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Inhibition of mitochondrial fission favours mutant over wild-type mitochondrial DNA

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Biased segregation of mitochondrial DNA variants has been widely documented, but little was known about its molecular basis. We set out to test the hypothesis that altering the balance between mitochondrial fusion and fission could influence the segregation of mutant and wild-type mtDNA variants, because it would modify the number of organelles per cell. Therefore human cells heteroplasmic for the pathological A3243G mitochondrial DNA mutation were transfected with constructs designed to silence Drp1 or hFis1, whose gene products are required for mitochondrial fission. Drp1 and hFis1 gene silencing were both associated with increased levels of mutant mitochondrial DNA. Thus, the extent of the mitochondrial reticular network appears to be an important factor in determining mutant load. The fact that the level of mutant and wild-type mitochondrial DNA can be manipulated by altering the expression of nuclear encoded factors involved in mitochondrial fission suggests new interventions for mitochondrial DNA disorders.

INTRODUCTION

Mendelian genetics do not apply to the DNA located in mitochondria: Mitochondrial DNA (mtDNA) is maternally inherited and its copy number can reach thousands per cell. Pathological mutations of human mtDNA have been known for many years (1,2) and mutant mtDNA molecules frequently co-exist with wild-type copies of mtDNA (1). Perhaps the most common point mutation is the adenine to guanine transition at nucleotide position (nt) 3243 of human mtDNA that is associated with the neuromuscular disease MELAS (3) and a diabetes and deafness syndrome (4). How deleterious mtDNA mutations become established, and how they wax and wane over the course of time are matters of intense investigation. Several reports have suggested that mutant mtDNAs become fixed purely by the stochastic process of genetic drift (5–7). However, recent studies in mouse suggest that a purification-selection process operates in the germline to weed out deleterious mtDNA variants (8,9). The surveillance system for the functional integrity of mtDNA may simply fail at low frequency enabling deleterious mutations to become established in rare cases, thereby producing pathological states; alternatively or additionally particular mtDNA variants, such as A3243G mtDNA, may evade the counter-selection measures acting against deleterious mutants. In somatic cells, the distribution of mutant mtDNA has been shown to be non-random in many contexts. Several studies have shown that particular mtDNA variants undergo biased segregation in human cell cultures (10–14). Tissue-specific biased segregation of mtDNA was also reported in mice engineered to contain a mixture of two mitochondrial genotypes (15), and this was subsequently attributed to differences in free radical production, which can seemingly stimulate mammalian mtDNA replication (16); a supposition that has been more firmly established for yeast (17). Moreover, the distribution of A3243G mtDNA in patients with mitochondrial disease was found to be non-random in one study (18). Thus, there is ample evidence that mutant load is not merely determined by random genetic drift.

The segregation and transmission of mtDNA is dependent on mitochondrial movement. Even in non-dividing cells mitochondria are dynamic undergoing fusion and fission; they range from small spherical structures to complex interconnected networks. The extent of the network depends on the balance between elongation and fragmentation of mitochondria (19). Mitochondrial fission is mediated by the dynamin-related GTPase, Drp1, which is located in the cytoplasm and is recruited to mitochondrial surfaces where it forms a ring around mitochondria and causes division via constriction. The recruitment of Drp1 to mitochondria requires hFis1,

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a tetratricopeptide domain protein anchored to the mitochondrial outer membrane, although the interaction between hFis1 and Drp1 is thought to be indirect. Neither increasing nor decreasing the expression of hFis1 affects Drp1 binding to mitochondria; nevertheless, down-regulation of hFis1, or Drp1 induces mitochondrial elongation, presumably by limiting mitochondrial fission (20–22). Fusion of mitochondria in mammals depends chiefly on the related GTPases, mitofusins 1 and 2 and OPA1. OPA1 also has a role in cristae maintenance, the folding of the inner mitochondrial membrane, which can be uncoupled from its role in mitochondrial fusion (23). The mitofusins are anchored in the mitochondrial outer membrane, whereas OPA1 is located in the intermembrane space; down-regulation/ablation of all three proteins causes mitochondrial fragmentation (21,24).

Mammalian mitochondrial DNA cannot readily be dislodged from mitochondrial membranes (25), suggesting it is tightly associated with the inner membrane. In budding yeast, mtDNA is linked to a transmembrane complex that is critical for its replication, distribution and transmission (26,27). And a variety of gene defects leading to aberrant mitochondrial morphology also cause mtDNA instability in yeast (28–30). In humans, mutations in Mfn2 associated with Charcot–Marie–Tooth disease have an adverse effect on mitochondrial DNA maintenance (31), and some mutations in OPA1 induce multiple mtDNA deletions (32,33), rather than the eponymous Optic Atrophy.

In light of the numerous links between mitochondrial morphology and mtDNA maintenance, we hypothesized that disturbing the balance between mitochondrial fusion and fission would perturb mtDNA distribution and dynamics, and might thereby affect the segregation of mtDNA. Therefore, three genes implicated in mitochondrial morphology maintenance were silenced in a muscle-like cell line carrying a mixture of A3243G and wild-type mtDNA. Two of the three shRNAs interfered with genes whose products mediate mitochondrial fission and both caused the level of A3243G mutant mtDNA to increase in human rhabdomyosarcoma cells.

