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OPA1 mutations cause cytochrome *c* oxidase deficiency due to loss of wild-type mtDNA molecules

Patrick Yu-Wai-Man[1,2], Kamil S. Sitarz[1], David C. Samuels[3], Philip G. Griffiths[1,2], Amy K. Reeve[1], Laurence A. Bindoff[4,5], Rita Horvath[1] and Patrick F. Chinnery[1,6,*]

[1]Mitochondrial Research Group, Institute for Ageing and Health, The Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, UK, [2]Department of Ophthalmology, Royal Victoria Infirmary, Newcastle upon Tyne, UK, [3]Department of Molecular Physiology and Biophysics, Center for Human Genetics Research, Vanderbilt University Medical Center, Nashville, TN, USA, [4]Department of Clinical Medicine, University of Bergen, Bergen, Norway, [5]Department of Neurology, Haukeland University Hospital, Bergen, Norway and [6]Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, UK

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Pathogenic *OPA1* mutations cause autosomal dominant optic atrophy (DOA), a condition characterized by the preferential loss of retinal ganglion cells and progressive optic nerve degeneration. Approximately 20% of affected patients will also develop more severe neuromuscular complications, an important disease subgroup known as DOA$^+$. Cytochrome *c* oxidase (COX)-negative fibres and multiple mitochondrial DNA (mtDNA) deletions have been identified in skeletal muscle biopsies from patients manifesting both the pure and syndromal variants, raising the possibility that the accumulation of somatic mtDNA defects contribute to the disease process. In this study, we investigated the mtDNA changes induced by *OPA1* mutations in skeletal muscle biopsies from 15 patients with both pure DOA and DOA$^+$ phenotypes. We observed a 2- to 4-fold increase in mtDNA copy number at the single-fibre level, and patients with DOA$^+$ features had significantly greater mtDNA proliferation in their COX-negative skeletal muscle fibres compared with patients with isolated optic neuropathy. Low levels of wild-type mtDNA molecules were present in COX-deficient muscle fibres from both pure DOA and DOA$^+$ patients, implicating haplo-insufficiency as the mechanism responsible for the biochemical defect. Our findings are consistent with the 'maintenance of wild-type' hypothesis, the secondary mtDNA deletions induced by *OPA1* mutations triggering a compensatory mitochondrial proliferative response in order to maintain an optimal level of wild-type mtDNA genomes. However, when deletion levels reach a critical level, further mitochondrial proliferation leads to replication of the mutant species at the expense of wild-type mtDNA, resulting in the loss of respiratory chain COX activity.

INTRODUCTION

Pathogenic mutations in the *OPA1* gene (OMIM 605290) account for ~60% of all cases of autosomal dominant optic atrophy (DOA), and the carrier rate in the general population is estimated to be at least 1 in 50 000 (1). The majority of patients are mono-symptomatic, with the onset of progressive central visual loss in early childhood invariably resulting in significant visual morbidity (2–4). Although optic nerve dysfunction is the pathognomonic feature of DOA, we have recently established that up to 20% of *OPA1* carriers will experience a more complicated disease course (5). These syndromal DOA$^+$ variants show a remarkable degree of phenotypic variability, but sensorineural deafness is a frequent neurological deficit,

*To whom correspondence should be addressed. Tel: +44 1912824375; Fax: +44 1912824373; Email: P.F.Chinnery@ncl.ac.uk

which develops from late childhood to early adulthood, followed by a combination of ataxia, myopathy, peripheral neuropathy and progressive external ophthalmoplegia (PEO) from the third decade of life onwards (5–9). These clinical observations are of major pathophysiological importance, as they highlight the deleterious consequences of *OPA1* mutations not only for retinal ganglion cells, whose axons constitute the optic nerve, but also for other central neuronal populations, peripheral nerves and skeletal muscle.

The marked inter- and intra-familial variability in disease severity seen in DOA is likely to be a reflection of the multiple, distinct roles played by the Opa1 protein in normal cellular function (10,11). Following proteolytic cleavage by various proteases (12), Opa1 assembles as polymeric structures within the inner mitochondrial membrane. As a result of its pro-fusion dynamin-like GTPase properties, Opa1 actively maintains a highly interconnected mitochondrial network and sequesters pro-apoptotic cytochrome *c* molecules within the mitochondrial cristae spaces (13,14). Opa1 is also thought to regulate oxidative phosphorylation by stabilizing the mitochondrial respiratory chain complexes and by facilitating the effective coupling of electron transport with ATP synthesis (15,16). In addition to these essential biological functions, there is now growing evidence supporting a novel role for Opa1 in mitochondrial DNA (mtDNA) maintenance. Central to this argument is the characteristic histochemical finding of cytochrome *c* oxidase (COX)-negative fibres in skeletal muscle biopsies from *OPA1*-positive patients, together with the presence of multiple mtDNA deletions on long-range PCR analysis of homogenate skeletal muscle DNA (5–8). Different mechanisms have been postulated to account for the formation of these deleted mtDNA species, including an imbalance of the intra-mitochondrial nucleotide pool, and the impaired interaction of the N-terminal domain of Opa1 with mtDNA nucleoids, which are anchored in close physical proximity to the inner mitochondrial membrane (6,7,17). Interestingly, the level of COX-deficient muscle fibres was found to be over four times higher in the DOA^{+} group compared with the pure optic atrophy group (5). Importantly, COX-deficient fibres were also significantly more frequent among patients with pure DOA compared with age-matched healthy controls (5). These observations strongly implicate a contributory role for these secondary mtDNA defects in triggering multi-system cellular dysfunction among affected *OPA1* carriers. However, the mechanisms involved have not been defined and the nature of the mtDNA deletions induced by *OPA1* mutations still remains to be clarified. To explore these fundamental research questions and how they relate to disease severity, we performed a quantitative and qualitative study of the mtDNA changes present in single skeletal muscle fibres from patients harbouring *OPA1* mutations.

