

Secondary mtDNA Defects Do Not Cause Optic Nerve Dysfunction in a Mouse Model of Dominant Optic Atrophy

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PURPOSE. The majority of patients with autosomal dominant optic atrophy (DOA) harbor pathogenic *OPA1* mutations and certain missense mutations, mostly within the GTPase domain, have recently been shown to cause multiple mitochondrial DNA (mtDNA) deletions in skeletal muscle. This raises the possibility that the optic neuropathy could be the result of secondary mtDNA defects accumulating within retinal ganglion cells (RGCs). To explore this hypothesis, the authors looked for evidence of mitochondrial dysfunction in a mouse model of DOA and documented the visual and neurologic progression in aging mutant mice.

METHODS. Visual function was assessed with a rotating optokinetic (OKN) drum at ages 13 and 18 months and neurologic phenotyping was performed using the primary SHIRPA screen at age 13 months, comparing mutant *Opa1*^{+/-} mice with wild-type C57Bl/6 mice. The presence of cytochrome *c* oxidase (COX) deficiency and multiple mtDNA deletions was investigated in gastrocnemius muscle and eye specimens harvested from 2- and 11-month-old *Opa1*^{+/+} and *Opa1*^{+/-} mice.

RESULTS. At age 13 months, *Opa1*^{+/-} mice had a statistically significant reduction in OKN responses compared to C57Bl/6 controls with both 2° and 8° gratings ($P < 0.001$). At age 18 months, the difference between the two groups was significant for the 8° grating ($P = 0.003$) but not for the 2° grating ($P = 0.082$). *Opa1*^{+/-} mice did not exhibit any significant neuromuscular deficits and no COX deficient areas or secondary mtDNA deletions were identified in skeletal muscle or the RGC layer. There was also no evidence of significant mtDNA depletion or proliferation in skeletal muscle from *Opa1*^{+/-} mice.

CONCLUSIONS. COX deficiency and mtDNA abnormalities do not contribute to optic nerve dysfunction in pure DOA. (*Invest Ophthalmol Vis Sci.* 2009;50:4561–4566) DOI:10.1167/iovs.09-3634

Dominant optic atrophy (DOA; Online Mendelian Inheritance in Man [OMIM] 165500) is one of the most common inherited optic neuropathies encountered in neuro-ophthalmological practice, and approximately 60% of affected individuals will harbor a pathogenic mutation in the *OPA1* gene (3q28-q29).^{1,2} DOA was thought to have a relatively limited ocular phenotype, with most patients experiencing an insidious onset of central visual failure starting in early childhood due to the focal loss of RGCs within the papillo-macular bundle.³ However, we and others have recently described DOA pedigrees where the visual loss also segregated with more severe neuromuscular deficits such as progressive external ophthalmoplegia (PEO), deafness, ataxia, myopathy, and peripheral neuropathy.^{4–6} Interestingly, skeletal muscle biopsies were available from some of the affected family members, and these showed unequivocal features of mitochondrial dysfunction with a mosaic pattern of COX-deficient muscle fibers and the presence of multiple mtDNA deletions on long-range polymerase chain reaction (PCR).^{4,5} The *Opa1* protein is part of the large, dynamin GTPase family of mechanoenzymes and is located within the inner mitochondrial membrane,⁷ providing intriguing causal links to the hitherto undescribed muscle changes in these “DOA plus” pedigrees. The underlying pathophysiology in DOA remains largely unexplained, and the accumulation of secondary mtDNA abnormalities could represent an important mechanism that triggers the downstream events leading to cellular dysfunction and loss of function, especially within RGCs. Basic research in DOA has been severely restricted by the lack of affected human specimens and for this reason, we have recently developed an *Opa1* mouse model to explore these fundamental molecular mechanisms in greater detail.⁸ To determine the role of COX deficiency and secondary mtDNA deletions in the RGC loss that characterizes DOA, we carried out a physiological, biochemical, and molecular genetic study in our mutant *Opa1* mice.

