

Mitochondrial quality control: Cell-type-dependent responses to pathological mutant mitochondrial DNA

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Abbreviations: A549.B2, adenocarcinoma cells; ACTB, actin beta; AR, androgen receptor; ATPJ, ATP synthase, H⁺ transporting, mitochondrial Fo complex subunit F6; B2M, beta-2-microglobulin; BNIP3, BCL2 interacting protein 3; BNIP3L, BCL2 interacting protein 3 like; BCL2L13: BCL2 like 13; MT-CO2, mitochondrially encoded cytochrome c oxidase II; CQ, chloroquine; CS, citrate synthase; $\Delta\psi_m$, mitochondrial membrane potential; EtBr, ethidium bromide; mtDNA, mitochondrial DNA; DNMI1, dynamin 1 like; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; FUNDC1, FUN14 domain containing 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HK2, hexokinase 2; mtDNA: mitochondrial DNA; MFN1/2, mitofusin1/2; LC3, microtubule associated protein 1 light chain 3 beta (MAP1LC3B/LC3B); OXPHOS, oxidative phosphorylation system; mtRFP, red fluorescent protein targeted to mitochondria; MT-TL1, mitochondrially encoded tRNA leucine 1 (UUA/G); PARK2, parkin RBR E3 ubiquitin protein ligase; PINK1, PTEN induced putative kinase 1; mtQC, mitochondrial quality control; mtRFP, red fluorescent protein targeted to mitochondria; siRNA: short interfering RNA; SQSTM1/p62, sequestosome 1; RD.Myo, rhabdomyosarcoma cells, RPLP0, ribosomal protein lateral stalk subunit P0; TOMM20, translocase of outer mitochondrial membrane 20; TMRM, tetramethylrhodamine methyl ester; WT, wild-type.

ABSTRACT

Pathological mutations in the mitochondrial DNA (mtDNA) produce a diverse range of tissue-specific diseases and the proportion of mutant mitochondrial DNA can increase or decrease with time via segregation, dependent on the cell or tissue type. Previously we found that adenocarcinoma (A549.B2) cells favored wild-type (WT) mtDNA, whereas rhabdomyosarcoma (RD.Myo) cells favored mutant (m3243G) mtDNA. Mitochondrial quality control (mtQC) can purge the cells of dysfunctional mitochondria via mitochondrial dynamics and mitophagy and appears to offer the perfect solution to the human diseases caused by mutant mtDNA. In A549.B2 and RD.Myo cybrids, with various mutant mtDNA levels, mtQC was explored together with macroautophagy/autophagy and bioenergetic profile. The 2 types of tumor-derived cell lines differed in bioenergetic profile and mitophagy, but not in autophagy. A549.B2 cybrids displayed upregulation of mitophagy, increased mtDNA removal, mitochondrial fragmentation and mitochondrial depolarization on incubation with oligomycin, parameters that correlated with mutant load. Conversely, heteroplasmic RD.Myo lines had lower mitophagic markers that negatively correlated with mutant load, combined with a fully polarized and highly fused mitochondrial network. These findings indicate that pathological mutant mitochondrial DNA can modulate mitochondrial dynamics and mitophagy in a cell-type dependent manner and thereby offer an explanation for the persistence and accumulation of deleterious variants.

INTRODUCTION

Mitochondria are dynamic organelles that continually undergo fusion and fission events in order to share their content and optimize their function.¹ Mitochondria generate much of the cell's ATP, house essential biosynthetic and metabolic pathways, participate in calcium and redox homeostasis and are key regulators of apoptosis. It is crucial for cell survival to maintain an efficient mitochondrial population through the elimination of dysfunctional mitochondria; hence there are multiple mechanisms acting at the molecular and organelle level to achieve mitochondrial quality control (mtQC).^{2, 3} At the organelle level, when mitochondria become dysfunctional they form discrete organelles that are unable to fuse with the mitochondrial network⁴ thereby facilitating their removal and destruction via autophagy, a process named mitophagy. Mitophagy is mainly mediated by 2 pathways, the PINK1 (PTEN induced putative kinase 1)- PARK2 (parkin RBR E3 ubiquitin protein ligase) and BNIP3 (BCL2 interacting protein 3)- BNIP3L (BCL2 interacting protein 3 like) systems.⁵ In the PINK1-PARK2 pathway mitophagy is usually triggered by an accumulation of PINK1 on mitochondria. PINK1 is imported and constitutively degraded in healthy mitochondria but after mitochondrial injury, PINK1 is stabilized and accumulates in the outer membrane, where it binds and recruits PARK2, an E3 ubiquitin ligase.⁶ PARK2 ubiquitinates various mitochondrial proteins of the outer mitochondrial membrane, including the mitochondrial fusion proteins MFN1 (mitofusin1) and MFN2. The subsequent proteasomal-dependent degradation of mitofusins prevents fusion of the PARK2-positive mitochondria.⁷ Finally, the ubiquitinated mitochondria are recognized by the ubiquitin-binding autophagic receptors, such as SQSTM1/p62 (sequestosome 1), NBR1, and OPTN (optineurin), that, by interacting with both poly-ubiquitin chains and LC3 (microtubule associated protein 1 light chain 3 beta), recruit the LC3-positive phagophore (the autophagosome precursor) on the surface of the dysfunctional fragmented mitochondria.⁸⁻¹⁰ The BNIP3-BNIP3L system works by directly

binding LC3 and therefore by docking the nascent phagophore on the mitochondria that accumulate BNIP3 or BNIP3L.⁵ Therefore these mechanisms of selective mtQC involve not only mitophagy but also the synchronous regulation of the mitochondrial shaping machinery.