RESULTS

To test the hypothesis that mitochondrial fusion and fission can influence mtDNA segregation, three genes whose products are involved in mitochondrial dynamics were silenced in cells carrying a mixture of mutant and wild-type mtDNA. The recipient or parental cell line was a muscle-derived rhabdomyosarcoma (RD) line carrying 80% A3243G mtDNA, whose mutant load remained unchanged throughout the study (Supplementary Material, Fig. S1). Stable transformation of cells with shRNAs targeting Drp1, hFis1 or OPA1 cloned in plasmids pRep4 or pSilencer (Fig. 1A) aimed to achieve persistent RNA interference. The network of mitochondria was more extensive in clonal cell lines transformed with Drp1 or hFis1 shRNA constructs than controls, whereas the mitochondrial network was fragmented in cell lines carrying OPA1 shRNA (Fig. 1B), in line with previous reports (21). These changes suggested that the shRNAs were having the desired effect on mitochondrial fusion and fission, and so the mitochondrial genotype was assessed in cells carrying the shRNAs and compared with sister clones transformed with the corresponding empty vector.

Drp1 down regulation induces an increase in A3243G mtDNA in an RD cybrid line

Drp1 protein level in seven clones transformed with pRep4.Drp1-shRNA was a third or more below the mean value of six control cell lines, 1 month after transfection, based on immunoblotting with anti-Drp1 antibody (anti-DLP1 BD Biosciences Pharmingen) (Fig. 2A). The effect of Drp1 shRNA on transcript abundance was measured also by real-time PCR analysis and the seven clones displayed a 50–80% decrease in Drp1 mRNA relative to the six empty vector control lines (Supplementary Material, Fig. S2A), confirming the shRNA strategy was effective.

The relative abundance of mutant and wild-type mtDNA was determined by last-cycle 'hot' PCR (Fig. 2B). The A3243G mutation creates an *Apa*I site and so PCR products spanning nucleotides 3033–3361 of human mtDNA yield a 328 bp fragment that is cleaved by *Apa*I into two fragments of 214 and 114 nt where the mutation is present, whereas products amplified from wild-type mtDNA have no such *Apa*I site. One cell line transformed with pRep4.Drp1-shRNA had a similar mutant load to the untransformed parental cell line (~80%), but in the other six lines mutant mtDNA was 13 or more percentage points higher than the untransformed cells (Fig. 2B; Supplementary Material, Fig. S2B). In contrast, there was little change in the proportion of A3243G mtDNA in any of the six cell lines transformed with empty vector (pRep4), which ranged from 72 to 82.1% of total mtDNA, with a mean of 77% $\pm$ 3.5 (Fig. 2B; Supplementary Material, Fig. S2B). The mean level of mutant mtDNA for all seven Drp1-shRNA clones was 92.1% $\pm$ 6.3, an average increase in mutant mtDNA of 15 percentage points, which was statistically significant ($P < 0.001$) when compared with the control cell lines (Fig. 2B). The lack of effect on the level of mutant mtDNA in the control group excludes the stress of transfection, or any aspect of the growth regime, as a causal factor in the increase in mutant mtDNA, rather it suggests that decreased mitochondrial fission favoured the selection of mutant mtDNA.

hFis1 down regulation induces an increase in A3243G mtDNA in an RD cybrid

If the increase in mutant mtDNA resulting from Drp1 gene silencing was a consequence of decreased mitochondrial fission, as postulated earlier, then hFis1 shRNA should also promote selection of mutant mtDNA, as it produced the same change in mitochondrial morphology (Fig. 1B). Analysis of the same RD cybrid carrying 80% A3243G mtDNA after transformation with pRep4.hFis1-shRNA revealed decreases in hFis1 protein (Fig. 3A) and mRNA (Supplementary Material, Fig. S3A), thereby confirming the effectiveness of the shRNA. The presence of the hFis1-shRNA construct was associated with an average increase in mutant mtDNA of 11 percentage points, among 11 clonal cell lines (Fig. 3B; Supplementary Material, Fig. S3B), with a *P*-value of <0.001.