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MATERIALS AND METHODS

Opa1 Mice

We have established a mouse model of DOA (B6;C3-*Opa1*^{Q285STOP}) with a heterozygous nonsense mutation in exon 8 (c.1051C>T) of the *Opa1* gene, which results in the introduction of a stop codon (Q285STOP) and a 50% reduction in the expression of the *Opa1* protein.⁸ Breeding, maintenance, and euthanatization were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Homozygous mutant mice (*Opa1*^{-/-}) died in utero during embryogenesis but heterozygous *Opa1*^{+/-} mice faithfully

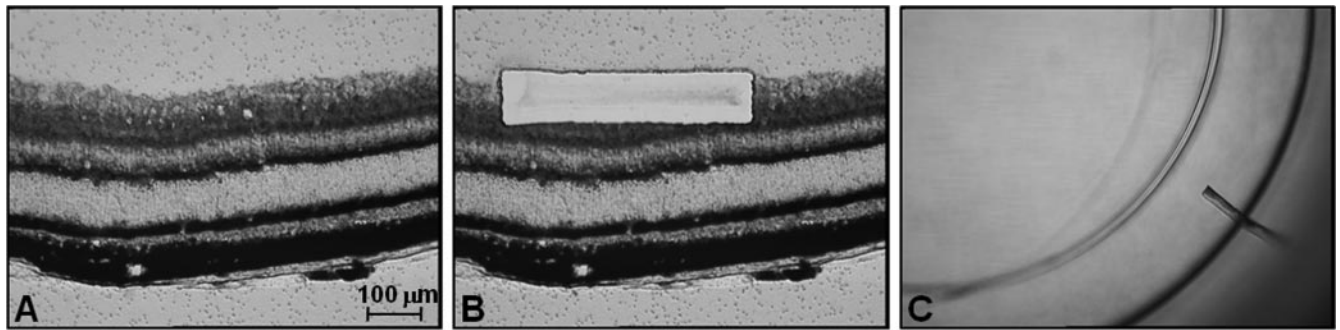


FIGURE 1. Collection of RGCs from an 11-month-old *Opa1*^{+/-} mouse. (A) A 12 μm cryostat section mounted onto a membrane slide. (B) Laser microdissection of a 100 μm \times 500 μm block from the RGC layer. (C) RGC block in the microcentrifuge cap before cell lysis.

replicated the human phenotype, exhibiting a slowly progressive optic neuropathy and demonstrating objective reduction in visual function on psychophysical testing.

Visual Assessment

Visual function was formally assessed with a rotating optokinetic (OKN) drum using high resolution 2° and low resolution 8° gratings, which correspond to 0.25 and 0.0625 cycles/degree respectively, and OKN responses were recorded using a validated protocol.^{9,10} We have previously reported visual data on 6- and 12-month-old *Opa1*^{+/-} mice⁸ and in this study, further visual assessment was performed by one investigator (VJD) on an older cohort of heterozygous mutant *Opa1*^{+/-} mice ($n = 14$ at 13 months and $n = 12$ at 18 months) and an age- and sex-matched group of C57Bl/6 wild-type mice ($n = 14$ at 13 months and $n = 12$ at 18 months). OKN data for both wild-type and mutant mice was collected on the same day, under the same experimental conditions.

Neurologic Evaluation

The primary SHIRPA examination is a comprehensive screening technique used to define abnormal mouse phenotypes in disease models.¹¹ It consists of 37 separate general health and neurologic measures and provides quantitative information on muscle, cerebellar, sensory, neuropsychiatric, and autonomic functions (<http://www.har.mrc.ac.uk/services/phenotyping/neurology/shirpa.html>). Using this assessment tool, our heterozygous mutant *Opa1*^{+/-} mice did not show any gross neuromuscular deficits at age 6 months and had a normal lifespan, similar to their *Opa1*^{+/+} control littermates.⁸ In this study, 14 *Opa1*^{+/-} mice and 14 age- and sex-matched C57Bl/6 controls were reevaluated at age 13 months with the primary SHIRPA protocol. This included tests of muscle tone, power and coordination, as well as hearing using the MRC standard click box (90 dB and 18–20 Hz).