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The human mitochondrial genome (mtDNA) encodes 13 polypeptides, all essential subunits of the oxidative phosphorylation system (OXPHOS). Mutations in the mtDNA are responsible for a wide range of diseases.¹² The most common point mutation is an adenine to guanine transition at nucleotide position 3243 (m3243G), in the *MT-TL1* gene (*mitochondrially encoded tRNA leucine 1 (UUA/G)*), responsible for several clinical phenotypes, including syndromes characterized by Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), cardiomyopathy, and Maternally Inherited Diabetes and Deafness (MIDD).^{13,14} Human cells have thousands of copies of mtDNA and individuals with pathogenetic mtDNA mutations often carry a mixture of mutated and wild-type (WT) mtDNAs in their cells, a situation referred to as heteroplasmy. A specific threshold level of mutant mtDNA must be exceeded to cause a respiratory chain defect in the cells.^{15,16} There is often a marked variation in the level of mutant mtDNA among tissues that is thought to be responsible for the diverse clinical phenotypes associated with the pathological mtDNA mutations.¹⁷ Therefore, the segregation to high level of mutant mtDNA is the major factor in determining the pattern and severity of the disease phenotype. Multiple studies have revealed cell and tissue specific segregation of mutant mtDNA.¹⁷⁻²⁴ Generally, in the same individual mutant mtDNA molecules accumulate in tissues, such as muscle, brain, heart; the opposite of what occurs in blood and lung where mutant load is lower.¹⁷⁻²⁰ In line with *in vivo* results, we found that mutated mtDNA was accumulated in RD.Myo cybrids,^{21,22} while WT mtDNA was selected in A549.B2 cybrids.^{23, 24} A better understanding of the molecular mechanisms

underpinning the selection of mutant or wild-type mtDNA would provide new therapeutic approaches for mitochondrial diseases that are currently largely incurable.²⁵

Because mtQC offers a means of degrading mitochondria whose energy-producing capacity is compromised,^{4, 5} it might account for the ability of A549.B2 cells to select wild-type mtDNA in preference to mutant m3243G. Therefore, we evaluated autophagy, mitophagy and mitochondrial dynamics in muscle and lung-derived tumor cell lines harboring different levels of m3243G, and related the findings to cellular energy status.

RESULTS

RD.Myo cells have a higher bioenergetic profile than A549.B2 cells

Considering the interplay of cellular bioenergetics and mtQC,^{26, 27} oxidative phosphorylation and glycolytic parameters were analyzed in A549.B2 and RD.Myo cybrids, carrying different, but stable, proportions of mutant (m3243G) and wild-type mtDNA (**Fig. S1A and B**). Mitochondrial ATP synthesis rates were similar in both WT and heteroplasmic A549.B2 and RD.Myo cybrids, when expressed relative to CS (citrate synthase), a widely used marker of mitochondrial mass (**Fig. 1A**).²⁸ However, the A549.B2 cybrids had only around one third of the citrate synthase activity of the RD.Myo cybrids (**Fig. 1B**), suggesting the capacity of their mitochondria to produce ATP was low, and this was reflected in the steady-state level of ATP (**Fig. 1C**). Despite the RD.Myo cybrids had greater mitochondrial ATP producing capacity, they were less dependent on their mitochondria than the A549.B2 cybrids based on a comparison of the pair of cybrids with 100% m3243G (**Fig. 1A,C**). Mutant load had no impact on ATP level in the RD.Myo cybrids, whereas the A549.B2 cybrid with 100% of the m3243G mutation had significantly less ATP than sister cybrids with lower mutant loads (**Fig. 1C**). Hence, the high level of ATP in the 100% m3243G RD.Myo cybrids must derive from glycolysis, and this was corroborated by the much higher levels of HK2 (hexokinase 2) (an index of glycolytic metabolism²⁹) in RD.Myo cybrids compared to A549.B2 cybrids (**Fig. 1D to G**). These results are concordant with the low activity of glycolytic enzymes of A549 cells compared with C2C12 muscle-derived cell line reported by Guha,³⁰ and the small glycolytic contribution (~5%) to the total ATP produced by A549 cells.³¹ The state 3 and 4 mitochondrial membrane potential and their ratio paralleled the mitochondrial ATP synthesis activity (relative to CS, **Fig. 1A**), with only the homoplasmic mutant cybrids displaying a significantly lower membrane potential than the controls (**Fig. 1H,I**).

