Mito-Nuclear Interactions Affecting Lifespan and Neurodegeneration in a *Drosophila* Model of Leigh Syndrome

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ABSTRACT Proper mitochondrial activity depends upon proteins encoded by genes in the nuclear and mitochondrial genomes that must interact functionally and physically in a precisely coordinated manner. Consequently, mito-nuclear allelic interactions are thought to be of crucial importance on an evolutionary scale, as well as for manifestation of essential biological phenotypes, including those directly relevant to human disease. Nonetheless, detailed molecular understanding of mito-nuclear interactions is still lacking, and definitive examples of such interactions *in vivo* are sparse. Here we describe the characterization of a mutation in *Drosophila* *ND23*, a nuclear gene encoding a highly conserved subunit of mitochondrial complex 1. This characterization led to the discovery of a mito-nuclear interaction that affects the *ND23* mutant phenotype. *ND23* mutants exhibit reduced lifespan, neurodegeneration, abnormal mitochondrial morphology, and decreased ATP levels. These phenotypes are similar to those observed in patients with Leigh syndrome, which is caused by mutations in a number of nuclear genes that encode mitochondrial proteins, including the human ortholog of *ND23*. A key feature of Leigh syndrome, and other mitochondrial disorders, is unexpected and unexplained phenotypic variability. We discovered that the phenotypic severity of *ND23* mutations varies depending on the maternally inherited mitochondrial background. Sequence analysis of the relevant mitochondrial genomes identified several variants that are likely candidates for the phenotypic interaction with mutant *ND23*, including a variant affecting a mitochondrially encoded component of complex I. Thus, our work provides an *in vivo* demonstration of the phenotypic importance of mito-nuclear interactions in the context of mitochondrial disease.

KEYWORDS Mitochondrial disease; Leigh syndrome; mito-nuclear interaction; neurodegeneration; *Drosophila*

HEALTHY neurons can remain viable and functional over the entire lifetime of an organism. Because neurons are nondividing cells, their ability to maintain structural and functional integrity over extended periods of time must depend on a variety of cellular and molecular mechanisms that enable them to withstand and repair damage from an array of environmental and biological insults. We still lack a full understanding of these neuroprotective mechanisms despite their fundamental biological and medical importance. To address this problem, we have performed minimally biased, forward genetic screens

to identify genes whose normal function is required to maintain neuronal integrity as a function of age; loss-of-function mutations in these “neurodegeneration suppressor” genes result in age-dependent neurodegeneration. We previously found that our collection of mutants, originally identified on the basis of temperature-sensitive paralysis or other locomotor defects, is enriched for those exhibiting age-dependent neurodegeneration (Palladino *et al.* 2002). These mutants have revealed important neuroprotective roles for a variety of cellular processes, including metabolism, innate immunity, and vesicular trafficking (Palladino *et al.* 2003; Gnerer *et al.* 2006; Miller *et al.* 2012; Cao *et al.* 2013; Babcock *et al.* 2015). Here, we analyze another mutant from this collection that exhibits shortened lifespan, mitochondrial abnormalities, and age-dependent neurodegeneration. We identify the causal mutation in the *ND23* gene, which encodes the *Drosophila* ortholog of human *NDUFS8*, a core subunit of mitochondrial complex 1.

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Complex 1 is one of five enzymatic complexes in the mitochondrial inner membrane that carry out oxidative phosphorylation (Bridges *et al.* 2011). Complexes 1–4 compose the electron transport chain, which uses energy derived from oxidative reactions to pump protons across the inner membrane, thereby establishing a proton gradient. Complex 5 uses this gradient to drive ATP synthesis. Oxidative reactions carried out by complex 1 result in electron transfer from NADH to ubiquinone via a series of iron-sulfur (Fe-S) clusters. Complex 1 is the largest enzyme in mitochondrial oxidative phosphorylation, containing ~45 subunits; however, it requires only 14 evolutionarily conserved “core” subunits to perform its enzymatic reactions (Efremov *et al.* 2010). Seven of these core subunits are encoded by mitochondrial DNA (mtDNA), whereas nuclear DNA (nDNA) encode the other seven. *ND23* is a nuclear gene that encodes one of the Fe-S core subunits of complex 1.

Mitochondrial diseases are the most frequently inherited metabolic disorder in humans (Smeitink *et al.* 2001), with an estimated incidence of at least 1 in 5000 births (Wallace and Chalkia 2013). A frequent cause of mitochondrial disease is complex I deficiency (Smeitink *et al.* 2001), which is the most common childhood-onset mitochondrial disorder (Fassone and Rahman 2012). Loss-of-function mutations in several different mitochondrial proteins, including *NDUFS8*, cause Leigh syndrome, which usually becomes apparent in the first years of life. Leigh syndrome is characterized by early, progressive neurodegeneration, intellectual and motor difficulties, and abnormal energy metabolism (Lake *et al.* 2016). However, as is true for many other inherited mitochondrial diseases (Lightowlers *et al.* 2015), Leigh syndrome is characterized by marked variation in phenotypic severity and age at onset, even when two individuals carry identical disease-causing mutations (Budde *et al.* 2003; Marina *et al.* 2013). Patients often die in the first years of life, primarily from respiratory failure; however, a number of associated medical issues complicate morbidity and mortality. Currently, treatment for Leigh syndrome is limited to palliative care.

The basis of the clinical heterogeneity of inherited mitochondrial disorders remains challenging (Lightowlers *et al.* 2015). Genetic heterogeneity and mitochondrial heteroplasmy are clearly involved (Wallace and Chalkia 2013). It has also been suggested that genetic background, including mtDNA polymorphisms, can modify disease susceptibility and severity (Wallace *et al.* 1999; Wallace and Chalkia 2013). Although most naturally occurring, mtDNA polymorphisms are relatively neutral, it has been proposed that certain mtDNA polymorphisms can interact epistatically with a nuclear mutation to enhance a disease phenotype (Wallace *et al.* 1999; Wolff *et al.* 2014). Although this notion is supported by epidemiological studies correlating human mitochondrial haplotype (Hofmann *et al.* 1997; Hudson *et al.* 2007; Strauss *et al.* 2013) or nuclear mutation (Guan *et al.* 2006; Jiang *et al.* 2016) with clinical expression of mitochondrial disorders, as well as analysis of transmitochondrial cytoplasmic hybrids (Potluri *et al.* 2009; Wilkins *et al.* 2014), direct *in vivo* evidence for this

association is rare. Work in *Drosophila*, however, has provided important support for this hypothesis. In *Drosophila*, the mitochondrial disease-like phenotype caused by a mutation in *technical knockout* (a nuclear gene encoding a mito-ribosomal protein) can be suppressed by a cytoplasmic factor that increases mtDNA copy number (Chen *et al.* 2012). Although this factor appears to be mitochondrial in nature, mtDNA changes that were present in all suppressor strains and absent from all nonsuppressor strains (or vice versa) could not be identified. Further, direct evidence exists for a mito-nuclear incompatibility that affects *Drosophila* development and fitness. The incompatibility was identified to be between a nuclear-encoded transfer RNA (tRNA) synthetase and the mitochondrially encoded cognate tRNA (Meiklejohn *et al.* 2013). However, the incompatibility occurred by mating two different *Drosophila* species. Thus, the relevance of within species incompatibility was not directly addressed in these studies. However, recent data suggest that an incompatibility between the two homologous human proteins may affect the phenotypic expression of Leber's hereditary optic neuropathy, the most common mitochondrial disorder (Jiang *et al.* 2016). Finally, *ATP6¹*, a mutation in an mtDNA-encoded gene, was shown to enhance the mutant phenotype of *sesB¹*, a mutation in a nDNA-encoded gene. However, the *ATP6¹* mutation alone results in significant defects (Celotto *et al.* 2006). Thus, even in *Drosophila*, direct *in vivo* evidence of a nonpathological mitochondrial background modifying mitochondrial disease manifestation within a species remains elusive.

In the course of characterizing our *ND23* mutant, we discovered that the shortened lifespan and neurodegeneration phenotypes were enhanced by a maternally inherited factor consistent with a mtDNA variant. Sequence analysis of the mitochondrial genome identified several mutations that are likely candidates for the interaction with *ND23*. In the absence of the *ND23* mutation, the mitochondrial variants exhibit no overt effect on lifespan or neuronal viability. Thus, the enhanced mutant phenotype appears to depend on a mito-nuclear genetic interaction that modifies the phenotypic manifestation of a nuclear mutation that affects complex I.

These studies provide a new model of Leigh syndrome in *Drosophila*. They also establish a powerful *in vivo* experimental system to further understand how nuclear and mitochondrial genotypes can interact to affect organismal phenotypes, as well as how these interactions can impact the pathophysiology of mitochondrial, and perhaps other disorders that are also characterized by variability in disease progression and severity.