Histochemistry

The gastrocnemius muscle was harvested from the leg area and both eyes were enucleated from the following mice: 2-month-old *Opa1*^{+/+} ($n = 3$), 2-month-old *Opa1*[±] ($n = 3$), 11-month-old *Opa1*^{+/+} ($n = 3$), and 11-month-old *Opa1*^{+/-} ($n = 3$). The muscle and eye specimens were immediately frozen in a melting isopentane bath (-150°C) and stored at -80°C . Serial sections were subsequently cut and mounted onto glass slides using a cryostat (Microm HM560; Thermo Fisher, Walldorf, Germany), at 20 μm thickness for muscle and 12 μm thickness for the eye specimens. The tissue sections were then stained using standard protocols for hematoxylin and eosin (H&E), cytochrome *c* oxidase (COX), succinate dehydrogenase (SDH) and dual COX-SDH.^{12,13} Myofibrillar adenosine triphosphatase (ATPase) staining, with a pre-incubation pH of 4.3, was also performed on serial muscle sections.^{14,15}

DNA Extraction

Muscle homogenate DNA was extracted using a hard tissue kit (Nucleon; Teqnel, Manchester, UK) but given the small quantities involved, an alternative method was devised to obtain an adequate yield of RGC DNA. *Opa1* mouse eyes were serially sectioned onto membrane slides and stained with dual COX-SDH to localize the RGC layer and cell bodies (Fig. 1A). RGC blocks (100 μm \times 500 μm) were then cut using a laser dissecting microscope (LMD 6000; Leica Microsystems, Wetzlar, Germany) and collected into a 1.5 mL microcentrifuge cap (Figs. 1B, 1C). A total of 200 RGC blocks were pooled together for each mouse, with 10 RGC blocks dissected from 10 serial sections of both right and left eyes. The dissection block included both the RGC cell bodies and the proximal axonal segment within the retinal nerve fiber layer. DNA extraction was performed using a purification kit (QIAamp DNA Micro Kit; Qiagen, Crawley, UK) and eluted in 20 μL of TE buffer.

Long-range PCR Assay

Six primer pairs, available on request, were specifically designed to amplify overlapping mtDNA regions and identify possible rearrangements, especially in the mutational hotspots along the major arc of the mitochondrial genome and involving *MTND4* (Fig. 2A).¹⁶ A long-range kit (Roche Expand Long-Range Kit; Roche Applied Science, Indianapolis, IN) was used with the manufacturer's recommended cycling procedures and the PCR products were electrophoresed in a 0.7% agarose gel at 40 V for 3 hours.

Quantitative PCR Assay

Relative copy number was determined in homogenate muscle DNA using a well-established iQ SYBR Green protocol on the real-time PCR detection system (MyiQ; BioRad Laboratories, Hercules, CA), with *MTND5* as the mtDNA reference gene and *GAPDH* as the nuclear DNA (nDNA) reference gene.^{17,18} Both assays were optimized and confirmed to be linear over an appropriate concentration range, and all measurements were done in triplicate. The mtDNA/nDNA ratio was derived from the difference in threshold cycle value (ΔCt) between *MTND5* and *GAPDH*, using the $2^{-\Delta Ct}$ method.

Statistical Analysis

Statistical analyses were performed using statistical software (SPSS v. 15; SPSS, Chicago, IL). An independent sample *t*-test and two-way ANOVA were used to compare the OKN responses, primary SHIRPA scores and mtDNA/nDNA ratio between mutant and wild-type mice, as required. The error bars in the figures provided represent the standard error of the mean (SEM).