In summary in our culture conditions, WT and heteroplasmic A549.B2 and RD.Myo cybrids had similar OXPHOS efficiency but a different bioenergetic profile. In line with previous *in vitro*³⁰⁻³² and *in vivo* results,³³ A549.B2 cells displayed low glycolytic capacity and reduced mitochondrial mass, on the contrary, RD.Myo cybrids showed high glycolytic and oxidative phosphorylation capacity.

Autophagy flux is not impaired in RD.Myo cybrids

Autophagy is an essential degradation pathway that clears the cell of redundant or damaged proteins and organelles.^{34,35} Moreover, the autophagy-lysosome system is also important for cell survival during stress conditions, such as in the absence of nutrients.³⁵ If the unusual ability of A549.B2 cells to eliminate mutant mtDNA^{23, 24} is underpinned by mitophagy, then A549.B2 cells might display increased autophagic flux, compared to RD.Myo cells. When we analyzed LC3-II and SQSTM1 amounts as well as the autophagic flux, i.e. the increase of these markers after chloroquine (CQ) treatment (**Fig. 2A to D**),³⁶ we found that A549.B2 cells without mutant mtDNA display greater autophagic flux than the equivalent RD.Myo cells. However, the situation is reversed in heteroplasmic cybrids (**Fig. 2A**). These data suggest that pathological mtDNA mutations can influence the process of autophagy but argue against changes in overall autophagy flux, being the explanation for the ability of A549.B2, and not RD.Myo, cybrids to purge their cells of mutant mtDNA.

Mitophagy flux is higher in A549.B2 than RD.Myo cybrids

Mitophagy is the specific degradation of mitochondria, or components thereof, by lysosomes via an autophagic process, and it has a mechanistically separate induction and regulation from total autophagy.⁵⁻⁶ PINK1, PARK2 and BNIP3 are the major players in the mitophagy process, that allow the recruitment of the phagophore to the damaged

mitochondria.^{5,6,37-38} To understand if mitophagy flux differed between A549.B2 and RD.Myo cells, with and without mutant mtDNA, we employed the following biochemical, molecular and imaging assays.

First, we carried out a biochemical analysis. Mitophagy was estimated by monitoring the recruitment of LC3-II, SQSTM1/p62, PINK1, PARK2, and BNIP3 on isolated mitochondria. The enrichment of these markers after CQ treatment was evaluated to provide an index of mitophagy flux³⁶ (**Fig. 2E to G; Fig. 3A to C, I to M; Fig. S2**).

Unlike general autophagy, mitophagy diverged in A549.B2 and RD.Myo cells. In WT and heteroplasmic A549.B2 cybrids with mitochondrial LC3-II (mtLC3-II) and SQSTM1/p62 (mtSQSTM1/p62) increased after CQ treatment 3.3-3.8 and 5.7 times while minor changes occurred in RD.Myo (**Fig. 2E, G; Fig. S2**). In A549.B2 cybrids these increases showed a significant positive correlation with m3243G mutant mtDNA accumulation (**Fig. 2H, M; Fig. S3A and B**). Conversely, in RD.Myo cells the amount of mtLC3-II negatively correlated with mutant load (**Fig. 2L**), while mtSQSTM1 did not display any correlation with m3243G (**Fig. 2N**). Since LC3-II is a key mediator of autophagy these results suggested A549.B2 cybrids utilized mitophagy as a mitochondrial degradation pathway significantly more than RD.Myo cybrids.

Consistent with mtLC3 and mtSQSTM1 data, the mitochondrial recruitment of PINK1 (mtPINK1), PARK2 (mtPARK2) and BNIP3 (mtBNIP3) was significantly enhanced in A549.B2 but not in RD.Myo cell lines (**Fig. 3; Fig. S2**). Indeed, the turnover rate of mtPINK1 was 2.4 to 3.7 times greater in WT and heteroplasmic A549.B2 than in RD.Myo cybrids (**Fig. 3A, 3I**), and there was no detectable signal of PARK2 (at around 50 kDa) in mitochondria of RD.Myo cybrids (**Fig. 3L**), probably for an insufficient PARK2 expression level (**Fig. 3P**) and consequent mitochondrial recruitment. Moreover, mtPINK1, mtPARK2, mtBNIP3 levels and

flux positively correlated with the increase of m3243G mutated mtDNA in A549.B2 cells (**Fig. 3D,F,G; Fig. S3C to E**) whereas an inverse trend for PINK1 (**Fig. 3E**) ($P < 0,001$) or no correlation (**Fig. 3H**) for BNIP3 was detected in RD.Myo cells. The opposite behavior of PINK1 in the 2 cell lines might reflect the fact that A549.B2 and RD.Myo cells express different species (**Fig. 3I**),³⁹ confirmed by an siRNA approach, done in parallel with PARK2 downregulation (**Fig S4**).