Materials and Methods

Drosophila genetics

Fly crosses and stocks were maintained on cornmeal-molasses medium. To eliminate the possibility that *Wolbachia* infection was the maternally inherited factor we discovered in these experiments that modifies *ND23* mutant phenotypes, we tested flies cured of *Wolbachia* by growing them for two generations at 25° on medium containing 30 mg/ml tetracycline

(Dobson *et al.* 2002). For aging studies, flies were maintained at 25° until adults were 0–2 days post eclosion. Adults were then transferred to 29° for further aging. Canton-S was used as the wild-type control. The *ND23⁶⁰¹¹⁴* line was generated by EMS mutagenesis in our previous screens for temperature-sensitive paralytic mutants. *ND23^{G14097}* deficiency stocks for mapping (including *Df(3R)Exel8162*), *C155-Gal4*, *Tubulin-Gal4*, and *Ddc-Gal4*, *UAS-MitoGFP* were obtained from the Bloomington *Drosophila* Stock Center. *Repo-Gal4* (second chromosome insert) was a gift from Brad Jones, University of Mississippi. *UAS-ND23^{WT}* was generated as described below.

DNA cloning

To generate *UAS-ND23^{WT}* flies, mRNA was isolated from Canton-S flies using TRIzol RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer instructions. Complementary DNA (cDNA) was synthesized from the isolated RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The following primers were used to amplify *ND23* from the cDNA: ND23-F: ATGTCGCTAACTATGCGAAT, ND23-R: TAACGATAGAGATGGTCGG. The PCR product was subcloned into pCR8/GW/TOPO with the pCR8/GW/TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and then cloned into a pUAS⁺ germ line transformation vector (pTW, provided by T. Murphy, Carnegie Institute, Troy, MI; <https://emb.carnegiescience.edu/drosophila-gateway-vector-collection>) with LR Clonase II Enzyme Mix (Invitrogen). DNA sequences were confirmed using standard Sanger sequencing protocols, followed by germ line transformation into *w¹¹¹⁸* flies using typical techniques for random genome integration.

Lifespan analysis

Adult flies were raised at 25° and collected 0–2 days post eclosion under CO₂. Males and females were separated, and aged at 29° at a density of 10–20 flies per vial. Flies were transferred to fresh vials every 2 days, and the number of dead flies in each vial recorded. The total number of flies used to determine lifespan for various genotypes ranged from 80 to 169 over 5–11 independent trials. Exact numbers for each experiment are reported in the figures or figure legends. OASIS2 (Han *et al.* 2016) was used to compute mean lifespans and perform log-rank tests for statistical comparisons.

Western blot

Three male flies (3–4 days old) were frozen at –80° until they were homogenized in 45 µl of 2× SDS sample buffer (1.52 g Tris base, 20 ml glycerol, 2.0 g SDS, 2.0 ml 2-mercaptoethanol, 1 mg bromophenol blue, H₂O to 100 ml, pH 6.8). The homogenized sample was boiled for 5 min and then spun at 14,000 rpm for 10 min. A total of 15 µl of the supernatant was loaded per well in a Bolt 4–12% Bis-Tris Plus Gel (Invitrogen). Western blots were probed with a mouse, anti-actin antibody (MAB1501; Millipore Sigma) at 1:10,000 and a mouse anti-NDUFS8 antibody (A-6, sc-515527, Lot # B1717; Santa Cruz Biotechnology). Primary antibodies were diluted in

blocking buffer [1:1 Odyssey Blocking Buffer (PBS) (LI-COR): PBS-Tween20–0.2%]. The secondary antibody was IRDye 800 donkey anti-mouse (LI-COR) at 1:5000 and diluted in blocking buffer plus 0.1% SDS. Blots were imaged on an Odyssey Imaging System, and bands were quantified using Image Studio version 3.1 software. For quantification, bands from two gels were averaged.

Histology

Fly heads were severed and placed in fresh Carnoy's fixative (ethanol/chloroform/glacial acetic acid at the ratio 6:3:1) for 24–48 hr at 4°. Heads were then washed and placed in 70% ethanol and processed into paraffin using standard histological procedures. Embedded heads were sectioned at 5 µm and stained with hematoxylin and eosin (H&E). Images were taken under a Nikon light microscope (Nikon, Tokyo, Japan), equipped with a QImaging camera (QImaging company). Images were generated using QImaging software and processed with Photoshop.

Neurodegeneration index

Neurodegeneration is indicated by the appearance of vacuolar lesions in the brain neuropil. To determine the neurodegeneration index for a brain, well-oriented 5 µm sections spanning the entire brain (~25 sections in total) were considered. Five levels of neurodegeneration (0, 1, 2, 3, and 4) were defined (Supplemental Material, Figure S1): 0 = no vacuoles; 1 = only a few, small vacuoles (mainly in the optic lobe) in only a few sections; 2 = many vacuoles in many sections (mainly in the optic lobe, but may also be a few in the central brain); 3 = vacuoles start to become prominent in the central brain; and 4 = many vacuoles in the central brain and some vacuoles in the optic lobes and central brain are large. Scoring of the neurodegeneration index was done blind with respect to genotype. The number of brains scored for each genotype is reported in the figures. Student's *t*-test values were used to determine statistical significance.

Immunohistochemistry

Brains were dissected and fixed in 4% formaldehyde in PBS for 20 min at room temperature. Samples were then placed in blocking buffer (PBS with 0.1% Triton X-100 and 0.1% normal goat serum) for 2 hr at room temperature. Samples were incubated in primary antibodies overnight at 4°. Samples were subsequently washed five times in PBS, and then incubated in secondary antibodies for 2 hr at room temperature. Finally, samples were washed five times in PBS and mounted in Vectashield. Primary antibodies were diluted in blocking buffer and included: rabbit anti-tyrosine hydroxylase (1:100; Millipore, Bedford, MA), and chicken anti-GFP (1:500; Invitrogen). Secondary antibodies used were: goat anti-rabbit Alexa-568 and goat anti-mouse Alexa-488 (Invitrogen) at 1:200 and diluted in blocking buffer.

Confocal imaging and quantification

Images were obtained on a Leica LSM 500 confocal microscope. Serial 0.34 µm *z*-stacks were obtained for each image

with a 2× zoom, using a Plan-Apochromat 100×/1.46 numerical aperture oil objective. For Figure 7, brightness and contrast were adjusted using Adobe Photoshop. Images were quantified with ImageJ software (Schneider *et al.* 2012), using brightest point projections of the acquired *z*-stacks. A circular region of interest (ROI) with a diameter of 1.2 μm was defined. Every GFP punctum within a tyrosine hydroxylase labeled cell was marked by the ROI if it was found to be larger than the ROI in any direction.

ATP assay

ATP levels were measured using bioluminescence with the Molecular Probes ATP Determine Kit (Thermo Fisher Scientific, Waltham, MA) following the recommended instructions. Preparation of experimental samples: seven flies were collected and each was rinsed several times in cold PBS. Heads were removed and homogenized in 140 μl of extraction buffer [6 M guanidine-HCL, 100 mM Tris (pH 7.8), 4 mM EDTA]. Then, 65 μl of homogenate (protein stock) was removed and frozen at –80° for protein quantification (see below). The rest of the homogenate was boiled for 5 min and then centrifuged at 14 K at 4° for 3 min. Next, 20 μl of the supernatant was diluted twice to a final dilution of 1:37.5 in dilution buffer [25 mM Tris (pH 7.8), 100 μM EDTA], and then centrifuged at 20,000 × *g* for 3 min at room temperature. A total of 5 μl of the experimental sample was diluted with 95 μl of the kit's standard reaction mixture. Plates were read on a Biotek multimode microplate reader. Three luminescence reads per well were averaged. Background luminescence of each well before the addition of experimental samples was subtracted. Three technical replicates per biological replicate were averaged. A standard curve was run with each experiment and used to determine ATP values, which were then normalized to protein content. Protein content was determined using fluorescence and the NanoOrange Protein Quantitation Kit (Thermo Fisher Scientific) following the recommended instructions. For preparation of experimental samples, the protein stock (see above) was diluted to 75 and 50% with the kit's 1× diluent. Plates were read on a Biotek Synergy 2 Multi-Mode microplate reader. Three technical replicates per biological replicate were determined. In order to easily compare values from multiple experiments, the average ATP value (normalized to protein content) was determined for all the controls in a given experiment. Each ATP value (normalized to protein content) within an experiment was then normalized to the control average from that same experiment. The reported means and SEMs were determined from these normalized values. Six biological replicates were performed with 2–4 day old flies, and seven biological replicates were performed with 17–19 day old flies. Statistical significance was determined using a two-tailed Student's *t*-test.

Behavioral assays

For climbing assays, flies were raised at 25° and maintained at 29° as described for lifespan analysis. Flies (9–13 per vial) were transferred from 29° into a climbing chamber at 25°. The

climbing chamber consisted of two empty vials stacked on top of each other, with the top vial inverted and taped to the bottom vial. After a 1 min rest period, flies were tapped down to the bottom of the vial and the number of flies that climbed above a line marking a vertical height of 8 cm within 10 sec was recorded. The climbing percentage for each vial was determined as the average of seven trials per vial, with 1 min rest periods between trials. The results from four to six vials were averaged to determine the climbing percentage for each genotype. Statistical significance was determined using a two-tailed Student's *t*-test.