RESULTS

MtDNA copy number density varies in normal control skeletal muscle fibres

The normal control muscles studied were collected from a 1-year-old female (C-1F), a 22-year-old female (C-22F), a 31-year-old female (C-31F), a 35-year-old male (C-35M) and a 52-year-old female (C-52F). The mtDNA copy number density was measured as the total number of mtDNA molecules in a muscle fibre section, divided by the fibre's cross-sectional area. There was a statistically significant variation in mtDNA copy number density among Type I skeletal muscle fibres from the five controls, except for C-1F versus C-22F (C-1F: mean = 3.20, SD = 1.67, $n = 18$; C-22F: mean = 2.50, SD = 0.87, $n = 24$; C-31F: mean = 5.62, SD = 1.97, $n = 19$; C-35M: mean = 10.63, SD = 3.22, $n = 17$; C-52F: mean = 4.36, SD = 0.92, $n = 21$) (Fig. 1A, Supplementary Material, Table S1A). Similarly, mtDNA copy number density varied significantly among Type II skeletal muscle fibres, except for C-1F versus C-31F, and C-1F versus C-52F (C-1F: mean = 2.62, SD = 0.98, $n = 13$; C-22F: mean = 1.57, SD = 0.60, $n = 24$; C-31F: mean = 2.96, SD = 0.85, $n = 17$; C-35M: mean = 4.13, SD = 1.54, $n = 14$; C-52F: mean = 2.45, SD = 0.50, $n = 22$) (Fig. 1B, Supplementary Material, Table S1B).

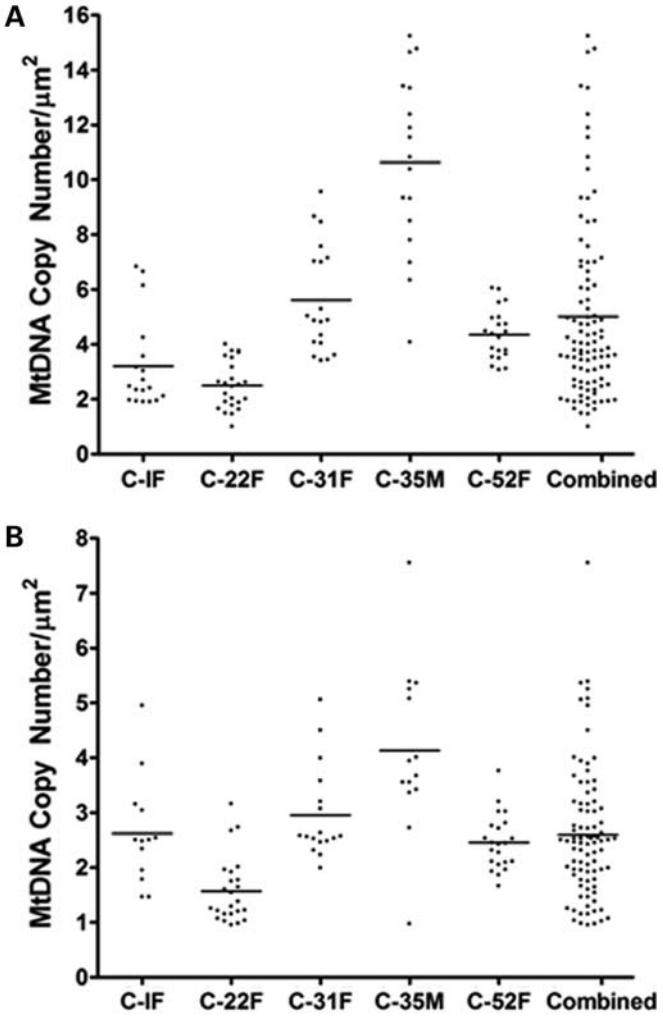

Figure 1. Total mtDNA content of single skeletal muscle fibres from normal controls: (**A**) Type I fibres, (**B**) Type II fibres.

Table 1. Clinical phenotype and *OPA1* mutational spectrum of our study cohort

Patient	Age (years)	Sex	Onset[a] (years)	*OPA1* mutation cDNA	AA change	Clinical phenotype Optic atrophy	Deafness	Ataxia	Myopathy	Neuropathy	PEO	Others
A	31	M	11	c.876-878del	p.V294fsX667	+						
B	59	M	5	c.876-878del	p.V294fsX667	+				+		HSP
C	44	M	2	c.1198C>T	p.P400S	+	+				+	
D	54	M	5	c.1212+3a>t	Splice defect	+	+					
E	43	M	5	c.1334G>A	p.R445H	+	+		+		+	
F	43	F	5	c.1334G>A	p.R445H	+	+			+	+	
G	54	M	–	c.1516+1g>t	Splice defect	+						
H	39	M	15	c.1516+1g>t	Splice defect	+						
I	58	F	–	c.2613+1g>a	Splice defect	+		+				MS-like illness
J	50	M	8	c.2613+1g>a	Splice defect	+						
K	43	M	5	c.2708_2711del	p.V903fsX3	+						
L	60	F	16	c.2713C>T	p.R905X	+						
M	40	F	15	c.2713C>T	p.R905X	+						
N[b]	60	M	–	c.768C>G c.854A>G	p.S256R p.Q285R	+		+	+	+		Spasticity
O[b]	64	F	–	c.768C>G c.854A>G	p.S256R p.Q285R	+		+	+	+		Spasticity

