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NDUFS4: Creation of a Mouse Model Mimicking a Complex I Disorder

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Abstract

The Complex I NADH dehydrogenase-ubiquinone-FeS 4 (NDUFS4) subunit gene is involved in proper Complex I function such that the loss of NDUFS4 decreases Complex I activity resulting in mitochondrial disease. Therefore, a mouse model harboring a point mutation in the NDUFS4 gene was created. An embryonic lethal phenotype was observed in homozygous (NDUFS4^{-/-}) mutant fetuses. Mitochondrial function was impaired in heterozygous animals based on oxygen consumption, and Complex I activity in NDUFS4 mouse mitochondria. Decreased Complex I activity with unaltered Complex II activity, along with an accumulation of lactate, were consistent with Complex I disorders in this mouse model.

Keywords

gene targeting; knock-in; Leigh syndrome; Leigh's disease

1. Introduction

NDUFS4 is a small 18kD protein of Complex I which is thought to be important in Complex I assembly or function. Mutations in NDUFS4 result in a loss of the last 10-15 amino acids of its final fifth exon and lead to mitochondrial diseases such as Leber's hereditary optic neuropathy (LHON), Leigh's syndrome (LS), and mitochondrial encephalomyopathy, lactic acidosis and stroke (MELAS). The mutation appears to affect oxidative phosphorylation and metabolism by either limiting electron transport chain (ETC) substrates (i.e., NADH + H⁺) or by creating a bottleneck in the ETC at Complex I. This decrease in energy production leads to the severe phenotypes characteristic of Complex I disorders. In cases of ETC impairment, this

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blockade also yields an overabundance of $\text{NADH} + \text{H}^+$ or FADH_2 (reduced) electron carriers leading to a drop in available NAD^+ . This situation readily mimics pyruvate dehydrogenase (PDH) deficiency, in that both lead to an increase in lactic acidosis (Pitkanen and Robinson, 1996; Yano, 2002). The main treatment for PDH deficiency is a high-fat, low-carbohydrate diet including thiamine (vitamin B_1 ; a co-factor of pyruvate dehydrogenase). Although thiamine aids in the conversion of pyruvate to acetyl-coA by PDH, the actual rate can only be increased with sufficient substrate (NAD^+) which is in short supply as it is in Complex I disorders. Thus, the treatment regimen of choice actually increases lactate production which in turn is resolved by oral sodium bicarbonate/citrate administration. Unfortunately, lactate accumulation continues as the actual defect in OXPHOS is maintained.

Traditional transgenic (nuclear-gene encoded) mouse models of mitochondrial disorders include the SURF-1 knockout mouse. Although this mutation occurs in cytochrome c oxidase, this is the only model that has lead to a phenotype similar to the NDUF54 point mutation in humans to date. Unfortunately ~90% of the mice homozygous for the SURF-1 knockout exhibited an embryonic lethality. This embryonic loss resulted in a 2.5% homozygous transmission rate from heterozygous crosses at birth (Agostino et al., 2003). These mice also lacked neurological symptoms; a surprising result mirrored in the majority of Complex I mitochondrial mouse models generated to date.

This paper examines the effect of mutating NDUF54 in a mouse model in order to truncate NDUF54 and selectively remove only the last 10-15 amino acids. Although homozygotes for the NDUF54 mutation were found to be non-viable, the heterozygotes displayed interesting biochemical changes. Complex I activity was found to be decreased in the absence of a change in Complex II activity in heart, brain, and skeletal muscle mitochondria of the NDUF54 heterozygotes. Also, the presence of mutant NDUF54 protein in properly assembled Complex I was found to be reduced in heart mitochondria along with a significant increase in lactate accumulation in total heart and brain cell cytosol samples. These data suggest that this NDUF54 mutation results in a Complex I mitochondrial disorder in heterozygous mice.

2. Materials and Methods

All chemicals were purchased from Sigma and all buffers were diluted in distilled deionized water (ddH_2O) unless otherwise noted.