To assay sensitivity to mechanical shock ("bang-sensitivity"), flies (9–13 per vial) were transferred from 29° to an empty vial at 25°. After allowing the flies to rest for 2 min in the vial, they were subjected to mechanical shock by vortexing the vial at maximum speed for 10 sec. The number of flies that subsequently climbed above 5 cm within 10 sec after cessation of vortexing was recorded. Results of two trials per vial (with 1 min rest interval between trials) were averaged to determine the climbing percentage per vial. Results from four to seven vials were averaged to determine the climbing percentage for each genotype. Statistical significance was determined using a two-tailed Student's *t*-test.

DNA sequencing

For DNA sequencing, a region of genomic (mitochondrial or nuclear) DNA from a single fly was first amplified using PCR and standard conditions. The primers used to make this template DNA are listed in Table S2. mtDNA templates were <3 kb in length, overlapping, and together covered a region of the mitochondrial genome that included all of the protein and tRNA coding genes. Template DNA was run on an agarose gel, and a single band of the correct size was purified from the gel for subsequent amplification by PCR with the BigDye-Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and sequencing primers, which are also listed in Table S2. These PCR products were cleaned using Axygen AxyPrep Mag DyeClean Up Kit (ThermoFisher Scientific) and sequenced using Sanger methods at the University of Wisconsin Biotechnology Center DNA sequencing facility. Excluding A-T rich alignments, mitochondrial pseudogenes in nuclear chromosomes (numts) are short and rare in *Drosophila melanogaster* (Rogers and Griffiths-Jones 2012). Estimates range from three to six numts that have an average length of ~200 bases, and together constitute ~800 bps (Bensasson *et al.* 2001; Richly and Leister 2004; Pamilo *et al.* 2007; Rogers and Griffiths-Jones 2012). Nevertheless, by sequencing a single band of purified template DNA of the correct size (predicted from published mtDNA sequence), we have avoided accidental amplification of numts.

Sequence analysis was done using ApE-a plasmid Editor version 2.0.45 by M. Wayne Davis. We compared our sequence data to a "reference sequence" (accession no. U37541.1). We used 2–10 sequencing reactions from at least two flies to confirm differences between the mtDNA sequence in ND23⁶⁰¹¹⁴ and the reference sequence, as well as differences between the mtDNA sequence in ND23⁶⁰¹¹⁴ and ND23^{Del} and ND23^{G14097}.

Figure 1 *60114* mutant flies exhibit shortened lifespan and neurodegeneration. (A) The lifespan of *60114/60114* is significantly shorter than that of *+/+* or *60114/+* (log-rank test $P < 0.0001$ for both males and females). Mean lifespan at 29° (days post eclosion) $\pm$ SEM *60114/60114*: males, 16.1 ± 0.4 ($n = 115$, 6 trials); females, 17.9 ± 0.3 ($n = 126$, 7 trials). *60114/+*: males, 51.5 ± 0.8 ($n = 107$, 7 trials); females, 51.6 ± 1.2 ($n = 101$, 7 trials). *+/+*: males, 39.0 ± 0.5 ($n = 108$, 6 trials); females, 39.4 ± 0.7 ($n = 169$, 9 trials). (B) Brain sections from *60114/60114* flies (aged at 29°) exhibit progressive, age-dependent vacuolar pathology not present in controls. The dotted ovals denote the area of extreme focal neuropathology in the posterior lateral protocerebrum. The age (days post eclosion) is indicated in the top right white box. The neurodegeneration index (*Materials and Methods*) score for each brain represented is indicated in the bottom left white

box. Bar, 100 μ m. (C) Quantification of neurodegeneration brain sections from *60114/60114* and *60114/+* flies using the neurodegeneration index. Error bars represent SEM.

mtDNA copy number

We used a published protocol for PCR-based determination of mtDNA copy number (Rooney *et al.* 2015). Briefly, 30 flies were homogenized in liquid nitrogen. DNA was isolated from the ground tissue using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The DNA samples were diluted 1:2 in TE buffer, and the DNA concentration was determined using the Quant-iT Pico Green double-stranded DNA Assay Kit (Thermo Fisher Scientific). PCR was then performed using “100% template” (60 ng of DNA in nuclease-free water with a final volume of 5 μ l), a “50% control” (30 ng of DNA in nuclease-free water with a final volume of 5 μ l), and a “no template control” (5 μ l of TE buffer in place of template DNA). A total of 20 μ l of PCR master mix was added to these 5 μ l samples for a total PCR volume of 25 μ l. The PCR master mix contained 9.25 μ l of nuclease-free water, 2.5 μ l of 10 $\times$ Ex Taq DNA polymerase buffer, 2.0 μ l of 10 mM dNTPs, 2.5 μ l of 10 μ M forward and reverse primers [primers for *D. melanogaster* published in Rooney *et al.* (2015)], 1 μ l of 25 mM MgCl₂, and 0.25 μ l of Ex Taq DNA polymerase. After PCR, the DNA concentration of the PCR product was determined by Quant-iT Pico Green double-stranded DNA Assay Kit. PCR conditions were optimized so that the PCR product concentration from the “50% control” was 40–60% of the PCR product concentration from the “100% template.” The following conditions were used for amplifying DNA from the mitochondrial genome: 94° for 2 min, 94° for 30 sec, 64° for 30 sec, 72° for 1 min, go to step 2 15 $\times$, and 72° for 5 min. The following conditions were used for amplifying DNA from the nuclear genome: 94° for

2 min, 94° for 30 sec, 65° for 30 sec, 72° for 1 min, go to step 2 19 $\times$, and 72° for 5 min. The ratio of mtDNA/nDNA was determined. For each genotype, four biological replicates were performed. For the data presented in Figure 12, ratios were normalized to the highest ratio in each genotype. Statistical significance was determined using a two-tailed Student’s *t*-test.

Data availability

Strains and reagents are available upon request. File S1 contains detailed descriptions of all supplemental files. File S2 contains a movie showing the bang-sensitivity of ND23 mutants and controls (<https://doi.org/10.6084/m9.figshare.5930281.v1>). Table S1 lists mtDNA variants discovered. mtDNA sequences are deposited in GenBank as accession numbers KX889415.2, KX889416.2, and KX889417.2. Table S2 lists primers used in these studies.

Results

Mutant 60114 exhibits neurodegeneration and shortened lifespan

Among our large collection of temperature-sensitive paralytic mutants previously shown to be enriched for mutations in genes required for maintenance of neuronal viability (Palladino *et al.* 2002) we identified mutant line *60114*, which exhibited a shortened lifespan (Figure 1A) and progressive, age-dependent neurodegeneration (Figure 1B).

Figure 2 *ND23*^{G14097} fails to complement 60114. (A) The lifespan of 60114/*ND23*^{G14097} is significantly shorter than 60114/+ and *ND23*^{G14097}/+ (log-rank test $P < 0.0001$ for both males and females). Mean lifespan at 29° (days post eclosion) ± SEM 60114/*ND23*^{G14097}: males, 19.022 ($n = 145$, 8 trials); females, 18.0 ± 0.2 ($n = 168$, 9 trials). *ND23*^{G14097}/+: males, 39.5 ± 0.7 ($n = 100$, 7 trials); females, 46.4 ± 0.5 ($n = 100$, 6 trials). Lifespans for +/+ and 60114/60114 are the same data shown in Figure 1. (B and C) 60114/*ND23*^{G14097} exhibit age-dependent neurodegeneration. (B) Brain sections from 60114/60114, 60114/*ND23*^{G14097}, and +/+. The neurodegeneration index score

for each brain represented is indicated in the white box. Bar, 100 μ m. (C) Average neurodegeneration index score ± SEM: 60114/60114: 3.6 ± 0.1 ($n = 12$, re-represented from Figure 1). 60114/*ND23*^{G14097}: 3.0 ± 0.1 ($n = 10$). *ND23*^{G14097}/+: 0 ± 0 ($n = 3$, re-represented from Figure 1). *ND23*^{G14097}/+: 0 ± 0 ($n = 3$). Error bars represent SEM.

Neurodegeneration was manifested by the appearance of spongiform vacuolar lesions in H&E-stained brain sections.

To quantify the neurodegeneration phenotype, we utilized a neurodegeneration index (ND Index, see *Materials and Methods* and Figure S1) that scores the severity of neurodegeneration based on the size and abundance of vacuolar lesions. In previous studies, this index has proved to be a useful and reliable metric for comparing neurodegeneration among different genotypes under different conditions (Cao *et al.* 2013). Vacuolar lesions in 60114 homozygotes are not seen at 5–7 days post eclosion, but are apparent at 10–12 days post eclosion (Figure 1C). Most often, lesions first appear in the optic lobes, lateral horn and/or mushroom body calyces. As 60114 mutants age, vacuolar pathology progresses and becomes prominent in the central brain. Many flies eventually exhibit an extreme focal neuropathology (often symmetrically on either side of the brain) in the posterior lateral protocerebrum (Figure 1B, 50% of males, $n = 20$; 86% of females, $n = 28$). The mutant phenotypes are recessive since 60114/+ heterozygotes did not exhibit shortened lifespan (Figure 1A) or neurodegeneration (Figure 1, B and C).