AA, amino acid; F, female; HSP, hereditary spastic paraparesis; M, male; MS, multiple sclerosis; PEO, progressive external ophthalmoplegia.
[a]Age of onset of visual failure.
[b]Norwegian siblings compound heterozygous for two novel *OPA1* mutations (5).

Table 2. Histochemical and mtDNA defects identified in skeletal muscle biopsies from our *OPA1*-positive patients

Patient	Skeletal muscle biopsy analysis Fibres (n)[b]	COX −ve (%)	RRF (%)	Long PCR[c]	Real-time PCR analysis Type I (*n*)[d]	Type II (*n*)[d]	COX −ve (*n*)	COX −ve single-fibre analysis[a] Deletion <70% (*n*)	(%)	Deletion ≥70% (*n*)	(%)
A	2826	0.1	0.0	+	15	14	2	1	50.0	1	50.0
B	482	2.1	0.6	++	14	14	8	3	37.5	5	62.5
C	1980	1.6	0.6	++	14	22	12	3	25.0	9	75.0
D	786	0.3	0.0	+	24	23	2	1	50.0	1	50.0
E	1272	3.1	0.7	++	19	12	20	3	15.0	17	85.0
F	1186	13.9	1.3	++	13	21	13	2	15.4	11	84.6
G	674	1.8	0.9	++	19	17	16	7	43.8	9	56.3
H	762	0.0	0.0	+	19	23	0	N/A	N/A	N/A	N/A
I	1304	1.4	0.3	++	21	21	15	1	6.7	14	93.3
J	848	0.0	0.0	–	14	19	0	N/A	N/A	N/A	N/A
K	832	0.6	0.1	+	14	13	6	2	33.3	4	66.7
L	1100	3.0	0.8	++	10	10	14	3	21.4	11	78.6
M	1126	0.5	0.1	+	14	15	6	0	0.0	6	100.0
N	1058	17.7	3.3	++	22	17	16	4	25.0	12	75.0
O	968	21.2	5.8	++	15	28	15	2	13.3	13	86.7

COX, cytochrome *c* oxidase; MtDNA, mitochondrial DNA; N/A, not applicable; PCR, polymerase chain reaction; RRF, ragged-red fibres.
[a]Level of mtDNA deletion detected in single COX-negative muscle fibres using our quantitative real-time PCR assay.
[b]Total number of muscle fibres present in the stained cryostat sections.
[c]Long PCR analysis of homogenate skeletal muscle DNA: (i) no deletion bands (−), (ii) smaller deletion bands in addition to the wild-type PCR fragment (+), and (iii) multiple deletion bands with no wild-type PCR fragment (++).
[d]Number of Type I and II COX-positive muscle fibres laser microdissected from 20 μm thick membrane sections.

OPA1 mutations lead to the accumulation of high levels of somatic mtDNA deletions in skeletal muscle fibres

The mean frequency of COX-negative fibres present in cryostat muscle sections from 15 *OPA1*-positive patients was 4.49% (SD = 7.00%, range = 0–21.20%, n = 15), with two patients, H and J, not harbouring any COX-negative fibres (Table 1). The presence and level of mtDNA deletion were determined for a total of 333 single skeletal muscle fibres from 15 patients: (i) Type I COX positive, $n = 94$; (ii) Type II COX positive, $n = 94$; and (iii) COX-negative, $n = 145$ (Table 2). The majority of COX-positive fibres harboured low deletion levels <30% (179/188, 95.2%), and there was no significant difference in mean deletion levels between Type I and II fibres (Type I: mean = 11.60%, SD = 7.99%; Type II: mean = 14.58%, SD = 11.51%, $P = 0.0723$). Among COX-negative fibres, 113 of 145 (77.9%) had deletion levels ≥70%, and the mean deletion level was significantly