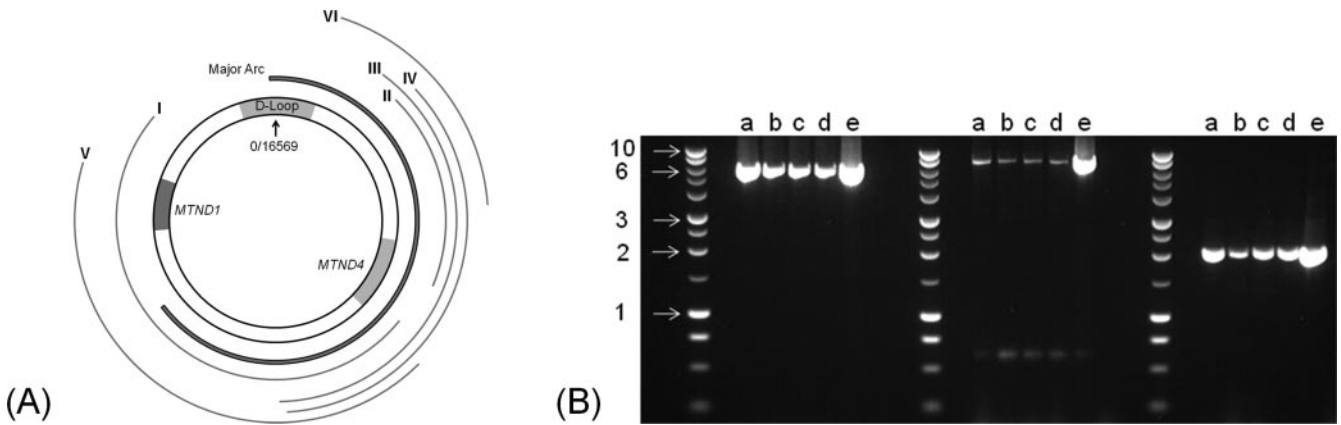


FIGURE 2. (A) Location of the mtDNA products amplified with our long-range PCR primer pairs: I (2,773–10,833; 8,060 bp), II (11,172–14,446; 3,274 bp), III (8,309–14,993; 6,684 bp), IV (8,381–14,446; 6,065 bp), V (3,106–10,595; 7,489 bp), and VI (13,053–15,053–2,000 bp). (B) Agarose gel (0.7%) showing the products generated with primer pairs IV, V, and VI, respectively. (a–c) RGC DNA extracted from our three 11-month-old *Opa1*^{+/-} mice; (d) RGC DNA extracted from one 11-month-old *Opa1*^{+/+} mouse; (e) muscle homogenate DNA extracted from the same 11-month-old *Opa1*^{+/+} mouse; and 1 Kb DNA ladder with 1, 2, 3, 6, and 10 Kb bands indicated).

RESULTS

Visual Function

Slit lamp and dilated fundal examinations did not reveal any gross ocular pathology such as anterior segment dysgenesis, cataracts, or retinal pigmentary changes in all *Opa1* mice studied. In this study, 13-month-old *Opa1*^{+/-} mice displayed statistically significant decreased mean tracking frequency at both 2° and 8° gratings ($P < 0.001$) compared to wild-type C57Bl/6 mice (main effect of genotype, $P < 0.001$; main effect of grating, $P = 0.435$; grating by genotype interaction, $P = 0.461$; Fig. 3A). At age 18 months, the difference between

mutant and control groups was significant for the 8° grating ($P = 0.003$) but not for the 2° grating ($P = 0.082$; Fig. 3B). There was also a statistically significant reduction in OKN responses between the 2° and 8° gratings at age 18 months (main effect of genotype, $P = 0.325$; main effect of grating, $P = 0.001$; grating by genotype interaction, $P = 0.880$). The ratio of mean tracking frequency (C57Bl/6 vs. *Opa1*^{+/-}) was 1.53 for the 2° grating and 1.70 for the 8° grating at age 13 months, and 1.05 for the 2° grating and 1.06 for the 8° grating at age 18 months.

Neurologic Phenotype

The primary SHIRPA neurologic examination did not reveal any functional neurologic deficits in the *Opa1*^{+/-} mice at age 13 months. No gait abnormalities or tremor were present at resting level and there was no statistically significant difference in limb tone, grip strength, and muscle coordination between mutant and wild-type mice. Compared to wild-type C57Bl/6 controls, *Opa1*^{+/-} mice had increased transfer arousal and locomotor activity scores, indicating that they were possibly more anxious and fearful in novel environments ($P < 0.05$). All the *Opa1*^{+/-} mice tested had normal hearing test results with the MRC standard click box.