Vector Construction

The point mutant NDUF54 targeting vector was constructed as shown in Fig. 1 (the neomycin resistance sequence remained in the targeting vector as the NDUF54 gene was located over 100kb from adjacent genes minimizing potential influences on gene expression). Children's Hospital of Oakland Research Institute (CHORI) bacterial artificial chromosomes filters (BACPAC) were screened to find three NDUF54 clones. A clone was used to amplify NDUF54 homology arms by polymerase chain reaction (PCR). Site-directed mutagenic PCR was used to delete one base on end of the 3' primer (5-ctctccacatcatgctccatcct-3) for homology arm 1 resulting in a premature stop codon effectively truncating the last 10-15 amino acids. The backbone vector was pPGKneobpA (Soriano et al., 1991).

ES Cell Targeting

PC1 embryonic stem (ES) cells were originally derived from 129S6/SvEvTac embryos (129S6; Taconic, Germantown NY)(Cassar et al., 2005). ES cell stocks were thawed at 37°C , plated and grown until there were at least 1.1×10^7 cells for selection. After G418 and DTA selection, ES cells that survived culture for 24 hr grew into colonies and were plated in individual wells

of a 96-well plate. Colonies were screened for homologous recombination by Southern blot analysis prior to propagation, re-screening, and microinjection into murine blastocysts.

ES Cell Transfer and Mouse Husbandry

ES cells ($\sim 1 \times 10^5$) were thawed and co-cultured for two days on mouse embryonic fibroblasts (MEFs) prior to injection into C57BL/6NTac (Taconic, Germantown NY) murine blastocysts using standard techniques (Pinkert, 2002; Pinkert and Trounce, 2002, 2005). The blastocysts were then surgically transferred into the oviducts of time-mated pseudopregnant female ICR mice and allowed to develop to term (Pinkert, 2002). Mice were housed in ventilated caging within a specified pathogen-free (SPF) barrier facility with *ad libitum* access to feed and water. Mice were mated to either wild-type (C57BL/6NTac) mice or to mice heterozygous for the NDUFS4 point mutation. Mice were genotyped by PCR for the vector sequence and for a fragment of wild-type DNA displaced following homologous insertion (Irwin et al., 2002; Pinkert, 2003). Mice were humanely sacrificed by cervical dislocation following CO₂ inhalation or i.p. anesthesia following Institutional Animal Care and Use Committee (IACUC) approved guidelines (Howell et al., 2003). The heart, brain and skeletal muscle were surgically removed and used for mitochondrial isolation and cytosol isolation. All mouse procedures including euthanasia conformed to IACUC guidelines (under OLAW assurance #A3292-01 and #A3152-01 in AAALAC-accredited barrier facilities).

Mitochondrial Isolation

Protocols for heart, brain and skeletal muscle mitochondrial isolation were adapted from a published skeletal muscle mitochondrial isolation procedure (Wisniewski et al., 1993). Hearts and skeletal muscle were removed, placed into 20 mL of buffer A (180 mM KCl, 10 mM EDTA-Na₂, pH 7.2), brain into 20 mL buffer MSE (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.4), finely minced with scissors and suspended in 20 mL of buffer A/MSE (skeletal muscle was supplemented with 1 mg/mL trypsin, brain with 5 mg bacterial proteinase XXIV). The resultant heart suspension was then stirred (~ 150 RPM) for 5-15 min until cloudy and skeletal muscle was allowed to sit on ice for 30 minutes. Another 20 mL of either Buffer A or MSE were then added and the solutions were homogenized using a Teflon-glass Potter homogenizer for 30 sec. at 600 rpm (200 rpm for heart). This mixture was centrifuged at $2\,000 \times g$ ($300 \times g$ for skeletal muscle) for 6 min., filtered through cheesecloth into a new tube and centrifuged at $12\,000 \times g$ ($3\,000 \times g$ for skeletal muscle) for 10 min. The supernatant was then discarded and the mitochondrial pellet was resuspended into buffer A (skeletal muscle), buffer B (180 mM KCl, pH 7.2, brain) or buffer MSE supplemented with 2 mg digitonin (brain) and centrifuged at $12\,000 \times g$ for 5 min ($3\,000 \times g$ for 2 minutes for skeletal muscle). The supernatant was again discarded and the pellet was resuspended in 100 μ L buffer B supplemented with 5 mM MgCl₂ (heart), 300 μ L buffer MSE (brain) or 50 μ L buffer A (skeletal muscle). All procedures were carried out at 4C.