We also tested the CQ mediated accumulation of the recently reported mitophagic receptors, FUNDC1 (FUN14 domain containing 1)⁴⁰ and BCL2L13 (BCL2 like 13)⁴¹ on isolated mitochondria of A549.B2 cells. CQ did not change the mitochondrial protein level of FUNDC1, a receptor for hypoxia-induced mitophagy⁴⁰ (**Fig. S5A and B**); on the contrary, CQ resulted in a significant 3-fold increase of mitochondrial BCL2L13 amount in both WT and heteroplasmic A549.B2 cells (**Fig. S5A, S5C**). In addition, mitochondrial BCL2L13 was 3-4 fold augmented in heteroplasmic mutant vs WT mitochondria. These results suggested that BCL2L13, but not FUNDC1, played a role in the active mitophagic flux in A549.B2 cells, probably both inducing fragmentation and/or cooperating with the PINK1-PARK2 system.⁴¹

Next we carried out a molecular analysis. To establish whether the difference of mitophagy between A549.B2 and RD.Myo cells might be ascribed to a transcriptionally-dependent regulation of these factors, we evaluated the expression of *PINK1*, *PARK2* and *BNIP3* by quantitative RT-PCR. To validate this comparison, the transcript level of the 2 housekeeping genes was estimated in a fixed number (10^7) of A549.B2 and RD.Myo cells. Both *RPLP0* (*ribosomal protein lateral stalk subunit P0*) and *B2M* (*beta-2-microglobulin*), the 2 housekeeping genes, were similar in the 2 cell types and were unaffected by m3243G mutant load (**Fig. 3N**). In WT and heteroplasmic mutant RD.Myo cells, the transcript level of *PINK1* was significantly lower than in A549.B2 cybrids (**Fig. 3O**). Similarly, *PARK2* expression was also decreased in RD.Myo cells (**Fig. 3P**), while *PINK1*, *PARK2* and *BNIP3* mRNAs were

significantly increased in heteroplasmic vs 0% A549.B2 cells (**Fig. 30 to Q**). Thus, A549.B2 but not RD.Myo cells, showed transcriptional induction of *PINK1*, *PARK2* and *BNIP3* in response to mutant mtDNA.

Subsequently, we examined mtDNA removal. To test mitochondrial disposal by mitophagy, mtDNA removal was determined by quantification of mtDNA copy number in WT and heteroplasmic mutant A549.B2 and RD.Myo cells untreated and treated with ethidium bromide (EtBr) (50 ng/ml) for 22 h with and without CQ, as described⁴² (**Fig. 4A and B**). EtBr, blocking the mtDNA synthesis,⁴³⁻⁴⁵ reduced the mtDNA copy number at 60% and 75% in WT and heteroplasmic A549.B2 cells respectively, as compared to the untreated cells. The concomitant addition of CQ increased significantly mtDNA amount of 21% in WT A549.B2 (EtBr+CQ vs EtBr $P<0.05$) and of 31% in heteroplasmic A549.B2 (EtBr+CQ vs EtBr $P<0.001$), showing the percentage of mtDNA degradation consequent to mitophagy (**Fig. 4A**). Similarly, in both WT and heteroplasmic RD.Myo, EtBr reduced the mtDNA amount at 58 to 60%, CQ treatment produced a slight and not significant increase of 14% and 3% in WT and heteroplasmic cells, respectively, indicating a reduced removal of mtDNA in RD.Myo cells (**Fig. 4B**). The differences between mtDNA copy number of control (untreated cells) and CQ + EtBr treated cells were proportional to the growth rate, expressed as duplication time (**Fig. S1C**) and represented the portion of mtDNA synthesized to fill up the dividing cells.

Finally, we used a morphological analysis. The inference, from the LC3-II (**Fig. 2**), *PINK1*-*PARK2* and *BNIP3* abundances and turnover (**Fig. 3**) and mitophagic mtDNA degradation (**Fig. 4A and B**), that RD.Myo cells largely eschew mitophagy compared to A549.B2 cells, was further corroborated by confocal microscopy analysis of cells transfected with GFP-LC3B and red fluorescent protein targeted to mitochondria (mtRFP). The LC3-positive puncta that colocalized with mitochondria were quantified and expressed as puncta/cell (**Fig. 4C to E**), in absence of GFP-LC3 aggregates, as tested in detergent-soluble

fraction of GFP-LC3B transfected cells immunoblotted for ubiquitin and LC3 (**Fig. S6C**). In line with the above data, also the morphological analysis showed that mitophagy was active in A549.B2 but not in RD.Myo cells. In fact, CQ treatment enhanced 3.3-4.6 fold the double positive vesicles in A549.B2 cybrids, but only 1.3 to 1.6 fold in RD.Myo cybrids (**Fig. 4E**). Moreover, the heteroplasmic A549.B2 cells carrying the m3243G mutation increased by 50% the mitophagic flux when compared to 0% A549.B2 cells (4.6 vs 3.3; **Fig. 4C and 4E**), in accordance with the biochemical data (**Fig. 2E, G**). Consistently, the high mitophagic flux in A549.B2 cells was confirmed by colocalization of endogenous LC3 puncta with immunostained mitochondria (by using anti-LC3B and anti-ATP5J antibodies) (**Fig. S6A and B**).