Unexpectedly, we found that 60114/+ heterozygotes have a longer lifespan than Canton-S, the nominal wild-type background strain (Figure 1A). To examine the genetic basis of this lifespan extension, we generated 60114/+ heterozygotes in reciprocal crosses. When the maternal contribution came from the wild-type strain rather than 60114 homozygotes [(♀)/+60114 vs. (♀)60114/+, where (♀) indicates which genotype made the maternal contribution], lifespan was still extended in female heterozygotes, but not in male heterozygotes (Figure S2). Thus, the lifespan extension follows inheritance of the sex chromosomes. Because 60114 maps to the third chromosome (see below), this result suggests that the lifespan extension is not associated with 60114. Instead, this lifespan extension is likely due to an X-linked dominant variant in the 60114 background that increases

lifespan, and/or a recessive X-linked variant in the Canton-S background that reduces lifespan, consistent with the well-documented phenomenon of hybrid vigor (Partridge and Gems 2007). In any case, it was distinct from the 60114-associated phenotypes that we investigated further.

60114 defines a new mutation of mitochondrial complex I protein ND23

We used standard deletion mapping to uncover the shortened lifespan and neurodegeneration phenotypes of 60114. *Df(3R)Exel1862* (*ND23*^{Del}) uncovered 60114, whereas *Df(3R)ED5705* and *Df(3R)Exel7327* did not, localizing the mutation to a region on chromosome 3 between sequence coordinates 15,793,796 and 15,901,434 that contains 17 genes (Figure S3). A recessive lethal mutation of one of these genes, *NADH dehydrogenase (ubiquinone) 23 kDa subunit* (*ND23*), *ND23*^{G14097}, which is also lethal over *ND23*^{Del}, failed to complement 60114 (Figure 2), indicating likely allelism. Western blot analysis confirmed that ND23 protein levels are strongly decreased in 60114 homozygous flies, as well as in 60114/*ND23*^{G14097} and 60114/*ND23*^{Del} flies compared to controls (Figure 3). To confirm 60114 as a mutant allele of *ND23*, we sequenced *ND23* in 60114 and Canton-S, and compared these sequences to a reference sequence (accession no. U37541.1). The *ND23* sequence in Canton-S did not differ from the reference sequence. However, there were six base changes in the *ND23* sequence from 60114 flies. Three of these base changes were synonymous. Two base changes occurred in an intron, but these were not in splicing or branch point consensus sequences. However, one base change was a G-to-A substitution 847 bases from the translational start site, which resulted in a glycine-to-aspartic acid amino acid substitution at amino acid position 199. We used the PROVEAN web server (Choi and Chan 2015) to predict the functional effect of this substitution. The PROVEAN score for this substitution is −6.919, and thus the substitution is deemed deleterious. Pairwise protein sequence alignment between ND23 and its

Figure 3 *ND23*⁶⁰¹¹⁴ and *ND23*^{G14097} are strong loss of function mutations. (A) Flies heterozygous for *ND23*⁶⁰¹¹⁴ have decreased levels of ND23 protein. ND23 levels are further decreased in *ND23*⁶⁰¹¹⁴ homozygotes. Decreased expression of ND23 protein in *ND23*^{G14097} is comparable to that of a deletion for the gene. (B) Quantification ($n = 2$). Error bars represent SEM.

human ortholog, NDUFS8, demonstrates that this protein is highly conserved (Figure S4), including the glycine at position 199. As confirmation, we found that both shortened lifespan (Figure 4 and Table 1) and neurodegeneration (Figure 5) in *ND23*⁶⁰¹¹⁴/*ND23*^{Del} could be rescued by ubiquitous expression of a *UAS-ND23*^{WT} transgene using a *Tubulin-Gal4* driver (Brand and Perrimon 1993). Together these results indicate that 60114 is a mutant allele of *ND23* and will be referred to hereafter as, *ND23*⁶⁰¹¹⁴.

Mitochondria are structurally and functionally aberrant in *ND23*⁶⁰¹¹⁴

ND23 is a core component of mitochondrial complex 1, required for mitochondrial electron transport and ATP generation. Morphological abnormalities in mitochondrial appearance have been described in patients with diseases affecting complex 1 (Pham *et al.* 2004; Koopman *et al.* 2005), as well as in *Drosophila* models of mitochondrial dysfunction (Celotto *et al.* 2006; Park *et al.* 2006; Mast *et al.* 2008; Xu *et al.* 2008; Burman *et al.* 2014; Hegde *et al.* 2014). Even under normal conditions, mitochondrial morphology is highly dynamic and is variable from cell type to cell type (Hoppins 2014).

In *Drosophila*, abnormal mitochondrial morphology can be detected using a green fluorescent protein with a mitochondrial import signal (*UAS-MitoGFP*) (Park *et al.* 2006; Wu *et al.* 2013; Hegde *et al.* 2014). To clearly visualize mitochondria in individual cells, we needed to express the *UAS-MitoGFP* transgene in relatively isolated neurons. We accomplished this by targeting its expression to neurons that express either serotonin or dopamine using *Dopa decarboxylase* (*Ddc*)-*Gal4*. We dissected brains from 15–17 day old adults and probed them with antibodies against tyrosine hydroxylase, which labels dopamine neurons, and GFP. This double-labeling approach allowed us to clearly visualize GFP (mitochondria) in a subset of neurons (dopaminergic). Tyrosine hydroxylase immunohistochemistry has identified eight clusters of dopaminergic neurons in the *Drosophila* brain (Mao and Davis 2009).

Figure 4 Shortened lifespan of *ND23* mutants is rescued by ubiquitous expression of a wild-type *ND23* transgene. Ubiquitous expression of *UAS-ND23*^{WT} using a *Tub-Gal4* driver in an *ND23*⁶⁰¹¹⁴/*ND23*^{Del} mutant background (*UAS-ND23*^{WT}/+; *Tub-Gal4, ND23*⁶⁰¹¹⁴/*ND23*^{Del}) rescues lifespan compared with *Tub-Gal4, ND23*⁶⁰¹¹⁴/*ND23*^{Del} controls (log-rank test $P < 0.0001$ for both males and females). Mean lifespan at 29° (days post-eclosion) $\pm$ SEM *Tub-Gal4, ND23*⁶⁰¹¹⁴/*ND23*^{Del}: females, 22.9 ± 0.2 ($n = 95$, 6 trials); males, 22.5 ± 0.2 ($n = 112$, 7 trials). *ND23*^{Del}/+: females, 39.2 ± 0.4 ($n = 112$, 7 trials); males, 34.9 ± 0.4 ($n = 141$, 10 trials). *UAS-ND23*^{WT}/+; *Tub-Gal4, ND23*⁶⁰¹¹⁴/*ND23*^{Del}: females, 34.9 ± 0.7 ($n = 80$, 5 trials); males, 30.8 ± 0.5 ($n = 84$, 5 trials). Error bars represent SEM.

Neurons in the protocerebral posterior medial 2 (PPM2) cluster were easy to identify. Furthermore, their location in the brain, as well as the spacing between them, made them ideal for imaging. For these reasons, and for consistency, we only imaged cells in the PPM2 cluster (Figure 6). Mitochondria in *ND23* mutant flies appeared grossly enlarged compared with controls. The number of neuronal cell bodies containing enlarged mitochondria (diameter $>1.2 \mu\text{m}$) increased nearly fourfold in *Ddc-Gal4, UAS-MitoGFP*/+; *ND23*⁶⁰¹¹⁴/*ND23*^{G14097} mutant flies compared with *Ddc-Gal4, UAS-MitoGFP*/+; *ND23*⁶⁰¹¹⁴/+ control flies (Figure 6D: control: 12 ± 5 (Avg. $\pm$ SEM), $n = 65$ cells from 10 PPM2 clusters in five brains; mutant: 47 ± 5 , $n = 79$ cells from 12 PPM2 clusters in six brains; $P < 0.001$). Furthermore, the number of enlarged mitochondria in the cells also increased significantly (Figure 6E: control: 0.75 ± 0.5 ; mutant: 3.3 ± 0.1 ; $P < 0.001$). The perturbation in mitochondrial morphology in *ND23* mutants was fully rescued by coexpressing the *UAS-ND23*^{WT} transgene in the marked dopaminergic neurons, *Ddc-Gal4, UAS-MitoGFP/UAS-ND23*^{WT}; *ND23*⁶⁰¹¹⁴/*ND23*^{G14097} (Figure 6D: rescue: 5.1 ± 2.6 ; and Figure 6E: rescue: 0.3 ± 0.2 , $n = 57$ from nine PPM2 clusters in five brains).