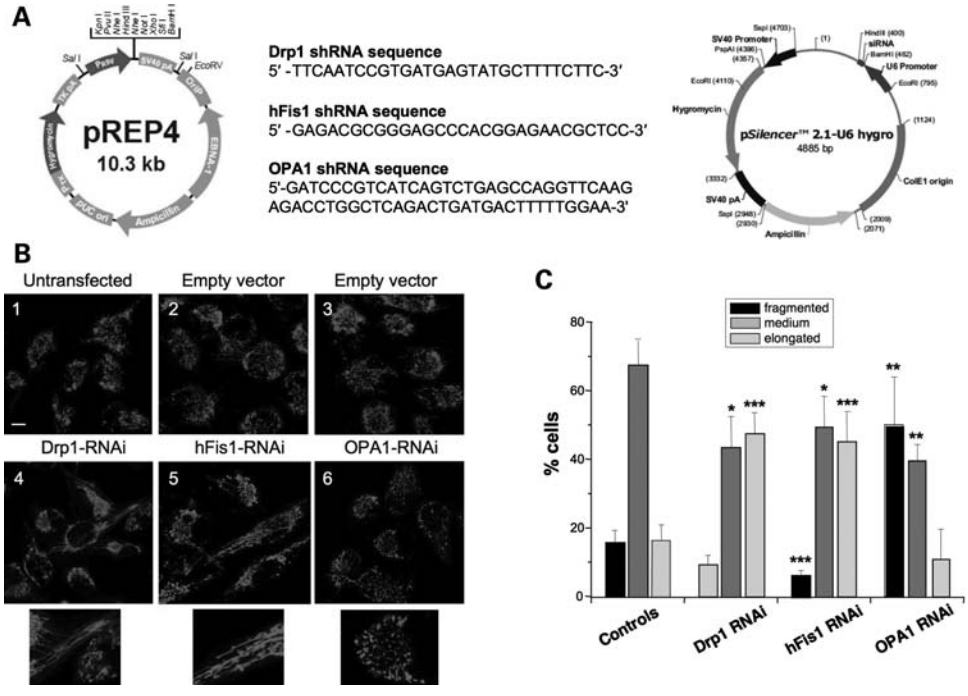

Figure 1. Gene-silencing of components of the mitochondrial fission and fusion apparatus alters mitochondrial morphology in RD cells with mutant mtDNA. (**A**) Plasmids specifying shRNAs targeting Drp1 (21), hFis1 (21) or OPA1 (58) were used to transform rhabdomyosarcoma (RD) cells carrying 80% A3243G mtDNA. Controls were untransformed (parental) recipient cells or transformants with pRep4 lacking an insert (empty vector). (**B**) After 1 month's growth in selective medium the mitochondrial morphology of Drp1, hFis1 and OPA1 RNAi cells was visualized with Mito-Tracker Red and analysed by confocal microscopy, and compared with controls. Enlargements show the detailed structure of mitochondria in shRNA transfected cells. Control cells (B1, B2 and B3) contained primarily 'intermediate' mitochondria; cells carrying shRNAs to Drp1 (B4) and hFis1 (B5) had many elongated mitochondria, whereas cells with OPA1 shRNA (B6) contained many more fragmented mitochondria than normal. Scale bar: 10 μm. (**C**) The percentage of cell population with fragmented, intermediate or elongated mitochondria in control cells or Drp1, hFis1 and OPA1 RNAi cells. Over 400 cells from multiple fields were scored for each category. Data represent the mean ± one SD of two clones chosen at random. Statistical significance $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Thus, RNA interference of both Drp1 and hFis1 increased the mutant load in cells carrying the A3243G pathological mtDNA variant and the combined probability that this chance was <1 in 100 000 ($P < 0.00001$).

Despite the strong association between RNAi targeting of the mitochondrial fission apparatus and segregation to mutant mtDNA, there was no strict correlation between reduced expression and segregation to mutant mtDNA. For example, the Drp1-shRNA clone with the lowest level of Drp1 protein was the only one not to show an increase in mutant mtDNA (Supplementary Material, Fig. S2B). And in the case of hFis1 shRNA, the most modest changes in mutant load were found in the two clones with the largest and smallest decreases in hFis1 protein compared with controls (Supplementary Material, Fig. S3B). Thus, while an appropriate level of hFis1 or Drp1 expression appears to be necessary, it is not sufficient, to induce the segregation of mutant and wild-type mtDNA.

Co-dependence of Drp1 and hFis1 expression

The expression of hFis1 was determined in cells where the Drp1 gene was silenced and vice-versa. Five of seven Drp1-shRNA clones had a marked reduction in the level of hFis1 protein, indeed hFis1p was more affected than Drp1p in four cases, yet the remaining two clones displayed no decrease whatsoever in hFis1p (Fig. 4A). The change in Drp1 expression was similar to hFis1 in eight of the 11 clones carrying pRep4.hFis1-shRNA, and the other three clones had *lower* levels of Drp1 protein than hFis1p (Fig. 4B). Taken together these results support the idea that Drp1 and hFis1 are in some circumstances co-regulated at the level of transcription. The regulatory system may not be functional in some rhabdomysarcoma cells, such as those that gave rise to Drp1-shRNA clones 5 and 6; or in HeLa cells, where Drp1 abundance did not alter appreciably after hFis1 shRNA (21). In any event, a *persistent* decrease in expression of both Drp1 and hFis1 was not required to induce an increase in mutant load. Clones D5 and D6 had normal levels of hFis1 protein (Fig. 4A), yet saw increases of 19 and 13% in A3243G mtDNA, respectively (Fig. 2B).

To test whether inhibition of mitochondrial fission might additionally affect the fusion apparatus, OPA1 and mitofusin (Mfn1) mRNA levels were measured in eight or nine clones carrying shRNAs targeting Drp1 or hFis1. The steady-state level of both transcripts was significantly lower than controls, $P < 0.05$ and $P < 0.01$, respectively (Fig. 5), which hints that there may have been a compensatory decrease in expression of the fusion proteins, to limit any adverse effect of a reduced capacity for mitochondrial fission.