These findings indicated a greater mitophagic capacity and flux in A549.B2 than RD.Myo cells. Moreover, m3243G enhanced mitophagy in A549.B2 but not in RD.Myo cybrids. These differences did not reflect a deficiency of the lysosomal degradation pathway in RD.Myo cells, as autophagy was active in heteroplasmic RD.Myo cells (**Fig. 2A and B, D**).

A fragmented mitochondrial network is permissive for mitophagy in A549.B2 cybrids

Modulation of mitochondrial shape plays a key role during mitophagy.^{3, 4} Mitochondrial fragmentation is necessary for the removal of damaged organelles, while mitochondrial fusion can complement damaged or dysfunctional units and possibly recover their activity, thereby maintaining metabolic efficiency.^{1, 46} Hence, one means by which RD.Myo cells might avoid or prevent mitophagy would be to organize their mitochondria in an interconnected reticular network. Conversely, A549.B2 cells might have a more fragmented mitochondrial population to enhance mitophagy. Therefore, we assessed whether

mitochondrial morphology was consistent with the differences in mitophagy of A549.B2 and RD.Myo cells.

Labeling of the mitochondria of A549.B2 and RD.Myo cybrids with mt-RFP indicated that the A549.B2 cells displayed many fragmented mitochondria, whereas RD.Myo cells showed tubular interconnected mitochondria (**Fig. 4F and G**) (RD.Myo vs A549.B2 $P < 0.001$). The difference was accentuated by the presence of m.3243G mutant mtDNA, with heteroplasmic A549.B2 cybrids having the most fragmented mitochondria, whereas elongated mitochondria were most extensive in RD.Myo cybrids with mutant mtDNA (**Fig. 4G**).

Three mammalian proteins are required for mitochondrial fusion.⁴⁷ Mitofusins (MFN1, MFN2) are important for outer membrane fusion while OPA1 is involved in inner membrane fusion.^{48, 49} Mitochondrial fission is mediated by DNM1L (dynamin 1 like), which is recruited to mitochondria via the receptor proteins MFF (mitochondrial fission factor), FIS1 (fission, mitochondrial 1), MIEF1 (mitochondrial elongation factor 1) and MIEF2 (mitochondrial elongation factor 2).^{50, 51} Besides the receptor recruitment, DNM1L needs also to be activated by a PPP3/calcineurin-dependent dephosphorylation.⁵² Consistent with the mitochondrial morphology, the DNM1L was significantly more enriched in the mitochondrial fraction of A549.B2 cells than those of the equivalent RD.Myo cells (**Fig. 5A,C**) (0% A549.B2 vs RD.Myo $P < 0.05$; 35 to 80% A549.B2 vs 70 to 80% RD.Myo $p < 0.005$). Consistently, the phospho-DNM1L:DNM1L ratio was reduced in the cytosol of A549.B2 compared to RD.Myo (**Fig. 5B,D**) (0% and 35 to 80% A549.B2 vs 0% and 70 to 80% RD.Myo $P < 0.005$), but was significantly increased in cytosol of heteroplasmic A549.B2 vs 0% A549.B2 cells ($P < 0.01$). Conversely, the steady state level of the fusion proteins, MFN1, MFN2 and OPA1, were significantly higher in isolated mitochondria of RD.Myo cells than those of A549.B2 cybrids (**Fig. 5E to H**). Thus, the fission and fusion machinery of the 2 cell lines predicted fragmented mitochondria in A549.B2 cybrids and an extensive reticular network in RD.Myo cells.

In summary A549.B2 cells had discrete mitochondria that were susceptible to mitophagy whenever dysfunctional, and they expressed sufficient PINK1 and PARK2 to execute mitophagy. Conversely, RD.Myo cells, that had a much lower capacity for mitophagy, maintained their mitochondria in a form that was resistant to mQC process.

Dysfunctional mitochondria are more evident in A549.B2 than in RD.Myo cybrids

Since the PINK1-PARK2 system is sensitive to mitochondrial membrane potential ($\Delta\psi_m$),^{53, 54} we inferred a different pattern of mitochondrial depolarization in A549.B2 and RD.Myo cells. To verify this, we investigated $\Delta\psi_m$ by flow cytometry of TMRM (tetramethylrhodamine methyl ester), a fluorescent probe that accumulates in polarized mitochondria and is released when $\Delta\psi_m$ decreases.^{55, 56} Since dysfunctional-depolarized mitochondria can be masked by the ATP synthase operating in reverse mode, such that $\Delta\psi_m$ is maintained by consuming ATP, we treated the cells with oligomycin, an inhibitor of mitochondrial ATP synthase.^{57, 58}