Blue Native Gel Electrophoresis

Blue native gel electrophoresis was performed using previously outlined procedures (Brookes et al., 2002; Tompkins et al., 2006). A 500 μ g mitochondrial pellet was resuspended in 225 μ L extraction buffer (0.75 M aminocaproic acid, 50 mM BisTris, 1% w/v lauryl-maltoside) and placed on ice for 30 min. Samples were then centrifuged for 5 min. at $14\,000 \times g$ and 200 μ L of supernatant were added to 12.5 μ L of 5% Coomassie blue (0.5 M aminocaproic acid, 5% wt/vol Coomassie blue). Aliquots (40 μ L vol.) were loaded into individual lanes of polyacrylamide gels containing a 4% wt/vol stacker layer, a 5-12% wt/vol gradient layer and lauryl maltoside. Gels were electrophoresed overnight at 4C and visualized the next day with Coomassie blue.

Silver Staining and Western Blotting

The blue native gel Complex I bands or the entire lanes (turned 90° and used for the whole gel) were used to generate second dimension gels (Brookes et al., 2002; Tompkins et al., 2006). The stacker and separator for these gels were 8% wt/vol and 16.5% wt/vol, respectively. Agarose (1% wt/vol), along with 1% wt/vol sodium dodecyl sulfate (SDS) and 0.01% wt/vol β -mercaptoethanol were mixed and used in the top of gels to fill the remaining space. Gels were subjected to standard voltages and either fixed or silver-stained (Silverquest, Invitrogen, Carlsbad, CA). Resolved proteins were transferred to nitrocellulose membranes with a semi-dry apparatus. Ponceau S staining was used to visualize the transfer of protein. Membranes for western blotting were blocked at 25C in Tris-buffered saline (TBS) with 0.05% vol/vol Tween-20 and 5% wt/vol nonfat dry milk for 2 hr. Primary antibody incubations were 2 hr at 25C (anti-NDUFS4 polyclonal; H00004724-A01, 1:1 000, Affinity Bioreagents, Golden CO). Secondary antibody incubations using horseradish peroxidase goat anti-mouse IgG (Pierce, 1:5 000) were 2 hr at 25C. Enhanced chemiluminescence (ECL) detection was then used for visualization (see Fig. 4).

Mitochondrial Respiration

Oxygen consumption of mitochondria was measured using a Clark-type oxygen electrode at 37C in a buffer containing: 125 mM KCl, 25 mM HEPES, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM KH_2PO_4 , 500 μ M EDTA, pH 7.2. For Complex I-driven respiration, 10 mM glutamate and 5 mM malate were added. For Complex II analyses, 10 mM succinate were used. For state 3 oxygen consumption ADP was added (33 μ M for glutamate/malate-driven respiration, 3.3 μ M for succinate-driven respiration). The state 3 rate was divided by the state 4 rate to determine the respiratory control ratio (RCR) which was normalized to control samples.

Complex I Activity. The Complex I activity was measured as previously described (Tompkins et al., 2006). **Complex II Activity.** Dichlorophenolindophenol (DCPIP) was used as a secondary dye as it oxidizes the CoQ reduced by Complex II and in turn becomes reduced itself (DCPIPH₂). The blank for this experiment consisted of 500 μ l $K^+PO_4^-$ Buffer (100 mM KH_2PO_4/K_2HPO_4 , pH 7.4), 40 μ l EDTA (2.5 mM) and 460 μ l ddH₂O. The samples were prepared by adding 500 μ l $K^+PO_4^-$ Buffer, 40 μ l EDTA, 40 μ l DCPIP (3 mM), 10 μ l KCN (100 mM in 500 mM HEPES), 10 μ l mitochondria (5 mg/ml protein), 2 μ l rotenone (1 mM in ethanol) and 350 μ l ddH₂O. Reactions were measured (at 600 nm) every 13.6 sec. for 10 min., with an addition (to the samples only) of 40 μ l succinate (500 mM) at 1 min. and 10 μ l 2-thenyltrifluoroacetone (TTFA, 100 mM in ethanol) at 5 min. The linear rate was subtracted from the inhibited (TTFA) rate to obtain the net rate. Final calculations were the same as for the Complex I activity.