Histology and mtDNA Analysis

In the 12 *Opa1* mice that were studied, H&E and myofibrillar ATPase staining showed normal muscle fiber morphology, with no inflammatory or neuropathic changes such as muscle fiber necrosis, regeneration, or fiber type grouping. There were no ragged red fibers (RRFs) on SDH staining and no COX deficient fibers were identified, with an average sampling of 3793 muscle fibers for each mouse (SD, 1049; range, 1690–5302; Fig. 4). Similarly, we found no evidence of COX-deficient regions within the RGC layer of both the 2-month and 11-month-old *Opa1*^{+/+} and *Opa1*^{+/-} mice (Fig. 5). The long-range PCR assay using all 6 primer pairs only amplified full mtDNA fragments with no evidence of smaller amplicons in both control and mutant groups (Fig. 2B). There was no statistically significant difference in mtDNA/nDNA ratio in muscle from *Opa1*^{+/-} (mean, 16.58; SD, 2.71; $n = 6$) and *Opa1*^{+/+} mice (mean, 15.17; SD, 4.51; $n = 6$; $P = 0.795$; Fig. 6).

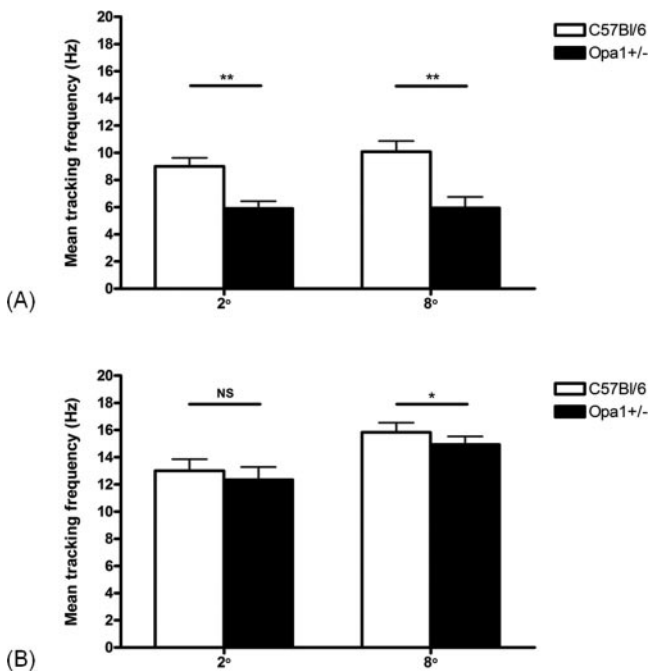


FIGURE 3. Performance on the optokinetic visual screening test expressed as the mean frequency of tracking a moving 2° and 8° grating for 2 minutes at (A) 13 months: *Opa1*^{+/-} mice ($n = 14$) and C57Bl/6 controls ($n = 14$); (B) 18 months: *Opa1*^{+/-} mice ($n = 12$) and C57Bl/6 controls ($n = 12$). ** $P < 0.001$; * $P = 0.003$; NS, non-significant at $P = 0.082$.