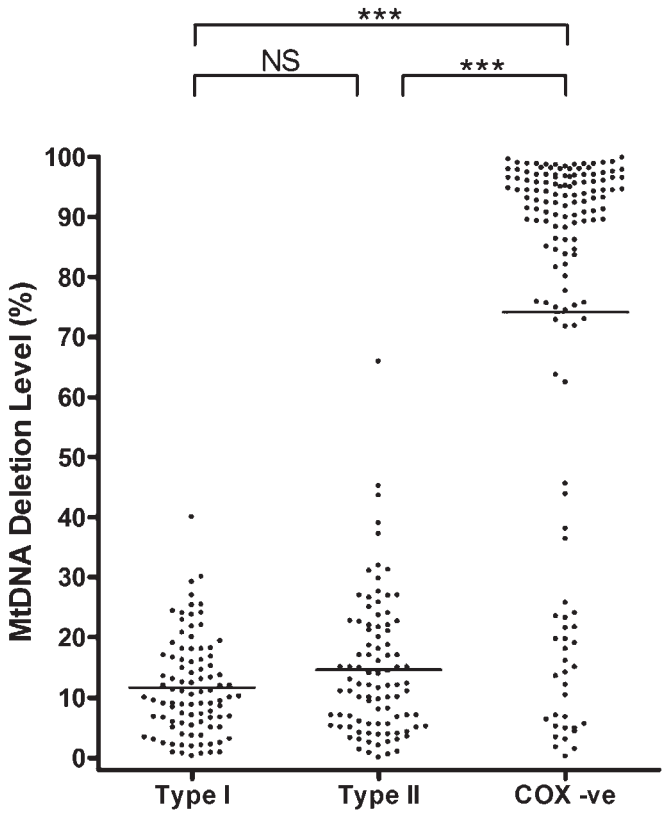

Figure 2. Level of mtDNA deletion in COX-positive and COX-negative single muscle fibres from *OPA1*-positive patients. NS at *P*-value = 0.0723; ***P-value <0.0001. Type I and Type II refer to COX positive fibres.

higher compared with both Type I and II COX-positive fibres (mean = 74.16%, SD = 33.04%, $P < 0.0001$) (Fig. 2).

COX-negative but not COX-positive muscle fibres are associated with significant mtDNA proliferation

There was no significant difference in mtDNA copy number density between COX-positive fibres from *OPA1*-positive patients and the combined control data set for both Type I fibres (*OPA1*: mean = 4.37, SD = 2.23; controls: mean = 4.91, SD = 3.17, $P = 0.0743$), and Type II fibres (*OPA1*: mean = 2.39, SD = 1.03; controls: mean = 2.60, SD = 1.20, $P = 0.1095$) (Fig. 3). However, there was evidence of significant mtDNA proliferation in COX-negative fibres when compared with adjacent COX-positive fibres from the same patient (Table 3, Supplementary Material, Fig. S1A–O), and COX-positive fibres from the combined control data set (Fig. 4). There was no significant difference in mean mtDNA copy number density between COX-negative fibres with mtDNA deletion levels ≥70% (mean = 11.55, SD = 8.83, $n = 113$) and <70% (mean = 9.10, SD = 5.32, $n = 32$, $P = 0.1385$). Of the 145 COX-negative fibres analysed, 44 were from patients with pure DOA (mean mtDNA proliferation ratio = 2.02, SD = 1.31, 95% confidence interval (CI) = 1.63–2.42) and 141 were from patients with DOA$^+$ phenotypes (mean mtDNA proliferation ratio = 2.66, SD = 1.52, 95% CI = 2.36–2.96), the difference being statistically significant ($P = 0.0172$) (Fig. 5).

MtDNA proliferation in COX-negative muscle fibres fails to maintain normal levels of wild-type mtDNA molecules

The mean wild-type mtDNA ratio was significantly lower for the COX-negative group (mean = 0.20, SD = 0.19, 95% CI = 0.17–0.24, $n = 112$) compared with the COX-positive group (mean = 1.00, SD = 0.32, 95% CI = 0.95–1.05, $n = 188$, $P < 0.0001$) (Fig. 6A). The relationship between the wild-type mtDNA ratio and the deletion level for COX-negative muscle fibres was in agreement with our previously established *in silico* model of mtDNA replication ($R^2 = 0.68$), and gave a predicted maximum mitochondrial proliferation factor (α) of 3.40 (95% CI = 2.60–4.80) (Fig. 6B).

COX-negative muscle fibres from *OPA1* patients harbour clonally expanded deleted mtDNA species

Deleted mtDNA species were detected in 31 COX-negative muscle fibres using our two-step long-range PCR protocol, and in none of them was the full-length 10 843 base pairs PCR product obtained. MtDNA deletions levels <30% were measured in 6 of 31 (19.4%) of these COX-negative fibres with our differential *MTND1–MTND4* real-time PCR assay. The deletion sizes ranged from 1.0 to 8.0 kb (mean = 5.2 kb, SD = 2.0 kb, $n = 33$), and two different deletions were amplified from the lysate of 2 of 31 (6.5%) COX-negative fibres. All these deletions were located within the major arc of the mitochondrial genome and they were predicted to result in the loss of critical segments containing both transfer RNA and protein-encoding genes.

DISCUSSION

Our study has revealed marked mtDNA proliferation in skeletal muscle from patients harbouring a range of pathogenic *OPA1* mutations. This observation is entirely compatible with the reported detrimental effect of *OPA1* mutations on mitochondrial oxidative phosphorylation (15,16,18), with the increased mtDNA copy number acting as a compensatory mechanism to maintain an adequate level of ATP for normal cellular function. COX-negative fibres were present in muscle biopsies from 13 *OPA1*-positive patients, and there was a consistent finding of mtDNA proliferation in these fibres, irrespective of disease severity. However, patients with DOA$^+$ phenotypes had significantly higher proliferation ratios in COX-negative muscle fibres when compared with those with isolated optic nerve involvement, indicating a possible important disease mechanism. Although we did not make any direct measurements of apoptotic markers in this study, mtDNA proliferation is linked with an increased susceptibility to undergo apoptosis in mitochondrial myopathies (19,20), and it is biologically plausible to postulate that such an effect would not be limited to muscle fibres, but would also extend to other post-mitotic tissues commonly affected in mitochondrial disorders. The relevance to *OPA1* disease becomes even more apparent in view of the fact that the level of COX deficiency in skeletal muscle is over four times higher in patients with DOA$^+$ compared with pure DOA (5). Even though the frequency of COX-negative fibres is lower among DOA patients with clinical features limited to the