We tested whether *ND23*⁶⁰¹¹⁴ disrupts mitochondrial function by quantifying ATP levels in the heads of mutant flies. At 2–4 days post eclosion, the ATP level in *ND23* mutant heads is decreased to $81 \pm 5\%$ of controls (Figure 6F, $P < 0.01$). We also measured ATP levels in the heads of 17–19 day old flies and found a similar decrease in *ND23* mutants compared with controls ($77 \pm 6\%$ of controls; $P < 0.05$, data not shown). Thus, *ND23*⁶⁰¹¹⁴ exerts deleterious effects both on mitochondrial morphology and function.

Table 1 Mean lifespan of flies examined in rescue experiments

Genotype	Mean restricted lifespan (days posteclosion)	
	Females	Males
<i>Tubulin-Gal4, ND23⁶⁰¹¹⁴/ND23^{Del}</i>	22.9 ± 0.2	22.5 ± 0.2
<i>ND23^{Del/+}</i>	39.2 ± 0.4	34.9 ± 0.4
<i>UAS-ND23^{WT/+}; Tubulin-Gal4, ND23⁶⁰¹¹⁴/ND23^{Del}</i>	34.9 ± 0.7	30.5 ± 0.5
<i>C155-Gal4; ND23⁶⁰¹¹⁴/ND23^{Del}</i>	24.5 ± 0.2	24.7 ± 0.3
<i>C155-Gal4; UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{Del}</i>	30.2 ± 0.7	28.5 ± 0.6
<i>Repo-Gal4/+; ND23⁶⁰¹¹⁴/ND23^{Del}</i>	21.1 ± 0.3	21.4 ± 0.3
<i>Repo-Gal4/UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{Del}</i>	20.8 ± 0.2	20.9 ± 0.3

ND23 mutants exhibit behavioral deficits consistent with nervous system dysfunction

ND23 mutants exhibit several behavioral phenotypes indicative of impaired neural function, including temperature-sensitive paralysis (Figure S5), age-dependent impairment in locomotor activity (Figure 7A), and bang-sensitive paralysis (Figure 7B and File S2). Although the bang-sensitive phenotype becomes more severe in older ND23 mutants, even 2–4 day old mutants are sensitive to mechanical shock as shown by the impaired climbing ability of young ND23 flies after vortexing for 10 sec (Figure 7, A and B and File S2).

ND23 is required in neurons for normal lifespan and neuronal maintenance

To investigate whether decreased lifespan and neurodegeneration in ND23 were due to loss of ND23 function in neurons

or glia, we tested whether neuronal-specific or glial-specific expression of the *UAS-ND23^{WT}* transgene could rescue these mutant phenotypes. Similar to ubiquitous expression by *Tub-Gal4*, neuronal-specific expression of *UAS-ND23^{WT}* by *C155-Gal4* rescued the shortened lifespan. This rescue was nearly complete in mutant males and partial in mutant females (Figure 8A and Table 1). Neuronal-specific expression of *UAS-ND23^{WT}* also delayed the onset of neurodegeneration, again more strongly in males than females (Figure 9, A and B). Although we have not further investigated the basis of this sex-dependent difference in rescue with the X-chromosome *C155-Gal4* driver, it is consistent with higher levels of *Gal4* expression in males than in females owing to dosage compensation (Warrick *et al.* 1999; Long and Griffith 2000). In contrast with neuronal expression of ND23, glial-specific expression of *UAS-ND23^{WT}* using *Repo-Gal4* did not rescue either lifespan (Figure 8B and Table 1) or neurodegeneration (Figure 9, C and D). These results suggest that the neurodegeneration observed in ND23 mutants results primarily from loss of ND23 activity in neurons rather than glia, and that neural dysfunction in ND23⁶⁰¹¹⁴ is predominantly responsible for shortened lifespan.

ND23 mutant phenotypes are modified by a maternally inherited factor

During the course of these investigations, we discovered that the severity of the mutant ND23 phenotypes was dependent on a maternally inherited factor that was independent of nuclear transmission. Figure 10A illustrates the crossing scheme used in the experiments that revealed the presence of this maternally inherited factor. We noticed that when ND23⁶⁰¹¹⁴

Figure 5 Neurodegeneration in ND23 mutants is rescued by ubiquitous expression of a wild-type ND23 transgene. Brain sections from (A) ND23^{Del/+}, (B) *Tub-Gal4, ND23⁶⁰¹¹⁴/ND23^{Del}*, and (C) *UAS-ND23^{WT/+}; Tub-Gal4, ND23⁶⁰¹¹⁴/ND23^{Del}*. (D) Average neurodegeneration index score ± SEM: ND23^{Del/+} (24–26 days): females, 0.7 ± 0.2 (*n* = 7); males, 0.7 ± 0.2 (*n* = 7). *Tub-Gal4, ND23⁶⁰¹¹⁴/ND23^{Del}* (21–23 days): females, 2.4 ± 0.2 (*n* = 8); males (*n* = 7), 1.9 ± 0.1. *UAS-ND23^{WT/+}; Tub-Gal4, ND23⁶⁰¹¹⁴/ND23^{Del}* (21–23 days): females, 1.0 ± 0.0 (*n* = 6); males, 1.0 ± 0.1 (*n* = 11). Student's *t*-test was used to determine statistical significance. ***=*p*<0.001, ****=*p*<0.0001. Bar, 100 μm.

Figure 6 Mitochondrial morphology and function is aberrant in *ND23* mutants. (A–E) Whole mount brains from 15–17 day old adults stained with an antibody against GFP and tyrosine hydroxylase. Tyrosine hydroxylase-positive cells in the PPM2 cluster were imaged. (A) *Ddc-Gal4, UAS-MitoGFP/+; ND23⁶⁰¹¹⁴/+*, (B) *Ddc-Gal4, UAS-MitoGFP/+; ND23⁶⁰¹¹⁴/ND23^{G14097}*, and (C) *Ddc-Gal4, UAS-MitoGFP/UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{G14097}*. (D) Percentage of imaged cells containing at least one enlarged mitochondria (GFP puncta): ND23 mutant: 47 ± 5% (imaged 49 cells in 12 PPM2 clusters from six brains); control: 12 ± 5% (imaged 65 cells in 10 PPM2 clusters from five brains); rescue: 5 ± 3% (imaged 57 cells in nine PPM2 clusters from five brains). (E) The number of enlarged mitochondria per cell: ND23 mutant: 3.3 ± 0.1; control: 0.75 ± 0.1; rescue: 0.3 ± 0.2. (F) At 2–4 days post eclosion, ATP levels in the heads of *ND23⁶⁰¹¹⁴/ND23⁶⁰¹¹⁴* flies is reduced compared controls (81 ± 5% of control values, $P < 0.01$). Student's *t*-test was used to determine statistical significance. **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$.

homozygous females were crossed to *ND23^{G14097}* males, the (♀)*ND23⁶⁰¹¹⁴/ND23^{G14097}* progeny (Figure 10A, gray cell) had a shorter lifespan than flies generated from the reciprocal cross that had an identical nuclear genotype, (♀)*ND23^{G14097}/ND23⁶⁰¹¹⁴*, but a different maternal contribution (Figure 10A, blue cell, and 10B, Table 2). The same result was observed in the male progeny from this crossing scheme (Figure S6A and Table 2). However, because the male progeny from these reciprocal crosses did not have identical sex chromosome genotypes, we limited our analysis to females. Similar results were also observed when *ND23^{Del}* was used instead of *ND23^{G14097}* in these reciprocal crosses (Figure S6B).

We subsequently found that climbing ability and neurodegeneration in *ND23* mutants was also dependent on a maternally inherited factor, independent of nuclear transmission. (♀)*ND23^{G14097}/ND23⁶⁰¹¹⁴* flies were stronger climbers than (♀)*ND23⁶⁰¹¹⁴/ND23^{G14097}* flies (Figure 10C), as was also the case for (♀)*ND23^{DEL}/ND23⁶⁰¹¹⁴* compared with (♀)*ND23⁶⁰¹¹⁴/ND23^{Del}* (Figure S7). Moreover, the onset of neurodegeneration was delayed in (♀)*ND23^{G14097}/ND23⁶⁰¹¹⁴* flies compared with (♀)*ND23⁶⁰¹¹⁴/ND23^{G14097}* (Figure 10, C and D).