Citrate Synthase Activity

Oxaloacetate and acetyl-CoA react to form citrate and CoASH. The CoASH can then be oxidized and bound to 5, 5' dithio-bis-2-nitrobenzoic acid (DTNB) to form DTNB-CoA which has a measurable absorbance of light at 412 nm. The blank for this reaction consists of 590 μ l buffer (100 mM Tris, 0.1% vol/vol Triton X-100, pH 8.0), 5 μ l acetyl-CoA (10 mM) and 5 μ l DTNB (20 mM in ethanol). Samples were prepared by addition of 580 μ l buffer, 5 μ l acetyl-CoA, 5 μ l DTNB and 2.5 μ l mitochondria (5 mg/ml protein). The absorbance was measured at 412 nm every 13.6 sec. for 3 min. with addition (to the samples only) of 10 μ l oxaloacetate at 1 min. The linear rate following the addition of oxaloacetate was calculated (as in the Complex I activity assay) and used to normalize all other mitochondrial assays.

Lactate Dehydrogenase

The lactate dehydrogenase enzyme converts pyruvate to lactate in the presence of NADH + H⁺ (decrease measured at 340 nM). Each sample contained 500 μ l Tra A buffer (100 mM triethanolamine, 10 mM EDTA, pH 7.6), 10 μ l NADH (20 mg/ml), 10 μ l cytosol, and 2 μ l rotenone (5 mM in ethanol). The reaction was initiated with 20 μ l pyruvate (50 mM) and tracked over 5 min. The net change in absorbance at 340 nm was calculated as a rate used to determine the enzyme activity according to the Lambert-Beer's equation.

Lactate Concentration

A lactate assay kit containing an L-lactate probe was used to measure the lactate concentration (BioVision, Mountain View, CA). The lactate standard was diluted according to the colorimetric assay from 0 to 10 nmoles/well of a 96-well plate. Each sample was measured with 1 and 10 μ l of cytosol solution. After determination of lactate concentration, total protein in the cytosolic solutions was determined and used to normalize the concentrations.

Statistical Analysis

Comparisons of NDUFS4 +/+;NDUFS4 +/- among the three tissues was performed using univariate ANOVA (2-way) with a *post hoc* Fisher's LSD. Tests between NDUFS4 +/+ and NDUFS4 +/- within tissues were performed with a paired, one-tailed Student's T-test. For each bar, n = 4-8. *P-value < 0.05.

3. Results

Targeting Vector

A knock-in point mutant NDUF54 targeting vector was designed with two homology arms and positive (neo) and negative (diphtheria toxin A; DTA) selection markers (Fig. 1). The NDUF54 point mutant construct was linearized with a restriction enzyme (SmaI) for subsequent electroporation into ES cells. This construct was designed such that a truncation in the NDUF54 gene would occur in the last exon prior to a conserved 10-15 amino acid sequence. This loss of the last segment of the NDUF54 protein containing this conserved region has been shown to occur in all identified NDUF54 cases of human Leigh disease and was chosen for that reason.

Embryonic Stem Cell Targeting and Screening

Recombinant clones were verified by Southern blot after restriction digestion of isolated DNA with EcoRI. The differential locations of the EcoRI site in the targeting vector, the NDUF54 gene and the homologously targeted construct are shown in Fig. 1. A probe 3' to the second homology arm was used to determine the size of the EcoRI-digested DNA fragment in that region to distinguish between homologous recombination and the wild-type NDUF54 gene. Heterozygous ES cells were identified by the presence of both a 4.8 kb wild-type NDUF54 gene and a recombinant 6.1 kb NDUF54 point mutation insertion. A representative Southern blot analysis of NDUF54 +/+ (wild-type) and NDUF54 +/- (heterozygous) mice is depicted in Fig. 2. NDUF54 point mutant heterozygous ES cells were then injected into the blastocoel of blastocysts. The blastocysts were then transferred to oviducts of pseudopregnant female mice to produce founder chimeras which were initially genotyped by polymerase chain reaction (PCR). A total of four mice (one for each positive clone) were injected with approximately 12 blastocysts each and resulting offspring were genotyped. All correctly targeted ES cells (based on Southern blot analysis) were subjected to karyotype analysis to confirm that there were 40 intact chromosomes. Second and third generation NDUF54 point mutant heterozygous mice were crossed (brother-sister matings) to generate homozygous offspring. Tissues from potential NDUF54 mutant homozygotes were genotyped by two separate PCRs. The first PCR amplified a fragment of leftover construct between the homology arms present in the NDUF54