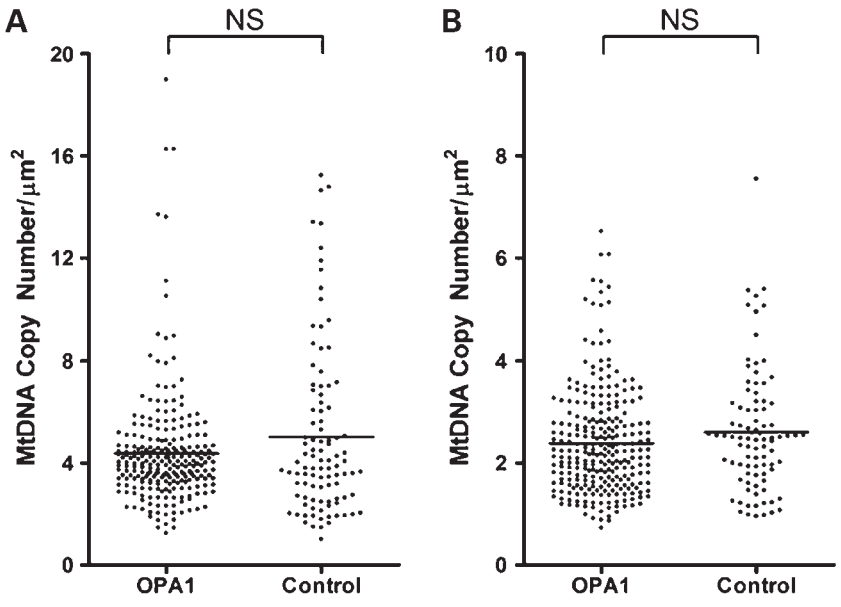

Figure 3. Comparison of total mtDNA copy number in single muscle fibres from *OPA1*-positive patients with normal controls. (**A**) Type I COX-positive fibres (NS at *P*-value = 0.0743), (**B**) Type II COX-positive fibres (NS at *P*-value = 0.1095). The control values represent the combined data set for C-1F, C-22F, C-31F, C-35M, and C-52F.

Table 3. Relative level of mtDNA proliferation seen in COX-negative muscle fibres

Patient	COX −ve fibres (n)[a]	MtDNA proliferation ratio[b]		
		Mean	95% confidence interval	
A	2	2.68	0.00	16.28
B	8	2.32	1.45	3.18
C	12	1.92	1.28	2.56
D	2	2.49	0.00	16.14
E	20	3.72	2.94	4.49
F	13	2.21	1.36	3.06
G	16	1.90	1.31	2.50
H	0	N/A	N/A	N/A
I	15	2.76	2.07	3.44
J	0	N/A	N/A	N/A
K	6	3.11	1.23	4.98
L	14	1.75	1.07	2.44
M	6	1.68	0.34	3.02
N	16	3.24	2.25	4.22
O	15	1.73	1.26	2.21

[a]Total number of COX-negative muscle fibres studied for each patient.
[b]The proliferation ratio for each COX-negative fibre was determined in relation to the mean mtDNA copy number (/μm^2) for COX-positive fibres from the same patient, with a value >1 indicating relative mtDNA proliferation (Supplementary Material, Fig. S1A–O).

optic nerve, this group still has significantly higher levels of COX-deficiency compared with age-matched healthy controls (5). The secondary mtDNA abnormalities, which precipitate this biochemical COX defect are therefore clearly linked to the underlying *OPA1* disease process. Although additional confirmatory studies are required, it is tempting to speculate that some *OPA1* mutations lead to multi-systemic manifestations and more severe visual failure as a result of their more pronounced effects on mtDNA deletion formation, and thus proliferation, with the consequent increase in apoptotic cell loss. The degree of mitochondrial network instability induced by different *OPA1* mutations also deserves further investigation, as the extent of fragmentation could have a crucial influence on the rate at which these somatic deletions become fixed at supra-threshold levels (21–23).

Our *in silico* model of mtDNA replication suggests that mitochondrial proliferation serves a useful biological purpose by maintaining wild-type mtDNA copy number at an optimal level (21,22). However, this compensatory mechanism only operates effectively up to a certain mutational load, beyond which it fails to prevent the loss of wild-type mtDNA genomes, and eventually becomes detrimental to cellular survival. The relationship observed in COX-negative *OPA1* muscle fibres between the level of wild-type and deleted mtDNA species is in agreement with our *in silico* prediction, and provides additional experimental evidence that the maintenance of wild-type mtDNA genomes is crucial for normal mitochondrial oxidative function. We previously showed that this was also the case in skeletal muscle fibres from a patient with PEO and mild proximal myopathy due to a 4.9 kb single deletion (23).

