively.

In previous work, we demonstrated that the A352V mutant has a lower level of ATP [17] and more recently, that it has a lower mitochondrial membrane potential in vivo [21]. We expected that Ndi1p expression would at least partially restore the mitochondrial membrane potential as NADH oxidation would be coupled to proton translocation, not through Ndi1p itself, but through complexes III and IV. We used a fluorescence based assay to measure the mitochondrial membrane potential-dependent accumulation of diS-C_3(3) in living animals. This assay exploits the spectral properties of diS-C_3(3); its fluorescence emission maximum (λ_{max}) and its fluorescence intensity at the maximum (I_{max}) respond to changes in membrane potential. The control strain, LB54 as well as the two original *nuo-1* mutants LB25 (A352V) and LB27 (A443F) have λ_{max} values of approximately 575 nm (Fig. 5). The λ_{max} values for LB52 and LB53 are 2 nm higher than that of LB54 (Fig. 5). The wild type strain, N2 also has a λ_{max} of 577 nm. These results indicate that Ndi1p functionally interacts with the nematode respiratory chain and contributes to the formation of the mitochondrial membrane potential in vivo.

4. Discussion

MRC biogenesis is a complex process involving over 70 genes encoded in 2 genomes. Mutations affecting MRC function produce a wide range of disorders, making the treatment of mitochondrial disease a challenge. Gene replacement therapy has been advocated as a strategy for treating mitochondrial disease [26–31]. One form of gene therapy is to express and deliver a protein to the damaged mitochondria that will functionally substitute for the affected MRC complex. In this work, we have utilized this strategy to deliver the *S. cerevisiae* Ndi1p to the mitochondria of complex I-deficient *C. elegans*.

Gene therapy experiments with the yeast Ndi1p have revealed its remarkable versatility. It can act as a functional member of the *Escherichia coli* respiratory chain [6]. It can restore NADH oxidase activity in complex-I deficient Chinese hamster cells [32] or in human C4T cells, which contain a homoplasmic frameshift mutation in the mitochondrial ND4 gene [26,33]. Ndi1p expression in human embryonic kidney cells does not interfere with the levels of endogenous complex-I [34]. An adeno-associated virus vector system has been used to successfully deliver and express the *NDI1* gene in non-proliferating human cells [35]. More recently, *NDI1* gene expression was shown to be beneficial in a murine model of chemically-induced complex I deficiency that mimics Parkinson disease [36]. Finally, complex I dysfunction or inhibition can lead to an elevated production of reactive oxygen species; *NDI1* expression can suppress ROS formation in neuronal cells experiencing rotenone-induced oxidative stress [37]. These latter results suggest that oxidative stress associated with neurodegenerative diseases may also be ameliorated with Ndi1p expression.

In this study, we have further explored the usefulness of Ndi1p expression in an animal model of complex I deficiency. The *C. elegans* strains LB52, LB53 and LB54 contain a point mutation in the *nuo-1* gene that alters Ala-352 to Val, reducing complex I activity to about 30% of wild type [11]. LB52 and LB53 express the *NDI1* gene under the control of the constitutive *let-858* and the *nuo-1* promoters, respectively. Ndi1p improves the overall fitness of these transgenic animals, increasing their fertility, their respiratory capabilities and their resistance to oxidative stress. Ndi1p significantly decreased the number of vacuolar structures that developed in aging animals. Further investigation will be required to determine whether the lesser number of vacuoles is a result of decreased necrotic or apoptotic cell death.

Ndi1p provides an alternative pathway for electrons from NADH to enter the respiratory chain. We have shown that Ndi1p expression increases the mitochondrial membrane potential. This is a highly significant finding because Ndi1p is not a proton pumping enzyme. The increases in mitochondrial membrane potential are due to the increased activity of the respiratory chain and the concomitant proton pumping at complexes III and IV. The *nuo-1* A352V mutation impairs the oxidation of NADH, the major respiratory substrate. The development of a method to assess in vivo mitochondrial membrane potential offers numerous advantages in understanding the complex relationship between genotype and phenotype in mitochondrial disease. The mitochondrial membrane potential is directly linked to important cellular processes such ATP synthesis, ion and metabolite transport and the production of reactive oxygen species.

The significant improvements in animal fitness we observed indicate that the *NDI1* gene can be expressed and that the Ndi1p precursor can be targeted to and imported into nematode mitochondria. Furthermore, Ndi1p can recruit its FAD cofactor and fold into a functional protein in the inner membrane, although the efficiency of these processes varied considerably between independently-derived transgenic lines. These observations attest to the extensive similarities between mitochondria

of extremely divergent species and confirm the versatility of Ndi1p.

Ndi1p expression cannot complement a complete loss of complex I. The *nuo-1(ua1)* allele is a lethal deletion that results in larval arrest [38]. We have attempted to directly rescue the *nuo-1 (ua1)* induced larval arrest by injecting *NDI1* under the control of a variety of promoters into *nuo-1(ua1)* heterozygotes and isolating *ua1* homozygous progeny. We have never succeeded in isolating a viable homozygous *nuo-1(ua1)* animal. Our best attempts have been with a heat shock-inducible promoter that resulted in the occasional production of L4 larvae or sterile adults. Why does Ndi1p expression fail to support viability in animals lacking complex I? One possible explanation is that we have not achieved sufficiently high expression levels of functional Ndi1p. We have noted that expression levels are highly variable between independently isolated lines (Fig. 3). A second explanation might be that Ndi1p is not functionally expressed in one or more tissues essential for viability in the absence of complex I. We have used constitutive promoters to drive ubiquitous expression, but we cannot rule out this possibility. Finally, a third possibility is that complex I performs one or more additional functions besides its role in electron transport and NADH oxidation. With 45 subunits in the human complex I, it seems likely that this may be the case. Complex I may have a significant role in regulating apoptosis [39,40] or in redox signaling [41]. One of the complex I accessory subunits is a homolog of acyl carrier proteins and may have a role in lipoic acid biosynthesis [42]. Finally, there is mounting evidence that complex I can be found in respiratory supercomplexes [42,43]; we have shown that complex I deficiency can affect the levels of complex IV [25]. Ndi1p expression produces significant benefits in our nematode model of complex I deficiency and may likewise produce benefits in patients. Our inability to replace complex I with Ndi1p should stimulate further investigations aimed at better understanding the structure and function of complex I.

Acknowledgements

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