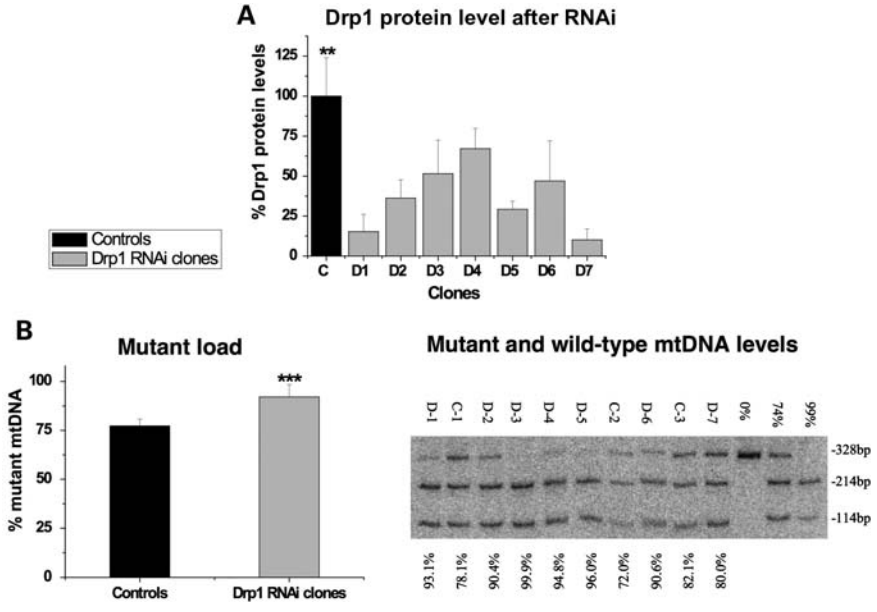

Figure 2. Drp1 protein (expression) is associated with an increase in the proportion of A3243G mutant mtDNA in RD cells. (**A**) Quantification of Drp1 protein level in total cell lysates from Drp1 shRNA clones (D-1 to D-7) and controls (pRep4 vector containing clones C-1 to C-6). The abundance of Drp1 relative to the mitochondrial outer membrane protein (VDAC) and the cytoskeletal element (actin) was determined by immunoblotting. Drp1 was decreased relative to VDAC and actin based on visual inspection, and this was confirmed by quantifying Drp1 and actin, as indicated in the graph. Data represent the mean $\pm$ one SE, $n \geq 2$. The mean Drp1 level among seven Drp1 RNAi clones was significantly lower than six control clones, $P < 0.01$ (**). (**B**) Mutant and wild-type mtDNA levels were analysed by phosphor-imaging, after LC-Hot PCR and *Apa*I digestion (see Materials and Methods). The percentage of mutant A3243G mtDNA is indicated at the base of the phosphorimage; quantification of the fragments indicated a higher mutant load (% A3243G mtDNA) among Drp1 RNAi clones, compared with controls. Each value represents the mean $\pm$ one SD, $n \geq 3$. The mean mutant load of the seven Drp1 RNAi clones was significantly higher than the six control clones, $P < 0.001$ (***).

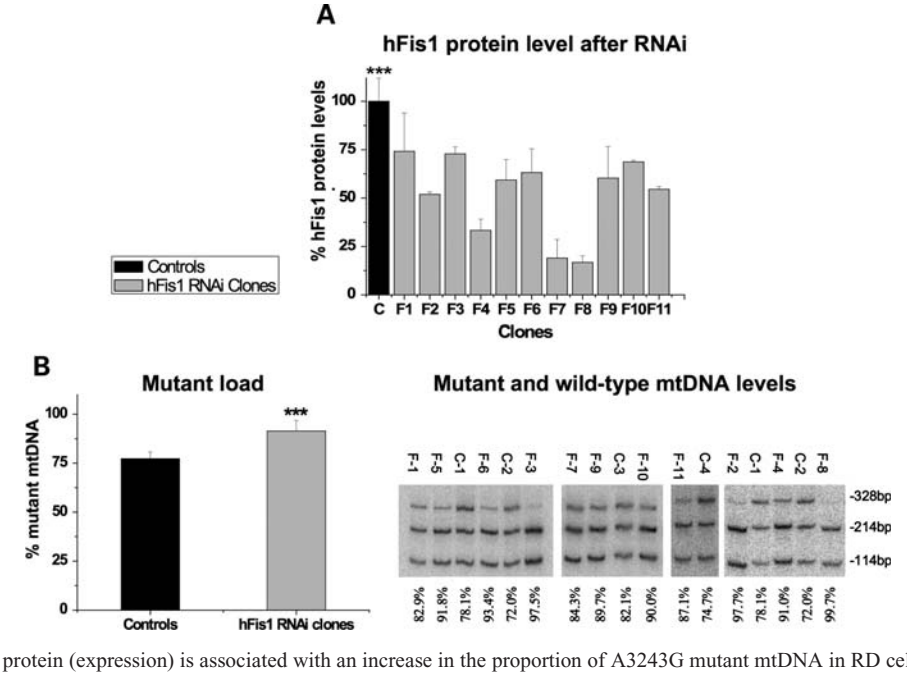

Figure 3. Decreased hFis1 protein (expression) is associated with an increase in the proportion of A3243G mutant mtDNA in RD cells. The layout is essentially as per Figure 2 except that the cells were transformed with pRep4 designed to yield clones (F-1 to F-11) with a shRNA targeting hFis1, rather than Drp1. Briefly, hFis1 abundance compared with VDAC and actin, indicated that the target gene was effectively repressed (**A**). The protein level of hFis1 of 11 hFis1 RNAi clones was significantly reduced compared with six controls, $P = 0.001$. (**B**) Analysis of the levels of mutant and wild-type mtDNA indicated an increase in mutant mtDNA in hFis1 RNAi clones, $P < 0.001$ (***).