MtDNA copy number was determined at the single-fibre level for five normal control skeletal muscle samples, and a wide variation was observed for both Type I and II fibres. A likely confounding variable is the level of physical activity, which was not documented for the individuals from whom the biopsies were taken. Skeletal muscle is a highly adaptable tissue and it is capable of pronounced metabolic and morphological changes in response to endurance training or disuse (24,25). It is well established that physical exercise enhances mitochondrial oxidative capacity *in vivo* (26–28), and this beneficial adaptation has been linked to an increase in mtDNA density (29,30). The regulatory pathways involved are complex but an important element is the up-regulation in the level of *TFAM* expression (31,32), which encodes for a

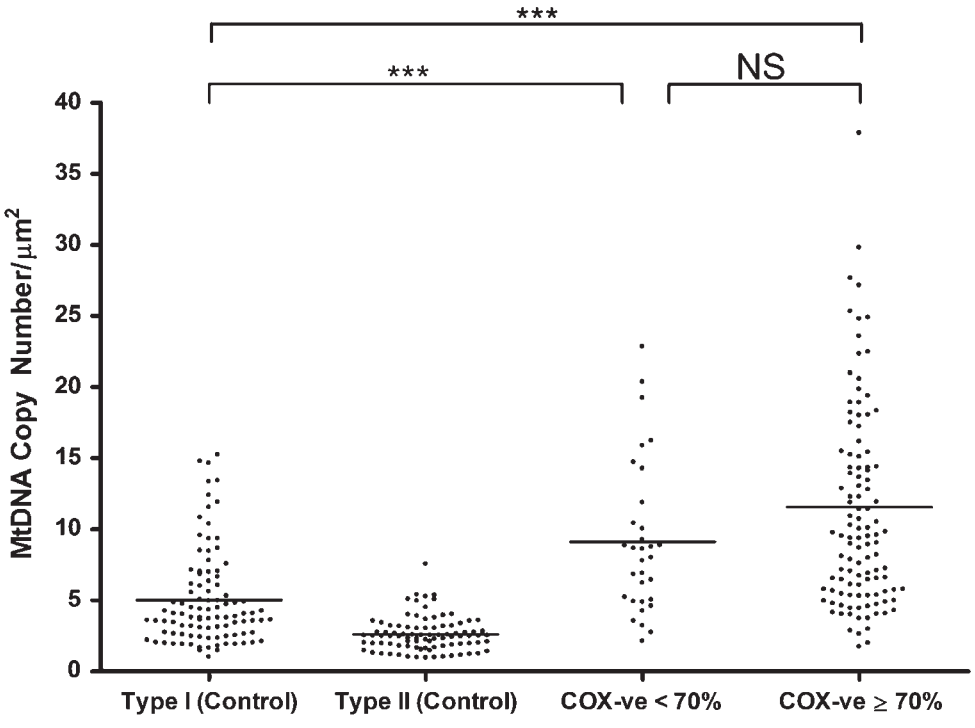

Figure 4. Comparison of total mtDNA copy number in COX-negative muscle fibres from *OPA1*-positive patients with COX-positive muscle fibres from the combined control data set. MtDNA deletion levels in COX-negative muscle fibres: low (<70%) and high (≥70%); NS at *P*-value = 0.1385; ***P-value <0.0001.

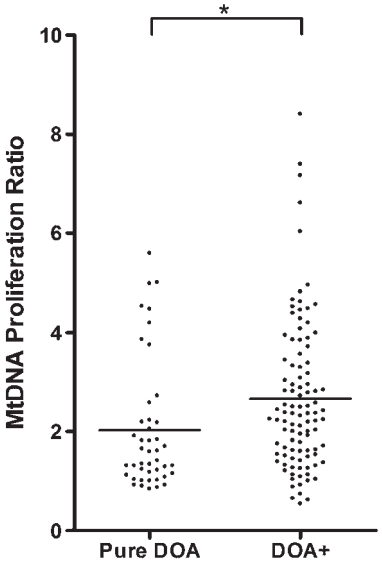

Figure 5. MtDNA proliferation ratio for COX-negative fibres from patients with pure DOA and DOA$^+$ phenotypes. The proliferation ratio for each COX-negative fibre was determined in relation to the mean mtDNA copy number ($/\mu m^2$) for COX-positive fibres from the same patient, with a value >1 indicating relative mtDNA proliferation. *P-value = 0.0172.

key activator of mtDNA transcription and replication (33,34). Our finding therefore highlights the need for caution when comparisons are made between individual patient muscle biopsies and controls, as erroneous conclusions could easily be reached, especially if only limited numbers are analysed. For this reason, we combined Type I and II fibres from our control data set, and used this normative range to interpret the results obtained from *OPA1*-positive muscle biopsies included in this study.