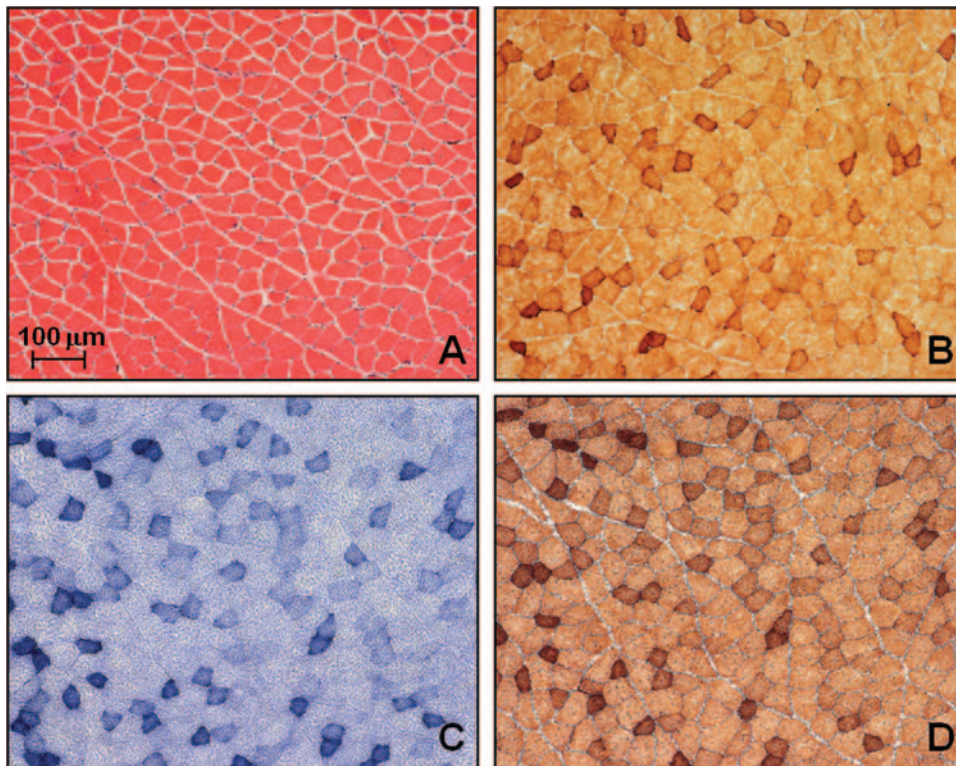


FIGURE 4. Serial 20- μm -thick limb muscle sections from an 11-month-old *Opa1*^{+/-} mouse stained with (A) H&E, (B) COX, (C) SDH, and (D) COX-SDH. The darker-staining muscle fibers are type I and reflect their higher mitochondrial oxidative capacity. A similar fiber type distribution was observed in all *Opa1* mice studied, irrespective of age or mutational status.

DISCUSSION

We previously reported a significant reduction in OKN responses in our heterozygous mutant *Opa1*^{+/-} mice age 12 months compared to their littermate controls. In this report, we extended our observations to a more aged colony and further confirm that the pathogenic variant in exon 8

(c.1051C>T) of the *Opa1* gene leads to optic nerve dysfunction in our heterozygous mutant mice. The optic neuropathy is relatively mild, as none of the *Opa1*^{+/-} mice completely failed to track the gratings, consistent with the better visual prognosis in DOA compared to other inherited optic neuropathies such as Leber hereditary optic neuropathy (LHON; OMIM 535000).³ There was a statistically significant reduction in