Lifespan reduction and neurodegeneration in *ND23* mutants is modified by mitochondrial background

The pattern of transmission of the mutant phenotype-modifying factor observed in these experiments was indicative of maternal inheritance. Because mitochondria are maternally

inherited, we hypothesized that mitochondria were the source of the maternally inherited factor that was interacting with mutations of *ND23* to determine lifespan and the age at neurodegeneration onset. To test this hypothesis, we replaced mitochondria in the *ND23⁶⁰¹¹⁴* line with mitochondria from the *ND23^{G14097}* line (Figure 11A, purple cell). To do this, we crossed *ND23^{G14097}* females with *ND23⁶⁰¹¹⁴* males. The *ND23^{G14097}* mutation is recessive lethal, so the stock is maintained using a balancer third chromosome, *TM6C*, allowing us to recover heterozygous F1 *ND23⁶⁰¹¹⁴/TM6C* male and female progeny. These flies were then intercrossed to generate a new balanced stock. As mitochondria are strictly maternally inherited, this new stock contained mitochondria derived entirely from the *ND23^{G14097}* line, but maintained the *ND23⁶⁰¹¹⁴* mutation. We refer to these flies as *ND23⁶⁰¹¹⁴-new MITO*. We then crossed *ND23⁶⁰¹¹⁴-new MITO* females to *ND23^{G14097}* males and measured the lifespan of (♀)*ND23⁶⁰¹¹⁴-new MITO/ND23^{G14097}* female progeny (Figure 11B, purple line, and Table 2). The mean lifespan of these flies was similar to the mean lifespan of (♀)*ND23^{G14097}/ND23⁶⁰¹¹⁴* flies (Figure 11B, blue line, and Table 2). Similar results were observed in male progeny (Figure S6A and Table 2), or when mitochondria in *ND23⁶⁰¹¹⁴* were replaced with mitochondria from *ND23^{Del}* (Figure S6B and Table 2). Thus, although the *ND23⁶⁰¹¹⁴/ND23^{G14097}* mutant genotype reduces mean lifespan by 38% in females (from 39.4 ± 0.7 to 24.4 ± 0.3 days) and 37% in males (from 39.0 ± 0.5 to 24.5 ± 0.2 days), flies that have mitochondria from the *ND23⁶⁰¹¹⁴* line have an additional reduction in mean lifespan.

Figure 7 *ND23* mutants exhibit impaired locomotor activity. (A) *ND23^{Del}/ND23⁶⁰¹¹⁴* flies show a more rapid age-dependent decline in climbing activity compared with *ND23^{Del/+}* controls. (B) *ND23^{Del}/ND23⁶⁰¹¹⁴* mutants do not climb as well as *ND23^{Del/+}* controls after a mechanical shock. Student's *t*-test was used to determine statistical significance. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

Mean lifespan is reduced an additional 15% for females (to 18.4 ± 0.3 days) and an additional 17% for males (to 17.9 ± 0.3 days).

We next tested whether neurodegeneration observed in (♀)*ND23⁶⁰¹¹⁴/ND23^{G14097}* was modified by mitochondria from *ND23^{G14097}*. We compared neurodegeneration in (♀)*ND23⁶⁰¹¹⁴/ND23^{G14097}* and (♀)*ND23^{60114-new MITO}/ND23^{G14097}* brains and found that mitochondria from *ND23^{G14097}* delayed the onset of neurodegeneration (Figure 11, C and D). Although brains from (♀)*ND23⁶⁰¹¹⁴/ND23^{G14097}* mutants exhibit extensive neurodegeneration at 17–19 days post eclosion (average neurodegeneration index score = 3.2), brains from (♀)*ND23^{60114-new MITO}/ND23^{G14097}* at this same age exhibit very little neurodegeneration (average neurodegeneration index score = 1.5). However, by 22–24 days of age (♀)*ND23^{60114-new MITO}/ND23^{G14097}* have developed extensive neurodegeneration (average neurodegeneration index score = 3.5).

Our results support the conclusion that the severity of the *ND23* mutant phenotype is dependent on background mitochondrial genotype. It is important to emphasize that the mitochondrial genotype in *ND23⁶⁰¹¹⁴* or *ND23^{G14097}* does not by itself shorten lifespan or cause neurodegeneration in *ND23/+* heterozygotes (Figure 1 and Figure 2). Thus, the ability of the mitochondrial background to modify lifespan and neurodegeneration apparently depends on a genetic interaction between a presumptive mitochondrial variant and the defect present in *ND23* mutants.

Phenotypic modification of *ND23* mutants correlates with a mtDNA variant

The data presented above suggest that the phenotypic severity of *ND23* mutants is subject to modification by a mitochondrially inherited factor. Specifically, mitochondria from the *ND23⁶⁰¹¹⁴* line enhance the mutant phenotype and/or mitochondria from *ND23^{G14097}* or *ND23^{Del}* partially suppress the mutant phenotype. Thus, we hypothesized that there would be differences in the mtDNA sequence between *ND23⁶⁰¹¹⁴*,

and *ND23^{G14097}* and *ND23^{Del}* that would be responsible for the phenotypic modification.

The mitochondrial genome in *D. melanogaster* contains 19,517 bp and codes for 13 proteins (all of which are subunits of the electron transport chain), 2 ribosomal RNAs, and 22 tRNAs. We used Sanger sequencing to sequence all 13 protein-coding genes and 22 tRNA genes in mitochondria from *ND23⁶⁰¹¹⁴* (KX889415.2), *ND23^{G14097}* (KX889416.2), and *ND23^{Del}* (KX889417.2). Although we saw evidence of heteroplasmy in all the mtDNA sequences, we did not attempt quantification and simply made base calls from the major variant.

mtDNA sequence from *ND23^{G14097}* and *ND23^{Del}* were nearly identical. They only differed at six bases in a noncoding region between ND3 and tRNA-Ala. In this region, *ND23^{Del}* had a deletion of two bases (at position 5963 and 5964), whereas *ND23^{G14097}* had a deletion of four bases (at 5967, 5969, 5970, and 5971). *ND23⁶⁰¹¹⁴* shared the four-base deletion with *ND23^{G14097}*. Although the mtDNA sequence from *ND23^{Del}* and *ND23^{G14097}* were nearly identical, there were 51 differences between these two sequences and the mtDNA sequence from *ND23⁶⁰¹¹⁴* (Table S1 and Table 3). One difference was a duplication of five bases (TTAAT) in *ND23^{G14097}* and *ND23^{Del}* that occurred in a noncoding region immediately 3' of the tRNA-Ala sequence and five bases upstream of the 5' start of tRNA-Arg. The other 50 differences were all SNPs found in coding regions: 38 were synonymous, 11 were nonsynonymous, and one was in the gene that codes for tRNA-Glu. We saw evidence of potential heteroplasmy in only one of the 51 changes we report; although the synonymous SNP in ND1 at position 315 is a G in *ND23⁶⁰¹¹⁴*, there is evidence that there may also be mtDNA with a T in this position, as is seen in *ND23^{Del}* and *ND23^{G14097}*. We used ARWEN (Laslett and Canbäck 2008) to determine that the A-to-C SNP in the tRNA-Glu occurs in the TΨC loop, immediately 3' of the stem, and is not predicted to significantly alter the structure of the tRNA. We used the PROVEAN web server (Choi and Chan

Figure 8 Neuronal-specific expression of a wild-type *ND23* transgene rescues lifespan, whereas glial-specific expression does not. (A) The lifespan of *C155-Gal4; UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{Del}* is significantly longer than *C155-Gal4; ND23⁶⁰¹¹⁴/ND23^{Del}* (log-rank test $P < 0.0001$ for both males and females). (B) In contrast, the lifespan of *Repo-Gal4/UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{Del}* is not significantly different from *Repo-Gal4/+; ND23⁶⁰¹¹⁴/ND23^{Del}* ($P = 0.45$, females; $P = 0.67$, males). Mean lifespan at 29° (days post eclosion) $\pm$ SEM *C155-Gal4; ND23⁶⁰¹¹⁴/ND23^{Del}*: females, 24.5 ± 0.2 ($n = 102$, 8 trials); males, 24.7 ± 0.3 ($n = 87$, 7 trials). *C155-Gal4; UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{Del}*: females, 30.2 ± 0.7 ($n = 84$, 5 trials); males, 28.5 ± 0.6 ($n = 85$, 6 trials). *Repo-Gal4/+; ND23⁶⁰¹¹⁴/ND23^{Del}*: females, 21.1 ± 0.3 ($n = 86$, 7 trials); males, 21.4 ± 0.3 ($n = 85$, 7 trials). *Repo-Gal4/UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{Del}*: females 20.8 ± 0.2 ($n = 148$, 10 trials); males, 20.9 ± 0.3 ($n = 124$, 8 trials). Lifespan data for *UAS-ND23^{WT}/Tub-Gal4, ND23⁶⁰¹¹⁴/ND23^{Del}* is from Figure 6. Error bars represent SEM.