targeting construct. The second PCR amplified a fragment of the NDUF54 genomic sequence, which would be removed if both NDUF54 alleles contained the NDUF54 point mutation. PCR analyses of greater than 200 adult animals only detected wild-type and heterozygous offspring. PCR analyses of fetuses from three E10-12 (embryonic day 10-12) litters (34 fetuses total) yielded only wild-type and heterozygous offspring.

BN-PAGE, Silver Staining & Western Blotting

Blue native polyacrylamide gel electrophoresis (BN-PAGE) for heart, skeletal muscle and brain mitochondria was performed to separate the mitochondrial complexes of the electron transport chain (ETC). As noted in Fig. 3a, BN-PAGE of samples from heart resolved the ETC complexes (Brookes et al., 2002; Tompkins et al., 2006). No differences were observed in the locations or intensities of the complexes between NDUF54 $+/+$ and NDUF54 $+/-$ mitochondria (Fig. 3b). This allowed for the mitochondrial electron transport chain complexes to be further separated into their protein subunits and analyzed via silver staining or by western blotting for NDUF54.

Ponceau S staining was used to verify that all protein was successfully transferred to nitrocellulose membranes. Second dimension gels were run and silver-stained to observe resolution of the ETC subunits and to ascertain whether there were any obvious differences between heart NDUF54 $+/+$ and NDUF54 $+/-$ mitochondrial proteins (Fig. 4 b, c). Additional second dimension gels were used for western blotting with the NDUF54 polyclonal antibody to visualize the shift in the mass of the mutant NDUF54 gene product in heterozygotes as the stop codon created a truncation in the protein from 18kDa to 14.4kDa (Fig. 4 a, d, heart mitochondria). The quantification of the 18kDa and 14.4kDa bands for both NDUF54 $+/+$ and $+/-$ is shown in Figure 4e. The heterozygotes appear to have less mutant protein than expected (20% in contrast to an expected 50% of wild-type levels).

Oxygen Consumption

Since the ability of the ETC to generate ATP is of concern in human patients afflicted with a Complex I disorder, oxygen consumption was used to determine the general health of the ETC in isolated mitochondria following addition of substrate (Budde et al., 2000; Iuso et al., 2006; Petruzzella and Papa, 2002; Petruzzella et al., 2001; Scacco et al., 2003; van den Heuvel et al., 1998).

The respiratory control ratio (RCR) was first determined following glutamate/malate addition which generates $\text{NADH} + \text{H}^+$ that feeds into the ETC via Complex I. Figure 5a shows a decrease in RCR of approximately 30% in heterozygous heart, skeletal muscle and brain mitochondrial samples as compared to their respective wild-type RCR, indicating a possible defect via Complex I-mediated oxygen consumption. To test whether this decrease was indeed due to Complex I or other ETC differences in the heterozygote mitochondria, a second assay using succinate as a substrate was performed (Fig. 5b). The results indicated that there was no difference in RCR in heterozygous tissue samples using succinate as a substrate (Complex II-mediated oxygen consumption) and that the NDUF54 defect mirrored metabolic deficiencies seen in Complex I disorders. This decrease in oxygen consumption via Complex I (glutamate/malate) which occurs in the presence of normal Complex II/III activity can also be observed in LHON, LS and MELAS when Complex I protein subunits are affected.

Complex I and II Activity

The results from the oxygen consumption assay suggested that Complex I activity may be altered in NDUF54 point mutant heterozygous mitochondria. The Complex I activity was therefore measured and normalized to citrate synthase activity (Fig. 5c). A decrease in heterozygous Complex I activity of around 25-30% was observed in heart, skeletal muscle and

brain mitochondrial samples. This correlated well with the Complex I-mediated oxygen consumption data and with what is seen in Leigh syndrome patient data (Budde et al., 2000; Iuso et al., 2006; Petruzzella and Papa, 2002; Petruzzella et al., 2001; Scacco et al., 2003; van den Heuvel et al., 1998) as well as with MELAS and LHON.