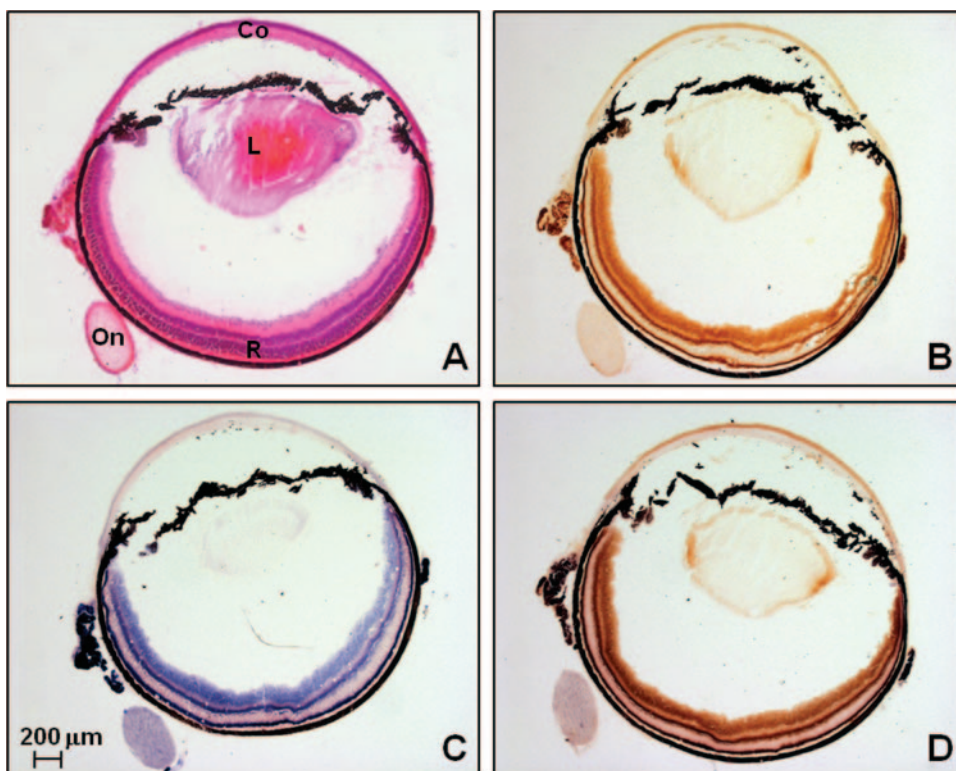


FIGURE 5. Serial 12- μm -thick eye sections from an 11-month-old *Opa1*^{+/-} mouse stained with (A) H&E, (B) COX, (C) SDH, and (D) COX-SDH. Co, cornea; L, lens; On, optic nerve; R, retina.

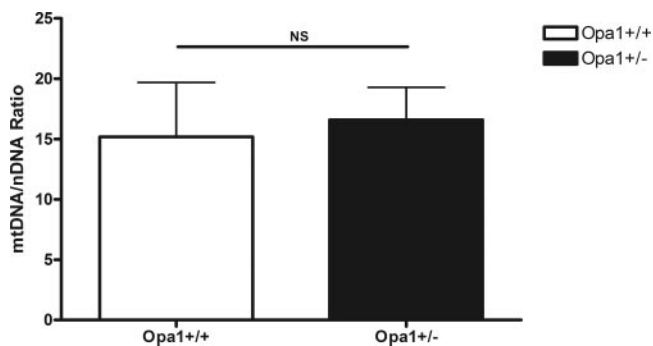


FIGURE 6. Comparison of mitochondrial copy number in homogenate muscle DNA extracted from *OPA1*^{+/+} and *OPA1*^{+/-} mice. NS, not significant at $P = 0.795$.

mean tracking frequency between *Opa1*^{+/-} mice and C57Bl/6 controls for both 2° and 8° gratings at age 13 months, but the difference was only significant for the 8° grating at age 18 months. The most likely explanation is a loss of visual acuity occurring with age for the C57Bl/6 mice, which reduced the difference between mutant and wild-type mice at the higher resolution 2° grating but not at the lower resolution 8° grating. The OKN ratios at age 13 and age 18 months also suggest that visual acuity does not deteriorate further with advancing age in our *Opa1*^{+/-} mice, which is similar to the natural history of DOA in humans, where less than 50% of affected patients experience further, albeit gradual, deterioration in their visual function on long-term follow-up.¹⁹⁻²¹

A comprehensive histochemical analysis of muscle and eye specimens failed to demonstrate any COX deficient areas and no mtDNA deletions were identified using our long-range PCR assay. Quantitative analysis on homogenate muscle DNA also showed no evidence of significant mitochondrial depletion or proliferation in the *Opa1*^{+/-} mice compared to *Opa1*^{+/+} controls. If the pathogenic *Opa1* mutation induced these secondary mitochondrial changes, we would have expected these to be apparent in the 11-month-old *Opa1*[±] mice, an age group in which visual dysfunction had already developed. In addition, we previously showed with electron microscopy that significant morphologic changes were clearly visible by the age of 9 months within the optic nerve, with abnormally swollen and distorted RGC axons, and irregular areas of demyelination and myelin aggregates along the nerve fiber bundles.^{8,22} A limitation of our experimental procedures is the inability to visualize individual RGC cell bodies and axons due to their small size, and it is possible that isolated COX deficient RGCs were present that have been missed. However, long-range PCR is a very sensitive molecular technique because of its preferential amplification of smaller mtDNA species and yet, enriching for RGCs by laser microdissection did not reveal any mtDNA deletions.²³ Another possible argument is that COX deficient RGCs were present in the pre-clinical stages but these were then lost coincident with the onset of optic nerve dysfunction. Although we cannot absolutely exclude this scenario, it is somewhat unlikely given the consistent observations of viable COX-deficient neurons in brain biopsies from patients with neurodegenerative disorders such as Parkinson's disease, where COX-deficient neurons accumulate both with increasing age and progression of the disease process.^{24,25} Furthermore, previous retinal histologic analysis of our *Opa1*^{+/-} mice did not show any significant reduction in total RGC count.⁸