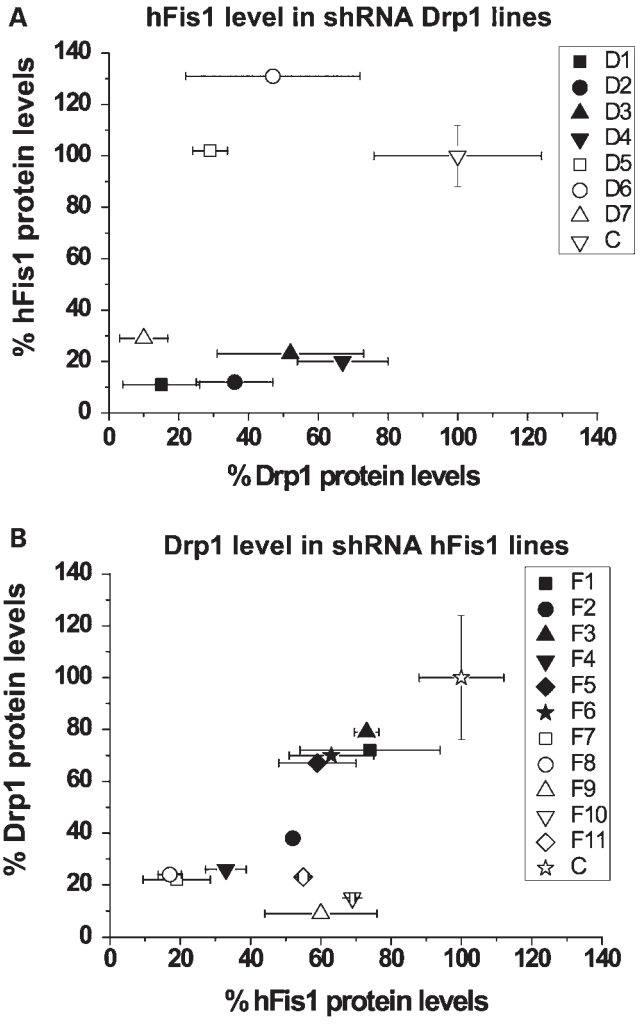

Figure 4. Drp1 and hFis1 expression are co-dependent in many clones. (**A**) hFis1 protein was quantified in Drp1 RNAi clones (D1–D7) and expressed relative to actin (i.e. the same reference as used for estimating the abundance of Drp1 protein). (**B**) The expression level of Drp1 in hFis1 RNAi clones (F1–F11).

Drp1 and hFis1 expression and mtDNA copy number

Drp1 gene-silencing has been linked to decreased mtDNA copy in HeLa cells (34), and mitochondrial morphology mutants in yeast often precipitate mtDNA loss (28–30). Therefore, mtDNA copy number was determined in RD transformants and untransfected cells. All the transformants had higher mtDNA copy numbers than the untransfected cells from which they derived (Fig. 6). The largest increase in copy number was around 10-fold in cells with 80% A3243G mtDNA, transfected with empty vector, whereas the increase was only 3-fold when the vector contained shRNA targeting Drp1 or hFis1. Cells containing wild-type mtDNA displayed a 2-fold increase in mtDNA copy number after transfection with empty vector, and yet there was no change in copy number when the same cells were transfected with shRNAs to Drp1 or hFis1. These results suggest transfection and drug selection promoted an increase in mtDNA copy number, which was attenuated when hFis1 or Drp1 was limiting.

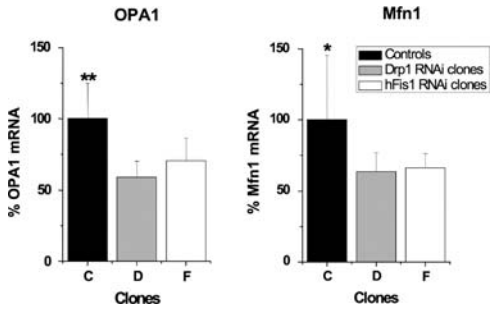

Figure 5. OPA1 and Mitofusin 1 (Mfn1) expression are reduced in RD cells carrying shRNAs targeting Drp1 or hFis1. OPA1 mRNA was significantly lower in four shRNA Drp1 clones (D) and in five clones downregulated for hFis1 (F) ($^{**}P < 0.01$) than controls (four pRep4 vector containing AP4 clones, C). Mitofusin 1 mRNA abundance was reduced to a similar extent in the same clones ($^{*}P < 0.05$). The values expressed are means ± one SD.

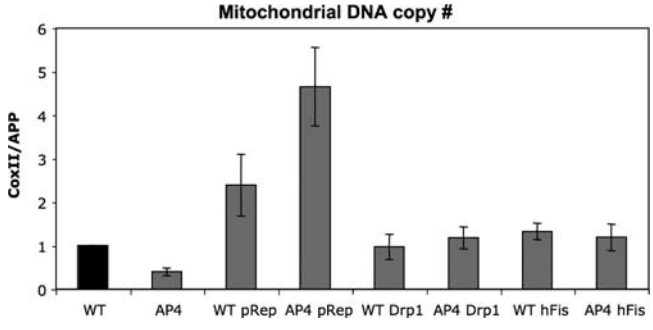

Figure 6. Mitochondrial DNA copy number of Drp1 and hFis1 RNAi clones. Mitochondrial DNA copy number was determined by real-time PCR, by comparing how rapidly a PCR product derived from the mitochondrial cytochrome *c* oxidase II gene (COII) was amplified compared with the nuclear amyloid precursor protein gene (APP). WT—three DNA samples from the RD cybrid with 0% A3243G mtDNA; AP4—three DNA samples from the RD cybrid with 80% A3243G mtDNA; WT pRep4—five clones of RD cybrids with 0% A3243G mtDNA transformed with empty vector; AP4 pRep4—six clones of RD cybrids with 80% A3243G mtDNA transformed with empty vector; WT Drp1—two clones of RD cybrids with 0% A3243G mtDNA transformed with vector containing an shRNA to Drp1; AP4 Drp1—seven clones of RD cybrids with 80% A3243G mtDNA transformed with vector containing an shRNA to Drp1; WT hFis1—five clones of RD cybrids with 0% A3243G mtDNA transformed with vector containing an shRNA to hFis1; AP4 hFis1—11 clones of RD cybrids with 80% A3243G mtDNA transformed with vector containing an shRNA to hFis1.

Thus, it appears that impaired mitochondrial fission impedes mtDNA replication in rhabdomyosarcoma cells, as for HeLa cells (34). The absence of components of the mitochondrial fusion apparatus affects mtDNA maintenance (31) and so the negative effect on mtDNA copy number reported here (Fig. 6) may be due at least in part to the decrease in expression of mitochondrial fusion factors that accompanied Drp1 and hFis1 shRNA (Fig. 5).