Under standard growth conditions depolarized mitochondria accounted for 6% of the total in the RD.Myo and 9% in A549.B2 cybrids, with no mutant mtDNA (**Fig. 6A, 6B**). In presence of m3243G mutant mtDNA, mitochondria of RD.Myo cells had a similar $\Delta\psi_m$; mitochondrial depolarization was obtained only after addition of the protonophore FCCP (carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone) (**Fig. 6B**). In A549.B2 cybrids m3243G mutated mtDNA induced a 2 fold higher depolarization (**Fig. 6A**) than in RD.Myo cells. Comparing the 2 sets of cybrids, the presence of m.3243G mtDNA was associated with significantly higher number of depolarized mitochondria in A549.B2 but not RD.Myo cell lines (**Fig. 6C**). Blocking autophagy by CQ treatment increased the levels of depolarized mitochondria in A549.B2, but not in RD.Myo, cells (**Fig. 6D; Fig. S7**), thereby, providing strong additional evidence that the mitophagy pathway is repressed in RD.Myo cells.

In summary, dysfunctional mitochondria were much more evident in heteroplasmic A549.B2 than RD.Myo cells, and mitophagy efficiently removed them to a much greater extent in A549.B2 than in RD.Myo cells.

DISCUSSION

The main findings of the present study indicate that general autophagy and mitophagy are dissociated and that regulation of mtQC (i.e.: mitochondrial dynamics and mitophagy) in response to m3243G mutant load is cell-type specific.

Our original idea was to find higher autophagy in A549.B2 than RD.Myo cells that would preferentially remove the mutated mtDNA.²¹⁻²⁴ However, there was no significant difference of autophagy flux between heteroplasmic A549.B2 and RD.Myo cells and actually we observed the opposite of what expected. In fact, autophagy flux was slightly higher in RD.Myo than in A549.B2 during accumulation of mutated mtDNA.

Differently from general autophagy, the specific elimination of dysfunctional mitochondria by mitophagy differs completely between the 2 cell types. In fact, A549.B2 cells show higher mitophagy and mitophagic mtDNA removal (**Fig. 4A and B**), when compared to RD.Myo cells. Molecular, biochemical and morphological analyses identified the involvement of PINK1-PARK2 and BNIP3 systems in the selective mitochondrial removal (**Figs. 2 to 4**). Furthermore, consistent with mitophagy data, morphological and biochemical analyses revealed a different organization of mitochondrial network in the 2 cell types being mainly fragmented in A549.B2 and fused in RD.Myo cells (**Figs. 4 and 5**). In A549.B2 cells, the fragmented mitochondrial network is enhanced by the m3243G mutant load (**Fig. 4F and G**), in parallel the mitochondrial depolarization (**Fig. 6**) and mitophagy (**Figs. 2 and 3**) are also increased. In fact, loss of mitochondrial membrane potential was found in A549.B2 heteroplasmic cybrids but not in the equivalent RD.Myo cybrids (**Fig. 6**). Selective mitophagy is predicted to clear damaged mitochondria (i.e. caused by high levels of pathological mtDNA mutations). In this model, fission events produce 2 functionally dissimilar mitochondria: one with high $\Delta\psi_m$ and the other with reduced/lost $\Delta\psi_m$. The depolarized mitochondria activate the PINK1-PARK2 system for the autophagy-dependent removal and destruction.⁴ It is well

known that asymmetrical fission results in uneven distribution of dysfunctional mitochondrial components that are expected to increase with the progressive accumulation of mutant mtDNA.^{4,5} Hence the increase of mtQC in response to higher percentage of m3243G mutated mtDNA in A549.B2 cells. In heteroplasmic RD.Myo cells this response is abolished, leading to a progressive accumulation of m3243G-mutant mitochondria. However WT RD.Myo cells had high protein levels of mtPINK1 (**Fig. 3A,I**), absence of the mtPARK2 signal at around 50 kDa (**Fig. 3L**), and a reduced mitophagic flux. These data are difficult to explain, even if it is not to exclude the presence of specific protein isoforms⁵⁹ and/or post-transcriptional modifications.⁶⁰ There is evidence of a PINK1 role in maintaining mitochondrial integrity in mild oxidative stress.⁶¹ Therefore, we can speculate that the increased levels of mtPINK1 in WT RD.Myo cells may be related to additional protective functions, which PINK1 carries out in the mitochondrial network.

It is still unclear why RD.Myo cells are unable to sustain selective form of autophagy even in face of an enhanced general autophagy. One potential explanation is the fact that RD.Myo cells privilege an elongated mitochondrial network for energy purpose, therefore blocking mitophagy (**Figs. 1; 4F and G**).