How can we then account for this apparent disparity between our *Opa1* mouse model and the recent reports of COX deficiency and multiple mtDNA deletions in "DOA plus" pedigrees? We recently investigated a cohort of 21 patients with

multi-systemic neuromuscular disorders, COX-deficient muscle fibers, and multiple mtDNA deletions.²⁶ Initial mutational screen for genes previously described in patients with multiple mtDNA deletions was negative: *POLG1*, *POLG2*, *SLC25A4*, and *PEO1*, but subsequent *OPA1* gene sequencing identified pathogenic variants in three probands who also had visual failure; that is, 14% of our study cohort. Although the underlying mechanisms are unknown, this very interesting finding provides additional robust evidence that some *OPA1* mutations do lead to the formation and clonal expansion of mtDNA deletions.

All the causative *OPA1* mutations in these "DOA plus" families have so far been missense mutations with most, but not all of them, within the catalytic GTPase site of the protein.⁴⁻⁶ Although functional studies are lacking, it has been speculated that the more severe phenotype is the consequence of the mutant Opa1 protein exerting a dominant negative effect, and the more severe cellular dysfunction becomes apparent not only within RGCs, but also in other "at-risk mitochondrial" tissues such as extra-ocular muscles, skeletal muscle, and brain.²⁷ If this hypothesis is substantiated, it is perhaps not surprising that we failed to detect these abnormalities in our *Opa1* mutant mice, where the pathology is limited to the optic nerve, that is, "pure DOA," with no evidence to suggest additional neurologic deficits such as muscle weakness, ataxia, or deafness, using the well-validated primary SHIRPA screen.¹¹ The heterozygous exon 8 mutation (c.1051C>T) in our *Opa1* mice is also truncative (Q285STOP) and overall results in a 50% reduction in the expression of the Opa1 protein, thereby representing a haploinsufficiency, and not a dominant negative, disease model. We previously showed the following percentage decrease in Opa1 protein levels in a panel of post-mitotic tissues harvested from *Opa1*^{+/-} mice: retina (55%), brain (50%), skeletal muscle (80%), heart (55%), liver (80%), kidney (35%), and spleen (65%).⁸

Although our results relate only to this specific mouse model, one could speculate that secondary mtDNA abnormalities do not contribute to optic nerve dysfunction among patients with truncative *OPA1* mutations and a "pure DOA" phenotype. It is perhaps also premature to conclude that COX deficiency and multiple mtDNA deletions are a sine qua non for a more severe disease progression in DOA. Spinazzi and colleagues²⁸ have recently described a large, multi-generational pedigree where the optic atrophy segregated with mild myopathy and an axonal sensory-motor peripheral neuropathy. In contrast to previous "DOA plus" pedigrees, COX deficient muscle fibers and multiple mtDNA deletions were absent and a novel *OPA1* deletion in the GTPase domain (c.1410_1443+4del38) was identified that reduced the level of protein expression by half; that is, led to haploinsufficiency. Functional studies performed on fibroblast and myoblast cultures showed no demonstrable respiratory chain defects, in contrast to the common c.2708_2711delTTAG *OPA1* deletion, which has been shown to inhibit mitochondrial oxidative phosphorylation in both in vitro fibroblast²⁹ and in vivo muscle assays.³⁰ However, the c.1410_1443+4del38 mutation led to marked fragmentation of the mitochondrial network, an effect also seen with other *OPA1* mutations³ and in fibroblasts derived from our mouse model,⁸ highlighting the additional important pro-fusion function of the Opa1 protein.

Opa1 is proving to be a multi-faceted protein with several crucial molecular roles relating to mtDNA replication, oxidative phosphorylation, maintenance of the mitochondrial network and apoptosis. The challenge ahead will be to unravel how *OPA1* mutations actually lead to loss of function, not only in RGCs, but also in other post-mitotic tissues to explain the expanding clinical spectrum being documented in DOA families. In this respect another *Opa1* mouse model with more

severe neurologic deficits in addition to the optic neuropathy would provide invaluable insights into these molecular mechanisms and how they interact to cause disease. Understanding these key pathophysiological steps will hopefully lead to the development of effective neuroprotective strategies with clinical benefits to patients with DOA.

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