RNA interference of OPA1 did not affect the level of A3243G mtDNA

Since decreased mitochondrial fission favoured mutant over wild-type mtDNA, an increase in mitochondrial fragmentation might have the opposite effect. As a test of this idea the OPA1

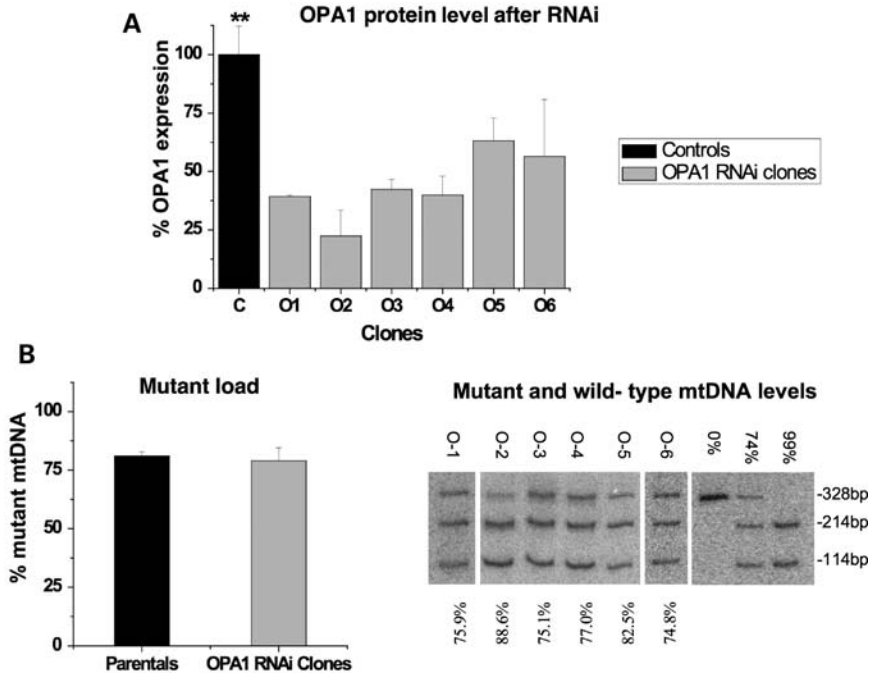

Figure 7. Decreased OPA1 expression has no effect on the proportion of A3243G mutant mtDNA in RD cells. (**A**) Quantification of OPA1 protein level, analysed by immunoblotting and quantified relative to actin in total cell lysates from OPA1 RNAi clones, along with control parental RD cells. Data represent the means ± one SE, $n \geq 2$. OPA1 was significantly reduced in the OPA1 RNAi clones compared with the parental RD cybrids, $P < 0.01$. However, there was no appreciable change in the proportion of mutant and wild-type mtDNA in any of the OPA1 RNAi clones (**B**).

gene was silenced in cells harbouring 80% A3243G mtDNA. OPA1 RNAi altered the mitochondrial morphology of transformants (Fig. 1C) and reduced the steady-state level of OPA1 protein by at least a third, in all six clones analysed (Fig. 7A), in line with a previous study employing pSilencer.OPA1-shRNA (35). However, the mutant load among OPA1-shRNA cell lines was very similar to the parental cell line suggesting that OPA1 RNAi had no effect on the segregation of mutant and wild-type mtDNA (Fig. 7B).

MATERIALS AND METHODS

Cell culture

A human ρ^0 rhabdomyosarcoma cell line lacking mtDNA (RD ρ°) (55) was used to generate transmitochondrial cell lines (cybrids) as previously described (56). Heteroplasmic cybrids harbouring 80% A3243G mtDNA were created from osteosarcoma cybrids with 74% mutant mtDNA by selection in medium lacking uridine and supplemented with 0.5 mg/ml G418. RD cybrids were transfected with pSilencer^{TM} 2.1-U6 hygro, pSilencer^{TM} 2.1-U6 hygro OPA1-RNAi (generous gifts of Dr L. Scorrano, Venetian Institute of Molecular Medicine, Padua), pRep4 hygro, pRep4.hygro Drp1-RNAi, pRep4.hygro hFis1-RNAi (generous gifts of Prof R. Youle, National Institute of Health, Bethesda). The transfection was performed using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in a six-well plate and transfected 24 h later with a mixture of 2 μg of DNA in 250 μl DMEM and 5 μl of Lipofectamine^{TM} 2000 in 250 μl of DMEM. Twenty-four hours after transfection cells were transferred to 60 mm plates and a day later, 0.5 mg/ml hygromycin was added to kill untransformed cells.

MitoTracker® Red staining

Cells were incubated with 125 nM MitoTracker Red probe for 30 min, then observed at confocal microscope (LEICA TCS SP5).

Biochemical analysis

Western blotting analysis. Total cell lysates were prepared by maintaining the cells on ice for 30 min with 200 μl of RIPA buffer (65 mM Tris, 150 mM NaCl, 1% NP-40, 0.25% Na-DOC, 1 mM EDTA, pH 7.4) and 7 μl of a cocktail of protease inhibitors (Sigma). After centrifugation at 14 000g_{max} for 15 min at 4°C, an equal amount of protein (30 μg) for each sample was separated by SDS–PAGE (12% acrylamide) and transferred to nitrocellulose membrane. The membrane was blocked in 5% (w/v) fat-free milk in 0.02 M Tris/HCl pH 7.5, 137 mM NaCl, and 0.1% (v/v) Tween-20 for 1 h at room temperature. The membrane was incubated overnight at 4°C in primary antibodies to anti-OPA1 monoclonal antibody (BD Biosciences, 1:1000), anti-Drp1 monoclonal antibody (BD Biosciences Pharmingen, 1:1000), anti-actin monoclonal antibody (Chemicon International, 1:4000); anti-hFis1 polyclonal antibody (Alexis, 1:1000); anti-VDAC/porin monoclonal antibody (Sigma, 1:1000). Bound antibody was visualized using an ECL reagent (GE Healthcare). Densitometric analysis of western blot signal was performed using Gel-Pro Analyser 3 software.