The majority of COX-positive muscle fibres from *OPA1*-positive patients had sub-threshold deletion levels <30% (179/188, 95.2%), i.e. within the detection limit of our quantitative real-time PCR assay (23,35,36). The mtDNA deletion level required to precipitate an overt biochemical defect at the cellular level is typically ≥70% in skeletal muscle (23,35,36), and using this threshold value, the histochemical status for over three-quarters of COX-negative muscle fibres could be accounted for (113/145, 77.9%). By applying a two-step long-range PCR strategy, clonal expansion of a single deleted mtDNA species was demonstrated for 29 of 31 (93.5%) COX-negative fibres, with the remaining fibres each harbouring two different deleted mtDNA species. Although long-range PCR is not quantitative, these results suggest that more than one deleted mtDNA species can propagate and reach high levels within a cell independently of each other. We recently reported the same observation in single substantia nigra neurones from three different groups: controls, patients with sporadic Parkinson's disease, and a patient with a heterozygous *POLG1* mutation leading to parkinsonism in association with PEO and multiple mtDNA deletions (37). It is also noteworthy that 6 of 31 (19.4%) COX-negative muscle fibres had mtDNA deletions levels <30% with our real-time PCR assay, which was designed primarily to detect deletions encompassing *MTND4* but not *MTND1* (23,38). It is therefore likely that

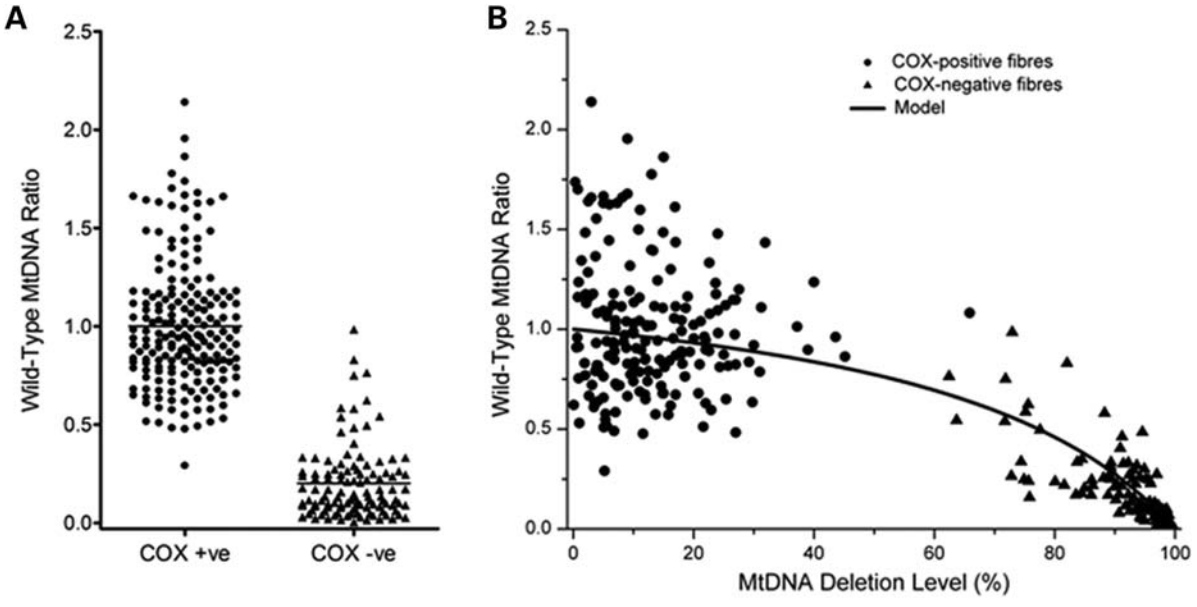

Figure 6. (**A**) Wild-type mtDNA ratio in single muscle fibres from *OPA1*-positive patients analysed according to COX status ($P < 0.0001$), (**B**) Variation in wild-type mtDNA ratio for COX-negative muscle fibres according to mtDNA deletion level. The simulated curve describes the relationship predicted by our *in silico* model, which is based upon the maintenance of wild-type mtDNA genomes ($R^2 = 0.68$). Circles indicate COX-positive fibres and triangles COX-negative fibres.

most of the COX-negative fibres with low deletion levels (32/145, 22.1%) in our study actually harboured smaller deletions, which spared both *MTND1* and *MTND4*, and therefore remained undetected. In support of this argument, Bua *et al*. (39) sequenced the breakpoints for 48 mtDNA deletions identified in aged normal skeletal muscle, and of these, 8 of 48 (16.7%) did not include *MTND1* and *MTND4*. We have not excluded the possibility that *OPA1* mutations could trigger COX deficiency via the accumulation of high levels of somatic mtDNA point mutations, but the evidence for this is weak, especially in post-mitotic tissues (40,41). Furthermore, previous studies of other mitochondrial maintenance disorders due to *POLG1* and *PEO1* mutations have only identified an increased burden of somatic mtDNA point mutations in the non-coding D-loop region (42–44). The significance of these mtDNA variants remains unclear but they would not be predicted to directly cause COX deficiency.

In summary, our study has provided some important insights into the consequences of *OPA1* mutations at the cellular level and has revealed possible important disease mechanisms. The accumulation of somatic, clonally expanded mtDNA deletions is a key pathological player, and the resulting mtDNA proliferation is likely to be an important mediator of apoptotic cell loss and disease severity. However, several intriguing features of *OPA1* disease remain to be clarified – its wide phenotypic spectrum, the marked variability seen within families segregating the same pathological variant, and the greater susceptibility of retinal ganglion cells. Dissecting the complex pathophysiological pathways involved will represent a major challenge, and future experimental paradigms will require the development of more sophisticated functional systems, such as animal models replicating the features seen in patients with DOA^+ phenotypes.