The activity of Complex II was assayed as a Complex I activity control and was measured and normalized to citrate synthase activity (Fig. 5d). The results show no difference in Complex II activity in heart, skeletal muscle or brain, correlating directly to the oxygen consumption data, and a variety of Complex I disorders (Budde et al., 2000; Iuso et al., 2006; Petruzzella and Papa, 2002; Petruzzella et al., 2001; Scacco et al., 2003; van den Heuvel et al., 1998).

Citrate Synthase Activity

The citrate synthase activity assay was used to normalize all data obtained from isolated mitochondria. The citrate synthase activity for the NDUF54 +/- mice was increased compared to the NDUF54 +/- controls although not significantly.

LDH Activity

In patients exhibiting homozygous NDUF54 mutations, the activity of lactate dehydrogenase (LDH) is increased two to three fold; therefore, the activity of LDH was assayed in the NDUF54 +/- (heterozygous) mice. Using both heart and brain cytosol samples, results in Figure 5e indicate that the levels of LDH activity were comparable in NDUF54 +/- and NDUF54 +/- samples. This is not surprising as NDUF54 +/- mice still have one wild-type NDUF54 copy and do not appear to be as impaired as human patients harboring (homozygous) NDUF54 -/- mutations. Also, the amount of LDH activity does not necessarily reflect the lactate concentration in NDUF54 +/- mice as the increase in lactate accumulation is responsible for leading to lactic acidosis and not necessarily by upregulation of the enzyme.

Lactate Concentration

A lactate concentration assay was performed and indicated a two to three fold increase in lactate concentration in both heart and brain tissue of NDUF54 +/- cytosol samples compared to NDUF54 +/- wild-type cytosol samples (Fig. 5f). Although the increase in lactate is not as high as that observed in some human disorders (e.g., Leigh syndrome), the consistency of lactate accumulation lends this model to be comparable to the majority of mitochondrial diseases. As such, the effects of this increase in lactate production and potential therapeutics can be readily tested as a model for many mitochondrial disorders.

4. Discussion

All NDUF54 gene mutations which correspond to human disease result in a truncation or absence of the last 10-15 amino acids of the NDUF54 protein (Budde et al., 2000; Iuso et al., 2006; Petruzzella and Papa, 2002; Petruzzella et al., 2001; Scacco et al., 2003; van den Heuvel et al., 1998). Therefore, a point mutant construct in which a stop codon was created via a single base deletion was developed to specifically mimic a NDUF54 truncation. A mouse model of this point mutation was then used to determine if the developmental consequences accurately reflected a human Complex I disorder. A point mutant NDUF54 mouse model was created and verified by: Southern blot analysis, to demonstrate homologous recombination; PCR, to differentiate homozygous/heterozygous animals; and western blot analysis to verify truncation of the NDUF54 protein.

The mutant NDUF54 band (6.1 kb) was less intense than the wild-type NDUF54 band (4.8kb) in all samples analyzed by Southern and although the truncation in NDUF54 protein size from 18 kDa to 14.4 kDa was observed in western blots, the NDUF54 +/- mutant band appeared of

faint intensity. The result is not surprising as NDUFS4 mutant mRNA may undergo nonsense-mediated decay (NMD) in light of the premature stop codon in the mutant NDUFS4 mRNA. This degradation may explain why the mutant NDUFS4 band (in western blots) is faint or not present in fully assembled Complex I. Alternatively, the mutant NDUFS4 protein may not be correctly imported into the mitochondria. With what is known about the import machinery (TIM, TOM complexes) this would not be expected. Yet, there is still a possibility that the 3' structure of the protein may hinder transport to mitochondrial Complex I.