2015) to predict the functional effect of the 11 nonsynonymous SNPs. 10 of the 11 were determined to cause neutral amino acid substitutions. However, the thymine-to-cytosine base change leading to a leucine-to-serine amino acid change at amino acid 12 in ATPase 6 was predicted to be deleterious. Importantly, 47 of the 51 identified differences are represented in at least one of 13 *Drosophila* mitochondrial haplotypes sourced from around the world (Australia; Spain, USA, Benin, Papua New Guinea, Chile, Sweden, and Zimbabwe) that have been sequenced (Wolff *et al.* 2016a), including the potentially deleterious leucine to serine substitution in ATPase6. However, the SNPs in the cytochrome B gene that results in an asparagine-to-aspartic acid amino acid change at position 217, and an arginine-to-glutamine

amino acid change at position 342 were not seen in any of the natural population haplotypes; nor was the SNP in the ND2 gene that results in a methionine-to-valine amino acid change position 280. The TTAAT duplication in the noncoding region between tRNA-Ala and tRNA-Arg was also not found in any of the natural populations. Thus, we identified several mtDNA mutations, one or more of which likely underlies the mitochondrial modification of the shortened lifespan and neurodegeneration phenotypes caused by mutant *ND23*.

The phenotypic modification of *ND23* mutants by mitochondrial background is not due to differences in mtDNA copy number

In humans, mtDNA genetic variants may modulate mtDNA copy number (Suissa *et al.* 2009). In various diseases modeled by cultured cell lines established by transmitochondrial cytoplasmic hybrid production, mtDNA haplogroups have been associated with changes in mtDNA copy number (Wallace 2015). And finally, in *Drosophila*, cytoplasmic suppression of a mutant phenotype caused by mutation in a nuclear encoded mitochondrial protein was correlated with increased mtDNA copy number (Chen *et al.* 2012). To determine whether the mitochondrial backgrounds that modify *ND23* mutant phenotypes are correlated with mtDNA copy number differences, we measured mtDNA copy number in (♀)*ND23^{G14097}/ND23⁶⁰¹¹⁴* and (♀)*ND23⁶⁰¹¹⁴/ND23^{G14097}* flies. Although these flies do have different mitochondrial backgrounds, they do not differ in mtDNA copy number (Figure 12).

Discussion

We have isolated a novel, recessive mutation, *ND23⁶⁰¹¹⁴*, adding to a collection of *Drosophila* mutants with defects in mitochondrial or nuclear genes encoding components of the mitochondrial oxidative-phosphorylation system, all of which shorten lifespan, cause neurodegeneration, and lead to mitochondrial abnormalities (Celotto *et al.* 2006; Liu *et al.* 2007; Mast *et al.* 2008; Xu *et al.* 2008; Burman *et al.* 2014). These features are shared with patients diagnosed with Leigh syndrome including those with mutations in *NDUFS8*, the human homolog of *ND23*.

Although there is a wide range of disease onset, Leigh syndrome is typically first seen before 12 months of age. It is characterized by multifocal spongiform degeneration localized diffusely throughout the brain, but particularly in the basal ganglia and/or brainstem. The lesions are often bilateral and symmetrical, with relative preservation of neurons, and are associated with demyelination and gliosis. Indeed, white matter lesions may be prominent (Lake *et al.* 2015). Similar to patients with Leigh syndrome, degeneration observed in *Drosophila ND23* mutants is also spongiform, most prominent in the neuropil, and mutant flies often develop a characteristic lesion in the posterior lateral protocerebrum that is symmetrical and bilateral.

The characteristic clinical features of Leigh syndrome are primarily neurological (e.g., seizures, hypotonia, ataxia, cognitive impairment), but can also be multisystemic (e.g., gastrointestinal,

Repo-Gal4/UAS-ND23^{WT}; ND23⁶⁰¹¹⁴/ND23^{Del}: females, 3.2 ± 0.2 (13); males, 2.8 ± 0.3 ($n = 10$). Student's *t*-test was used to determine statistical significance. **= $p < 0.01$, ****= $p < 0.0001$. Bar, 100 μ m.

pulmonary, cardiac, metabolic). The cellular and molecular mechanisms responsible for this clinical variability are not fully understood. Here, we demonstrate that the shortened lifespan and neurodegeneration in *ND23* mutants can be substantially rescued by expression of wild-type *ND23* in neurons, but not in glia. Thus, *ND23* dysfunction in the nervous system, and specifically in neurons rather than glia, is responsible for much of the neurodegeneration and early death. This is likely due to the increased energy demands of the nervous system, particularly neurons, compared with glia and other tissues, which may explain why glia can survive without the citric acid cycle, using only glycolysis to satisfy their energy demands, whereas neurons cannot (Volkenhoff *et al.* 2015). Furthermore, our data suggests that although brain lesions in Leigh syndrome are often accompanied by demyelination, this is not a direct consequence of *ND23* defects within glia, but instead a complication from disrupted homeostasis from compromised neurons. However, the fact that ubiquitous expression of wild-type *ND23* provides a somewhat greater degree of rescue (of both lifespan and neurodegeneration) than neuronal-specific rescue suggests that *ND23* dysfunction may also have important phenotypic consequences in cells other than neurons. Although a variety of cells that support nervous system function could be impaired, the most likely candidates are glia. Alternatively, the somewhat greater degree of rescue seen with ubiquitous expression of wild-type *ND23*

than with neuronal-specific expression may be due to differences in the relative level of transgene induction by *Tub-Gal4* vs. *C155-Gal4*.

Interestingly, there are some differences between our results and a recently published study on *ND23* using RNA interference (RNAi) (Cabirol-Pol *et al.* 2018). Although neuronal knockdown of *ND23* caused some phenotypes similar to those observed in *ND23* mutants, including a shortened lifespan and impaired climbing ability, it did not lead to obvious brain degeneration as is seen in *ND23* mutants. Glial knockdown of *ND23* RNAi did, however, cause severe brain vacuolization, leading to the suggestion that mitochondrial dysfunction in glia may contribute to mitochondrial disease pathology. However, our data suggest that normal function of *ND23* in neurons is most critical for maintaining neuronal integrity. Thus, the loss-of-function phenotypes seen in *ND23* mutants and in flies expressing *ND23* RNAi are not identical. Although these differences may be due to off-target effects of the RNAi, they may also reveal the complexity of the precise role of this protein in mitochondrial function and nervous system integrity.

This complexity likely underlies much of the heterogeneity in Leigh syndrome, and other mitochondrial disorders. Mutations in over 75 genes are associated with Leigh syndrome (Lake *et al.* 2016). However, as with other mitochondrial disorders, individuals with the same mutation can have variable disease presentation and life expectancy, suggesting that

($n = 6$), and 3.0 ± 0.6 ($n = 3$) at 23–25 days]. Student's *t*-test was used to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

other genetic or environmental conditions modify the disease and contribute to disease heterogeneity (Budde *et al.* 2003; Marina *et al.* 2013).

It has been hypothesized that mito-nuclear interactions can account for some of the unexplained phenotypic variability of mitochondrial disease (Wallace *et al.* 1999; Wolff *et al.* 2014). Because mtDNA has a significantly higher mutation rate than nDNA, there is a substantial background of existing mitochondrial variants within populations (Ballard and Whitlock 2004; Lynch 2007). As the nuclear and mitochondrial genomes must work in concert, mutations in either genome create the possibility of genetic epistasis. For example, substituting mtDNA from *D. simulans* into the closely related *D. melanogaster* can lead to a mito-nuclear mismatch between a mitochondrial-encoded tRNA and a nuclear-encoded tRNA synthetase, causing large maladaptive effects on development and fitness (Meiklejohn *et al.* 2013). Within species, increasing evidence suggests that natural sequence polymorphism in mtDNA can affect development, fitness, lifespan (Wolff *et al.* 2014), gene

expression (Innocenti *et al.* 2011), respiratory capacity, and mitochondrial number per cell (Kenney *et al.* 2014; Wolff *et al.* 2016b). The magnitude and direction of these effects can depend on the nuclear genetic background. Furthermore, some evidence suggests that naturally occurring mtDNA polymorphisms can modify severity of nuclear-encoded mitochondrial disorders. For example, mtDNA haplotype correlates with the severity of cardiomyopathy associated with

Table 2 Mean lifespan of *ND23* mutants varies with mitochondrial background

Genotype	Mean lifespan (days post eclosion)	
	Females	Males
<i>Canton-S</i>	39.4 $\pm$ 0.7	39.0 $\pm$ 0.5
$(ND23^{60114})_{\text{maternal}}/ND23^{G14097}$	18.4 $\pm$ 0.3	17.9 $\pm$ 0.3
$ND23^{60114}/(ND23^{G14097})_{\text{maternal}}$	22.2 $\pm$ 0.3	23.4 $\pm$ 0.1
$(ND23^{60114})_{\text{new MITO-maternal}}/ND23^{G14097}$	24.4 $\pm$ 0.3	24.6 $\pm$ 0.2