Controversial data concerning the modulation of mtDNA heteroplasmy by mitophagy are present in literature. Suen and Dai demonstrate that genetic⁶² or pharmacological⁶³ stimulation induces mitophagy and the progressive removal of pathogenetic mtDNA mutations in heteroplasmic cybrids. Hamalainen et al. describe a tissue-specific PINK1-PARK2-mediated positive mitophagy related to MELAS pathogenesis in neuronal cells.⁶⁴ Recently Valenci has provided *in vivo* evidence for the PARK2-mediated change of mtDNA heteroplasmy.⁶⁵ However, other reports fail to find specific PARK2-mediated mitophagy in cells with genetically- and/or chemically-induced mitochondrial depolarization.^{66, 67} These discordances may be explained by considering that interaction of the mitochondrial mutation

pattern with mtQC-mitophagy is a complex process dependent on multiple factors, such as cell bioenergetics, culture conditions, level of PARK2 expression, cell-type and specific mtDNA mutations.⁶⁸ Noteworthy, our data were obtained in cells containing the same mutation, that were grown in identical culture conditions therefore minimizing the factors that strongly influence bioenergetic status, morphology and dynamics of mitochondria.^{28,69} Therefore, the differences observed in mtQC and bioenergetics are due to the respective cell-type specificity.

A549.B2 cells had reduced glycolysis and mitochondrial mass (**Fig. 1**) compared to RD.Myo cells. Moreover the A549.B2 cells relied prevalently on OXPHOS activity for their energy purposes.^{30-32, 70} Therefore, it would be expected a preferential selection of fully functional mitochondria by an active mtQC process. RD.Myo cells display a greater bioenergetic capacity^{30, 32} than A549.B2 cells. Consistently, our data show higher glycolysis, mitochondrial mass and ATP amount (**Fig.1**). RD.Myo mitochondrial network appears as long filaments (**Fig 4F and G**). A highly fused mitochondrial network is important for proper mitochondrial calcium buffering and for the optimal production of ATP because of a higher cristae density and an ideal organization of electron transfer chain components in super complexes.^{51,71} In summary, mitochondrial fusion is advantageous under conditions of high energy demand, and optimizes mitochondrial function in stress conditions.⁷¹

Moreover, elongated mitochondria are protected from mitophagy, as recently shown in hearts lacking the fission protein DNM1L.⁷² Importantly, mitochondrial fusion allows the transfer of soluble and membranous components, favoring functional complementation that reduces the deleterious effects of mtDNA mutations to preserve respiratory function.^{73,74} In this way, the entire mitochondrial compartment works as a single dynamic unit to maximize ATP synthesis.⁷⁵ In this scenario, the reduced mitophagy and elimination of mutant mtDNA variants, together with replicative advantage of the m3243G mutation,⁷⁶ might favor mutant mtDNA accumulation until the WT mtDNA cannot further compensate for the effect of the

mutant one. Consistently, a genetically-induced fusion mimics and accelerates a similar physiological process.²¹ Another key element in modulating mitochondrial dynamics is the redox state. Fusion is induced by oxidized glutathione^{77, 78} and regulated by the redox-sensing protein ROMO1.⁷⁹ Fusion, and the consequent sharing of antioxidant defense, is considered a protective response to oxidative stress, preserving mitochondrial redox balance.^{78, 80} Indeed, mitochondrial fragmentation is associated with increased mitochondrial ROS levels, conversely tubular morphology prevents ROS production.^{51, 80} A549.B2 cells, but not RD.Myo cells, are equipped with a robust antioxidant defenses⁸¹ that maintain redox homeostasis and may protect them from the oxidative stress arisen by mitochondrial fragmentation (data not shown).

We might conclude that high-energy demand, functional complementation and redox homeostasis dictate the mitochondrial fate, favouring mitochondrial fusion, the consequent maintenance of high $\Delta\psi_m$ and reduced mitophagy in RD.Myo cells. Conversely, the reduced bioenergetic profile and the increased antioxidant defenses favour mitochondrial fission, mitochondrial depolarization and the increased selective mitophagy, that should be balanced by a limited biogenesis, in A549.B2, considering the reduced mitochondrial mass.

To our knowledge this study provides the first instance of a cell-type difference in mtQC, driven by mitophagic activity and mitochondrial dynamics. Whereas a cell-type dependent regulation of mitochondrial morphology is well documented and usually linked to cell-type-specific functions and physiological energy demands.⁸² Only 2 *in vivo* studies in rat report a gender-associated tissue difference in total autophagy, but not in mitophagy.^{83, 84}

The cell lines used in this investigation are cybrids obtained from immortalized cells: respectively from adenocarcinoma-A549²³ and from rhabdomyosarcoma.⁴⁵ Although these cells have obvious differences from their normal counterparts and mammalian tissues *in vivo*, they do maintain varying degrees of lineage specific traits. For example, A549 cells, derived

from human lung carcinoma, retain characteristics of type-II alveolar epithelial cells,⁸⁵ while rhabdomyosarcoma-derived cells retain biochemical characteristics of skeletal muscle cells,^{86, 87} and functional capacity to differentiate into polynuclear myotubes.²² Moreover the A549.B2 and RD.Myo cybrids largely mirrored the bioenergetics profile of parental tissues.³³

In summary the present data reveal a novel relationship among genetic background and mtQC, they strengthen the link between cell-type specific segregation of the m3243G mtDNA mutation and mtQC and might contribute to understand mtDNA fate in different cell-types.