A significant decrease in oxygen consumption via Complex I of about 30% with no difference in Complex II activity was observed in the heterozygous mutants as reported in human patients (Budde et al., 2000; Iuso et al., 2006; Petruzzella and Papa, 2002; Petruzzella et al., 2001; Scacco et al., 2003; van den Heuvel et al., 1998). As performed by others using human patient samples, these results were divided by the concentration of citrate synthase (ranges from 1.8-2.2 $\mu\text{g}/\text{min}/\text{mg}$) to normalize mitochondrial protein content (Bonora et al., 2006; Procaccio and Wallace, 2004). The concentration of citrate synthase was slightly higher in heterozygous mitochondria than in wild-type mitochondria in all 3 tissues (brain, heart and skeletal muscle) which is suggestive of a compensatory mechanism in the NDUFS4 +/- mice. It is also known that a number of patients with metabolic diseases such as Leigh syndrome or MELAS display a hyper-proliferation of mitochondria as a possible compensatory mechanism. This phenomenon has also been shown to occur in Complex IV (COX) mitochondrial disorders and most likely leads to heart failure (Aure et al., 2006; Goffart et al., 2004).

The results obtained from the oxygen consumption assay correlated with data from human Leigh syndrome patients. This provided an initial indication that the NDUFS4 +/- mutant phenotype was similar to a generalized mitochondrial disorder. The activities of Complexes I and II were normalized to citrate synthase activity and correlated well with the oxygen consumption assay data. The activity via Complex I directly or via glutamate/malate resulted in a decrease of approximately 30% in heterozygous NDUFS4 point mutant mitochondria with no difference observed in Complex II activity directly or via succinate. This finding was used to illustrate that our model accurately depicts a mitochondrial Complex I disorder.

The second observation compared in human patients reflected increased lactate levels leading to lactic acidosis (Budde et al., 2000; Iuso et al., 2006; Petruzzella and Papa, 2002; Petruzzella et al., 2001; Scacco et al., 2003; van den Heuvel et al., 1998). Therefore, the activity of lactate dehydrogenase (LDH) was investigated to discover if there was a similar increase of 200-300% in our NDUFS4 +/- mice as reported in humans. An increase in LDH activity was not observed in NDUFS4 heterozygotes which is not surprising since the mutant NDUFS4 gene product was found in some of the fully assembled Complex I in heterozygotes by western blotting. Since heterozygous Complex I is functioning at 70% of the wild-type Complex I activity, the accumulation of NADH might not be high enough to raise the levels of LDH. However, this accumulation could still lead to an increase in lactate production (although milder than observed in human patients). The observed lactate concentration in NDUFS4 heterozygotes was two to three fold higher than wild-type mice. This increase in lactate may be attributable to a lack of functional NDUFS4 genes (one instead of two) limiting Complex I activity to cause a rate limiting step (no NAD^+) at pyruvate resulting in lactate build-up. Alternatively, a dominant-negative effect of the mutant NDUFS4 could cause a decrease in Complex I activity and again lead to lactate accumulation by causing incorrect Complex I assembly which would not be detected by western blotting. To elucidate the mechanism for this increase and the possible dominant negative effect of NDUFS4 +/- mice, a vector could be made with a promoter driving over-expression of the mutant NDUFS4 gene. Nonetheless, our results corresponded to the three-to ten-fold increases in lactate concentrations observed in human Complex I disorders (including Leigh syndrome). These findings further illustrated and mirrored human pathological hallmarks observed in Complex I disorders.

Following initial characterization, an embryonic lethal phenotype was observed in homozygous *NDUFS4*^{-/-} mutant fetuses. In contrast, using heterozygous *NDUFS4*^{+/-} mutant mice, viable heterozygous offspring were obtained for further study. Recently, a knockout of *NDUFS4* was shown to cause lethality as mice only survived to 7 weeks of age following a complete loss of Complex I activity (Kruse et al., 2008). When compared to the fact that our homozygotes do not survive, this suggests a potential dominant negative aspect of the *NDUFS4* point mutant. This may be independent or in conjunction with a decrease in available mutant *NDUFS4* to form Complex I and therefore not be as lethal as dominant negative proteins tend to be. Interestingly, *NDUFS4* knockout mice also displayed similar muscular impairment to human patients and decreases in Complex I activity as seen in our mouse model.