Figure 11 Mitochondrial background can delay onset of neurodegeneration in *ND23* mutants. (A) Mating scheme to replace the mitochondria in the *ND23*⁶⁰¹¹⁴ line (gray mitochondria) with mitochondria from the *ND23*^{G14097} line (blue mitochondria) is shown at the top. The purple circle represents an F1 zygote from the last cross in the scheme. The purple F1 zygotes have the same nuclear genotype as the gray zygotes (from Figure 10) but the same mitochondria as the blue zygotes (from Figure 10). (B) The longer lifespan of (♀)*ND23*^{G14097}/*ND23*⁶⁰¹¹⁴ (blue line) compared to (♀)*ND23*⁶⁰¹¹⁴/*ND23*^{G14097} (black line) first shown in Figure 10 depends on mitochondrial rather than nuclear genotype. The lifespan of (♀)*ND23*⁶⁰¹¹⁴/*ND23*^{G14097} females is extended when their mitochondria come from the *ND23*^{G14097} line (♀)*ND23*⁶⁰¹¹⁴-new MITO/*ND23*^{G14097} (purple line, 24.4 ± 0.3 days, *n* = 124, 8 trials, *P* < 0.0001). (C) Brain sections from (♀)*ND23*⁶⁰¹¹⁴-new MITO/*ND23*^{G14097} at 17–19 and 22–24 days post eclosion. (D) Neurodegeneration observed in (♀)*ND23*⁶⁰¹¹⁴/*ND23*^{G14097} at 17–19 days (average neurodegeneration index ± SEM = 3.2 ± 0.29, *n* = 12) is delayed in (♀)*ND23*⁶⁰¹¹⁴-new MITO/*ND23*^{G14097} females (1.5 ± 0.2 at 17–19 days; *n* = 6, and 3.5 ± 0.2; *n* = 8 at 22–24 days). Student's *t*-test was used to determine statistical significance. ***=*p* < 0.001, ****=*p* < 0.0001. Bar, 100 μm.

adenine translocator-1 deficiency and the clinical expression of Leber's hereditary optic neuropathy (Hudson *et al.* 2007; Strauss *et al.* 2013). Finally, in *Drosophila*, mitochondrial disease-like phenotypes can be suppressed by a maternally inherited factor, likely mitochondria (Chen *et al.* 2012).

Here we directly demonstrate that shortened lifespan and neurodegeneration in *ND23* mutants are modified by a maternally inherited factor. Thus, our data strongly suggest that this factor is mitochondrial background, although we cannot rule out some other currently unknown maternally inherited factor. Specifically, *ND23* mutants with mitochondria from *ND23*⁶⁰¹¹⁴ die sooner and show neurodegeneration earlier than *ND23* mutants with mitochondria from the *ND23*^{G14097} or *ND23*^{Del} lines. We sequenced all 13 protein coding genes and 22 tRNA genes from all three *ND23* mutant lines to determine whether there were mtDNA coding differences that

could account for the mitochondrial modification of these phenotypes. We found 51 potential candidates; one of these differences was a duplication of five bases in a noncoding region between two tRNAs, the rest were SNPs located in gene coding regions.

As targeted manipulation of the mitochondrial genome is not yet feasible, we cannot directly test which of the identified changes is responsible for modifying the *ND23* mutant phenotype. However, some of the mitochondrial variants appear to be more likely candidates than others. Of the 50 mitochondrial SNPs we identified, 38 lead to synonymous amino acid changes. These SNPs are also naturally occurring, making these 38 changes less likely candidates for epistatic interactions with nuclear variants. Although it is difficult to predict the consequences of a base substitution on tRNA function, the SNP in tRNA-Glu is not expected to have large structural

Table 3 The *ND23*⁶⁰¹¹⁴ mtDNA sequence differs from the *ND23*^{G14097} and *ND23*^{Del} consensus sequence at 51 sites

Effected gene	Effected complex	Type of change	Number of synonymous changes	Number of nonsynonymous changes	Amino acid substitution due to nonsynonymous change
ND1	1	SNP	3	1	V190M
ND2	1	SNP	5	2	I277L, M280V ^a
ND3	1	SNP	1	0	
ND4	1	SNP	4	1	V161L
ND4L	1	SNP	1	0	
ND5	1	SNP	2	1	M520I
ND6	1	SNP	1	1	Y21F
CYT8	3	SNP	2	2	N73D ^a , R342Q ^a
COX1	4	SNP	10	0	
COX2	4	SNP	2	0	
COX 3	4	SNP	5	0	
ATPase 6	5	SNP	2	3	L12S ^b S180P M187V
tRNA-Glu		SNP			
3' of tRNA-Ala		five base duplication ^a			

^a Not yet found in a natural population.

^b Predicted to be deleterious.

effects. Furthermore, this SNP is also found in natural populations. Thus, we cautiously suggest it is also an unlikely candidate. However, we want to emphasize that the mitochondrial background that modifies the homozygous mutant *ND23* phenotype does not have much, if any, effect on flies heterozygous for mutant *ND23*. Thus we do not expect the mitochondrial variant alone to cause severe phenotypic effects. Nevertheless, we believe the best candidates are the 11 non-synonymous SNPs and the five-base duplication in the noncoding region between tRNA-Ala and tRNA-Arg. We can further speculate on which of these 12 candidates are most likely to underlie the phenotypic interaction with *ND23* mutants to modify lifespan and neurodegeneration.

The nonsynonymous SNP causing an isoleucine-to-methionine substitution at position 502 in the ND5 subunit of complex I in *ND23*⁶⁰¹¹⁴, but not in *ND23*^{G14097} or *ND23*^{Del} is an intriguing candidate. PROVEAN predicts this to be a neutral mutation and the SNP is found in natural populations, which suggests it is unlikely to have significant functional effects *in vivo*. However, this *ND5* SNP may be detrimental in an *ND23* mutant background, especially because the ND5 subunit is likely to interact physically with the ND23 subunit in complex 1 (-Sazanov and Hinchliffe 2006). Such a possibility would be consistent with our observation that the mitochondrial variant responsible for modifying lifespan and neurodegeneration in *ND23* mutants does not have significant effects on these phenotypes in a wild-type background.

Four of the other 11 changes are also plausible candidates for a mito-nuclear interaction with *ND23* mutants: The leucine-to-serine substitution in the ATPase 6 subunit of complex V that is predicted by PROVEAN to be deleterious, and the two coding variants in cytochrome B and the duplication of five bases in the noncoding region between tRNA-Ala and tRNA-Arg that have not yet been found in natural populations, possibly owing to deleterious effects on their own or in

combination with other nuclear-encoded variants. Although ATPase 6, cytochrome B, tRNA-Ala, and tRNA-Arg do not physically interact with ND23, mutations in any of these molecules could conceivably alter the physiological environment in which ND23 acts, thereby exacerbating the phenotypic manifestation of ND23 mutants.

It is important to note that we have not measured the degree of heteroplasmy at any location in any of the mitochondrial backgrounds. Base calls were made from the major variant. Thus, it remains possible that differences in the presence of a minor mtDNA variant could be responsible for modification of the mutant *ND23* phenotype and further analysis will be required to investigate this possibility.

In conclusion, we have isolated a mutation of *ND23* that causes phenotypes in flies that closely parallel those of Leigh

Figure 12 Modification of *ND23* mutants by mitochondrial background is not due to differences in mtDNA copy number. The amount of mtDNA relative to nuclear DNA was measured in (♀)ND23^{G14097}/ND23⁶⁰¹¹⁴ and (♀)ND23⁶⁰¹¹⁴/ND23^{G14097} flies. Normalized values are graphed. Although these flies do have different mitochondrial backgrounds, they do not differ in mtDNA copy number ($P = 0.54$). Student's *t*-test was used to determine no statistical significance.

syndrome in humans, one of the most commonly inherited mitochondrial disorders. Further characterization of this mutant should help elucidate the cellular and molecular mechanisms that underlie the pathophysiology of Leigh syndrome, which may reveal new avenues for therapeutic intervention. Moreover, we discovered that the phenotypic severity of *ND23*⁶⁰¹¹⁴ varies depending on the mitochondrial background. Sequence analysis of mitochondrial genomes identified several mitochondrial variants, one or more of which are likely candidates for the phenotypic interaction with *ND23*⁶⁰¹¹⁴. Although mito-nuclear interactions have long been suggested to explain unexpected variation in phenotypic severity of mitochondrial disorders, there are still relatively few examples where such an interaction can be convincingly demonstrated *in vivo*. Although further work is needed to resolve the remaining details of the interaction we discovered, it provides a compelling *in vivo* demonstration of the phenotypic importance of mito-nuclear interactions in the context of mitochondrial disease.

A deeper understanding of the mito-nuclear interaction we report here should not only clarify its mechanism, but also enhance our general understanding of mito-nuclear interactions and their effect on mitochondrial function in both normal and disease conditions. Future experiments are aimed at determining the specificity of the mito-nuclear interaction described here. Do the mitochondrial backgrounds we have identified that modify *ND23* mutations also modify other mitochondrial mutant phenotypes? Can the mutant *ND23* mutant phenotype be modified by other mtDNA backgrounds? Furthermore, given that mitochondria seem to have a prominent role in the pathology of neurodegenerative diseases in general (Johri and Beal 2012), it will be important to test whether mutant phenotypes observed in *Drosophila* models of other neurodegenerative diseases, such as Alzheimer's and Parkinson's, can also be modified by mtDNA backgrounds.

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