Mitochondrial genotyping by last hot-cycle PCR. The amount of heteroplasmy for the A3243G mutation was measured in DNA samples extracted from cells using the materials and as described previously (57). Briefly, the regions around the mutation sites were PCR amplified using mtDNA-specific primer pairs: F 5′- GTTCGTTTGTTCAACGATT-3′; R 5′-GGTAAGATTACCGTTACCG-3′, with addition of [α-^{32}P] dCTP in the last synthesis cycle. Labelled PCR products were then digested with the restriction enzymes diagnostic for the site polymorphisms created by the mutations (*Apa*I), and products of replicate digestions were separated by polyacrylamide gel electrophoresis and phosphorimages were analysed with Gel-Pro Analyser 3 software.

Real-time PCR

RNA extraction was performed using TRIzol Reagent (Invitrogen, life technologies). First-Strand cDNA synthesis was performed using SuperScript™ II Reverse Transcriptase from Invitrogen. The levels of OPA1, Drp1 and hFis1 transcripts were quantified by real-time PCR, with reference to the expression of the housekeeping ribosomal gene RPLPO (acidic ribosomal phosphoprotein PO).

The mtDNA copy number was estimated by amplifying a portion of the cytochrome *c* oxidase (COII) gene of mtDNA and comparing it to the amplification profile of a nuclear single copy gene, Amyloid Precursor protein (APP). ABI PRISM 7000 Sequence Detection System and Platinum SYBR green qPCR SuperMix-UDG (Invitrogen, USA) or AmpliTaq gold (Applied Biosystems) were used. The primers and probes used are tabulated below. Probes were labelled with FAM and TAMRA.

APP	F5′-TTTT TTTTTGTGTGCTCTCCCAGGTCT-3′
	R 5′-TGGTCACTGGTTGGTTGGC-3′
	P 5′- CCCTGAACTGCAGATCACCAATGTGGTAG-3′
COII	F 5′- CGTCTGAACTATCCTGCCCG-3′
	R 5′- TGGTAAGGGAGGGATCGTTG-3′
	P 5′- CGCCCTCCCATCCCTACGCATC-3′
OPA1	F 5′- GGAAAAGGGAACAGCTCTGA-3′
	R 5′- CACTTGGTGTGCCTTTAG CA-3′
Drp1	F 5′- CTGACGCTTGTGGATTTACC-3′
	R 5′- CCCTTCCCATCAATACATCC-3′
hFis1	F 5′- GGAGGACCTGCTGAAGTTTG-3′
	R 5′- ACGATGCCTTTACGGATGTC-3′
RPLPO	F 5′-GTGATGTGCAGCTGATCAAGACT-3′
	R 5′-GATGACCAGCCCAAAGGAGA-3′

Statistical analysis

Data were expressed as mean $\pm$ SD or mean $\pm$ SE. Statistical analysis of group differences was examined using the unpaired Student's two-tailed *t*-test. The differences were considered significant at the 95% confidence level ($P < 0.05$).

DISCUSSION

Here we show that suppressing the expression of genes involved in mitochondrial fission has an impact on the relative amounts of mutant and wild-type mtDNA in human cultured cells. These findings not only provide further evidence of biased segregation of mtDNA, they show that interventions can influence this process, with important implications for mitochondrial DNA diseases.

Intracellular versus intercellular selection of mutant mtDNA

In theory the observed increases in mutant load could have reflected selection of a subset of cells with high levels of mutant mtDNA; however, the number and time of appearance (growth rate) of transformants was similar, based on daily visual inspection, whether or not the vector carried an shRNA targeting the fission apparatus. Therefore, selection of mutant mtDNA is adjudged to have occurred at the intracellular level. This was certainly the case for A3243G mtDNA in osteosarcoma cells, where an increase in mutant load was tracked in a clonal cell line and shown to have no effect on the growth rate of the cells (13).

Mitochondrial autophagy, proliferation or direct replicative advantage could explain biased mtDNA segregation

Mitochondrial autophagy is presumed to recycle defective mitochondria and this process is intimately linked to mitochondrial fission (36). Hence, impaired fission could limit the clearance of mitochondria with high levels of mutant mtDNA, which might in part explain the increases in mutant mtDNA reported here, where fission was inhibited. The expression of mitochondrial fusion genes is more pronounced in differentiated cells and tissues than in rapidly dividing cells (37,38). Therefore, the cultured cells with a much-reduced capacity for mitochondrial fission studied here, may recapitulate the behaviour of mutant mtDNA in post-mitotic tissues. That said, the accumulation of defective mitochondria in muscle takes many years and is largely restricted to the subsarcolemma, and so the increase in mutant mtDNA achieved after a matter of weeks in the proliferating cells documented here must differ in some way.