Taken in the aggregate, multiple analyses verified a Complex I disorder phenotype in the *NDUFS4*^{+/-} heterozygous mice. This correlation with Complex I disorders was reflective of the importance of this model. Future experiments which utilize this model are expected to illustrate further similarities with Complex I disorders and to play a definitive role in elucidating the etiology and treatment of such disorders. These results provide several lines of evidence supporting the role of the *NDUFS4* point mutation in Complex I-linked mitochondrial disease in the heterozygous *NDUFS4*^{+/-} mice. This model will be useful in the study of mechanisms of mitochondrial Complex I-derived disease such as Parkinson's disease, cardiac ischemia-reperfusion and Leigh syndrome as well as possible treatment and prevention.

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a **Fig. 1.**

Schematic of the NDUFS4 Point Mutant Construction. The NDUFS4 point mutant construct gene targeting strategy is depicted. Successful homologous targeting to the NDUFS4 gene of ES cells is determined by Southern blotting of EcoRI-digested DNA using a probe specific for the region of DNA just downstream of the NDUFS4 gene (4.8kb band for wild-type, 6.1kb band for the recombinant mutant).

Fig. 2. Southern Blot Analysis of the NDUFS4 Targeting Vector. (a) A representative Southern blot analysis of mouse DNA digested with EcoRI. Lanes 1-2 represent wild-type mice (NDUFS4 +/+) as there is a single band at 4.8 kb. Lanes 3-4 represent heterozygous recombinant (NDUFS4 +/-) mice as there are two bands (NDUFS4 wild-type at 4.8 kb, NDUFS4 recombinant at 6.1 kb). (b) The NDUFS4 recombinant band is notably of lesser intensity than the wild-type band as shown by quantification.

Fig. 3.

Blue Native Gel Electrophoresis and Silver Staining for NDUF54. (a) Heart mitochondrial proteins were separated on blue native gels to identify mitochondrial complexes I-V (complex II not visible as it is very close to the dye front). NDUF54 $+/+$ and NDUF54 $+/-$ lanes were turned 90° counter-clockwise in order to generate a second dimension gel used for silver staining and NDUF54 western blot shown in Figure 4. (b) Quantification of Complex I from the blue native gel shows equal intensity in all lanes.

Fig. 4.

Western Blot Analysis for NDUF54 in Heart Mitochondria. As noted in Figure 3, a second dimension gel of a complex I band was generated for western blot analysis of NDUF54 +/+ and NDUF54 +/- heart mitochondria. (b, c) NDUF54 +/+ and NDUF54 +/- heart mitochondrial second dimension silver staining are respectively shown. Identification of the NDUF54 wild-type band (18kDa) and the point mutant NDUF54 truncated band (14.4kDa) in the silver stains is not discernable as there are many Complex I bands which overlap these two bands. (a, d) NDUF54 +/+ and NDUF54 +/- heart mitochondrial second dimension western blotting are respectively shown. The wild-type NDUF54 band (18kDa) is identified in both the NDUF54 +/+ and NDUF54 +/- Complex I whereas the point mutant NDUF54 truncated band (14.4kDa) is only detected in (d) the western blot of NDUF54 +/- Complex I. (e) Quantification of the results displayed normalized to +/+ = 100. Wild-type and mutant respectively refer to the 18kDa and 14.4kDa bands.

Fig. 5. Mouse Mitochondrial and Cytosolic Biochemical Analyses in NDUF54 +/+ and NDUF54 +/- Mice. (a) Mitochondrial respiration using glutamate/malate as substrates or (b) succinate as a substrate. (c) Complex I activity, expressed as a RCR normalized to citrate synthase activity. (d) Complex II activity, expressed as a RCR normalized to citrate synthase activity. (e) Lactate dehydrogenase enzymatic activity calculated using Lambert Beer's equation. (f) Lactate concentration using an L-lactate probe and colorimetric quantification against a standard lactate control set. RCR, Complex I and Complex II activity comparisons among three tissues were performed using a 2-way ANOVA with a *post hoc* Fisher's LSD. Tests between NDUF54 +/+ and NDUF54 +/- were performed with a paired, one-tailed Student's T-test. For each bar, n

= 4 to 8. *P-value < 0.05. LDH and lactate comparisons were performed for heart and brain samples using a 2-way ANOVA with a *post hoc* Fisher's LSD. Tests between NDUFS4 +/+ and NDUFS4 +/- were performed with a paired, one-tailed Student's T-test. For each bar, n = 4 to 8. *P-value < 0.05.