MATERIALS AND METHODS

Patients and controls

Quadriceps or tibialis anterior muscle biopsies from 15 *OPA1*-positive patients (mean age = 49.5 years, SD = 9.8 years, range = 31.0–64.0 years) (Table 1), and five normal controls with no evidence of ocular or neuromuscular pathologies (mean age = 28.2 years, SD = 18.7 years, range = 1.0–52.0 years) (Fig. 1) were included in this study. Among the DOA group, seven patients had isolated optic nerve involvement whereas eight patients manifested additional neuromuscular features. The clinical descriptions of these patients have been detailed previously (5), and muscle biopsies were obtained at the time of their initial diagnostic investigations. This study had the relevant institutional approval and complied with the Declaration of Helsinki.

Mitochondrial histochemistry

Skeletal muscle samples were snap frozen in melting liquid isopentane (−150°C) within 6 h of the biopsy being taken and then stored at −80°C. The specimens were mounted on OCT (VWR BDH Prolabo, UK) before being sectioned at 20 μm thickness onto glass and membrane slides using a Microm™ HM560 cryostat (Thermo Fisher, Germany). The serial muscle sections were then stained for COX, succinate dehydrogenase (SDH), and sequential COX-SDH activities, using standard histochemical protocols (45).

Single-muscle fibre analysis

Individual skeletal muscle fibres were cut using an LMD 6000™ laser dissecting microscope (Leica Microsystems, Germany) and collected into a 0.5 ml microcentrifuge cap

(Eppendorf, UK). Cross-sectional fibre area (μm^2) was recorded from the LMD 6000^{TM} operating software (Leica Microsystems) prior to laser microdissection. The fibres were incubated overnight in a 30 μl lysis solution containing proteinase K at 55°C for 16 h, followed by heat inactivation at 95°C for 10 min (23). The molecular investigations described below were then performed on the single-fibre lysate on the following day.

Quantitative real-time PCR

Our quantitative real-time PCR assay for single muscle fibres was originally designed based upon the fact that the *MTND1* gene is only rarely involved in large-scale rearrangements, whereas the *MTND4* gene is removed in the majority of reported mtDNA deletions (38,46). Absolute quantification of mtDNA content was performed on the MyiQ^{TM} real-time PCR detection system (Biorad, USA), with iQ SYBR Green and primers pairs designed to amplify short fragments spanning *MTND1* and *MTND4* (23). The primer sequences for the various real-time PCR templates used have been provided in the online Supplementary Material (Table S2). The total number of mtDNA molecules (total copy number) present in muscle fibre sections was determined from the *MTND1* Ct values, and the number of wild-type mtDNA molecules (wild-type copy number) from the *MTND4* Ct values, using the linear regression equations generated by serial dilutions of the relevant standards. Both assays were optimized and confirmed to be linear over an appropriate concentration range by the standard curve method, and all single-fibre lysates were measured in triplicates. The mtDNA deletion level was calculated with the $2^{-\Delta Ct}$ method from the obtained *MTND1*–*MTND4* ΔCt value.

Long-range PCR

Multiple mtDNA deletions in homogenate skeletal muscle DNA was investigated with either southern blot and/or long-range PCR (6,7). The detection of deleted mtDNA species in single skeletal muscle fibres was achieved using a previously optimized two-step long-range PCR strategy (37). Two rounds of PCR were required to achieve adequate amplification from the single-fibre lysate, and the primer pairs were specifically designed to allow the identification of large-scale rearrangements in the mutational hotspots along the major arc of the mitochondrial genome (Supplementary Material, Table S3). The Expand Long Template PCR System^{TM} (Roche, UK) was used with the following cycling procedures for both the first and second rounds of PCR: 3 min at 93°C; 10 cycles of 93°C for 30 s, 58°C for 30 s, 68°C for 12 min; 20 cycles of 93°C for 30 s, 58°C for 30 s, 68°C for 12 min + 5 s per additional cycle; and a final extension step of 11 min at 68°C. The PCR products were electrophoresed in a 0.7% agarose gel containing ethidium bromide at 40 V for 3 h, before being visualized under ultra-violet light.

Wild-type mtDNA ratio

For each patient, the mean wild-type mtDNA copy number density for COX-positive muscle fibres was determined by averaging the values obtained for all Type I and II fibres separately. To remove the variation among individuals, the wild-type mtDNA ratios for COX-positive and COX-negative fibres were normalized to each patient's mean wild-type mtDNA copy number density. The wild-type mtDNA ratio for each fibre was calculated by dividing the copy number density for that fibre with the patient's mean value for COX-positive fibres, either Type I or II.

In silico modelling

Our mathematical simulation of mtDNA replication is based upon recessive loss-of-function mutations, with the total amount of wild-type mtDNA being the primary determinant of mitochondrial function (21,22). Our modelling equation predicts that the wild-type mtDNA copy number (w) is a function of the mutation level (m) within a cell, and the mtDNA proliferation parameter (α):

$$w = \frac{1 - m}{1 - (\alpha - 1/\alpha)m}$$

The proliferation parameter (α) was determined from the wild-type and mutant copy number data by a non-linear least squares fitting routine using the Levenberg–Marquardt algorithm in Origin 7^{TM} (Northampton, MA). The proliferation parameter (α) represents the maximum proliferation that should be observed when the mutation level approaches 100%. The actual proliferation level observed will be smaller than the predicted α value.

Statistical analysis

Groups were compared using an independent sample *t*-test with GraphPad^{TM} v.4 statistical software (San Diego, CA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflicts of Interest statement. None declared.

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