Inter-mitochondrial competition based on function might be expected to occur more readily when there is little in the way of a reticular network, as the proliferation of discrete organelles could well be coupled to respiratory competence. If true, mitochondrial fission should favour mitochondria containing wild-type mtDNA over organelles with high levels of mutant mtDNA. Here RNAi targeting Drp1 and hFis1 decreased fission, resulting in mitochondrial elongation and a shift to higher levels of A3243G mtDNA. Selection based on mitochondrial function may be inoperable when mitochondria are organized in a reticular network, perhaps because the membrane potential is more evenly distributed within elongated organelles than among fragmented mitochondria. Alternatively, complementation may be facilitated in mitochondrial networks compared with discrete organelles. In either case, the question of why the level of mutant mtDNA increases at the expense of wild-type mtDNA remains. The proliferation of dysfunctional mitochondria is the hallmark of mitochondrial myopathy, and runs counter to the idea of selection based on respiratory competence. It has been attributed

by many to a feedback system, whereby mitochondria sequester factors required for biogenesis according to respiratory demand. The system works well ordinarily, but defective mitochondria will corrupt the process, as they fail to satisfy respiratory demand in spite of the resources they consume. Clearly there must be constraints on such a mechanism otherwise each and every defective mitochondrion would be positively selected, leading to an energy crisis at every turn.

Free radical (ROS) production might well contribute to the selection of A3243G mtDNA. ROS is known to act as a signal transducer for cellular proliferation (39), and ROS has been implicated in stimulating mtDNA replication, in mammalian and yeast cells (16,17). Furthermore, ROS production is often elevated when mitochondrial respiration is impaired (40), including RD cells carrying A3243G mtDNA (38). The presumption is that the effects of ROS on the proliferation of A3243G-containing mitochondria are restricted to mitochondrial networks; perhaps because high ROS production triggers autophagy of discrete organelles, while sparing elongated mitochondria. In which case, fragmented mitochondria could escape autophagy even if defective, where ROS is low. Thus, the selection of mtDNA variants is likely to depend on the complex interplay of the fusion and fission apparatus, autophagy regulation, ROS production and antioxidant defences.

The situation may be yet more complex. Mitochondria appear to operate more than one mechanism of replication (41,42), and the changes in Drp1 and hFis1 expression may favour one or other mechanism of replication. Nucleotide 3243 of human mtDNA is located within the binding region of the mitochondrial transcription termination factor (mTERF), and mTERF binding in this region promotes replication pausing (43). The affinity of mTERF for its canonical binding site is reduced when a template harbours the A3243G mutation (44). Thus, if replication pausing at nt 3243 is associated with a particular mechanism of replication that is induced when hFis1 or Drp1 is limiting, then templates harbouring the A3243G mtDNA mutation will be less prone than wild-type mtDNA to replication pausing and so will have a replicative advantage, which could explain the selection of mutant over wild-type mtDNA.

Does increased expression of the mitochondrial fission apparatus favour wild-type mtDNA?

Several outstanding questions could be addressed in the cell culture system used here. The most urgent would seem to be whether or not over-expression of Drp1 or hFis1 has the opposite effect to RNA interference; i.e. does increased expression of Drp1 or hFis1 promote the selection of wild-type mtDNA? Preliminary experiments using the RD cell line transformed with Drp1 cloned in pcDNA3 failed to elicit an effect on Drp1 protein level or mutant load among 10 clones analysed (data not shown); but this type of unregulated approach typically increases gene expression by one to two orders of magnitude, in the short term, and so can perturb normal cell function. Certainly stable expression in human cultured cells often fails due to transgene loss, despite retention of the drug resistance marker (45–47). Therefore there is a need to develop an inducible system in cells heteroplasmic for the A3243G mutation, in order to investigate the effect of increased expression of the mitochondrial fission apparatus. Moreover, co-ordinated increased expression of Drp1 and hFis1, or even the entire fission apparatus, may be necessary to exert the opposite effect on mtDNA segregation to that observed after Drp1 and hFis1 gene silencing.

The failure of OPA1 RNAi to induce segregation to wild-type mtDNA (Fig. 6) would seem to imply that increased mitochondrial fission, at least as a result of limited fusion capacity, does not have the opposite effect to decreased fission. However, OPA1 occurs as eight splice variants/isoforms and has multiple roles in mitochondrial biogenesis and function, which are only now beginning to be unravelled (48). The known pathogenic variants of OPA1 affect mitochondrial morphology and apotosis, making it difficult to differentiate between the two roles. Mitofusin 2 makes a critical contribution to linking mitochondria and the ER, as well as mitochondrial fusion (49), and so it might also produce myriad effects that counteract or mask those pertaining to segregation. Thus, RNAi of Mitofusin1 may be the best means of increasing fission, by limiting the capacity for mitochondrial fusion.

The cell culture system can also be used to address the issue of whether other pathological mtDNA variants behave similarly; and other cells can shed light on the importance of nuclear background. Determining the fate of A3243G mtDNA in A549 adenocarcimoma cells where fission is limited by RNA interference would be of particular interest, as unlike HOS cells, these cells favour wild-type over A3243G mtDNA (11).

In vivo models and the prospects for intervention in mtDNA diseases

Animal models will be needed to elucidate the effects of modulating the expression of the fusion and fission machinery upon mutant and wild-type mtDNA in whole organisms. There is no direct equivalent of the A3243G mutation in an animal model with which to recapitulate our study, but other mouse models of mitochondrial disease have been generated (8,50,51), and the cybrid mouse with a mixture of BALB and NZB mtDNA (15) is another possible test-bed for the idea that modulating the expression of components of the mitochondrial fission apparatus influences mtDNA segregation *in vivo*.

The identification (52) and characterization of RNA interference (53) has made enormous strides in the past decade, and the technique is already being used in gene therapeutic studies (54) and in stage III clinical trials. Hence, the prospects for manipulating mitochondrial fusion and fission are good; the key outstanding issue is to identify changes in expression that favour wild-type, rather than mutant, mtDNA.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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