MATERIAL and METHODS

Cell culture.

Homoplasmic and heteroplasmic cybrids, from rhabdomyosarcoma (RD.Myo) and adenocarcinoma-A549 (A549.B2) parental lines, harboring the MELAS mutation m3243G, were established as previously described.²¹⁻²³ We used 3 0% A549.B2 and 3 0% RD.Myo clones; one A549.B2 and one RD.Myo clone with 100% m3243G mtDNA; heteroplasmic A549.B2 clones: one with 35%, one with 70%, one with 80% m3243G mtDNA; heteroplasmic RD.Myo clones: one with 70% and one with 80% m3243G mtDNA. Cells were grown in Dulbecco modified Eagle medium (4500 mg glucose/l, 110 mg pyruvate/l) (Gibco-ThermoFisher Scientific, 41966-029), supplemented with 10% fetal bovine serum (FBS; Gibco-ThermoFisher Scientific, 10270-106), amino acids (Gibco-ThermoFisher Scientific, 11130-036), L-glutamine (Sigma-Aldrich, G5763), vitamins (Biochrom, K0373), amphotericin B (Bristol Myers-Squibb, 6116) and antibiotics (Biochrom, A2213). For the different treatments, cells were seeded at a density of 1×10^4 cells/cm². After 48 h the standard medium was supplemented with 50 μ M chloroquine (Sigma-Aldrich, C6628) and the cells were collected 6 h later. The growth rate was evaluated as duplication time as described.⁸⁸

Mitochondria isolation.

For mitochondria isolation, cells were seeded in 10 dishes (24 150-mm) at the density previously reported and treated with or without 50 μ M CQ. After 6 h cells were collected and mitochondria were isolated as described.⁸⁹ Briefly, cells were scraped on ice, pelleted at 600 g for 5 min and washed twice in cold PBS (Gibco-ThermoFisher Scientific, 14190). The cell pellets were broken by adding one volume of hypotonic homogenization buffer (IB 0.1X: 3.5 mM Tris-HCl, pH 7.8, 2.5 mM NaCl, 0.5 mM MgCl₂), and homogenized by 10 or 20 strokes for RD.Myo or A549.B2 cybrids respectively, using a Velp Scientifica homogenizer (Italy). Soon after 1/10 of the packed cell volume of hypertonic buffer IB 10X was added. This homogenate

was centrifuged at 1200 g for 3 min at 4°C twice. The supernatant was centrifuged at 15000 g for 2 min at 4°C. The pellets containing the mitochondria were washed using homogenization buffer A (0.32 M sucrose (Sigma-Aldrich, S9378), 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4) and the pellets were stored at -80°C until use. CS was quantified. 1000, 2000 or 5000 nm/min/mg prot of CS activities were loaded for the different analysis. Mitochondrial enrichment was tested by western blot (WB) as the ratio between GADPH (cytosolic marker) and TOMM20 (mitochondrial marker) in the total lysate (TL) and isolated mitochondria from A549.B2 and RD.Myo cells.⁸⁹ As shown in **Fig. S8**, in total lysate the ratio was 1.45 ± 0.24 whereas in isolated mitochondria 0.08 ± 0.02 . This procedure yielded 18-fold enrichment of mitochondrial fraction compare to total homogenate, indicating a good purification of mitochondria (**Fig. S8**).

Preparation of detergent-soluble and -insoluble fractions.

Twenty-four h after GFP-LC3B transfection, 0 and 80% A549-B2 cells were treated as described.⁹⁰ Briefly, cells were scraped on ice and dissolved in ice-cold lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.5% Triton X-100 (Sigma-Aldrich, T9284), protease inhibitor cocktail (Sigma-Aldrich, P8340)); then were centrifuged at 500 g for 10 min at 4°C to separate supernatants (fractions soluble in 0.5 Triton X-100) and pellets. Pellets were resuspended in 1% SDS in PBS Triton X-100 as insoluble fractions. The fractions were analyzed by WB.

Molecular analysis

Quantitative Real Time PCR (RT-PCR). Total RNA was isolated from cybrids using TRIzol Reagent (Life Technologies-ThermoFisher Scientific, 15596026). First-Strand cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems-ThermoFisher Scientific, 43688814) and transcript levels were quantified with SYBR Green Real-Time PCR (Life Technologies-ThermoFisher Scientific, 11733-046) using the

ABI PRISM 7000 sequence detection system (Applied Biosystem-ThermoFisher Scientific, Foster City, CA, USA).

List of primer sequences: *B2M*: F 5'-ATGAGTATGCCTGCCGTGTGA-3', R 5'-GGCATCTTCAAACCTCCA-3', *BNIP3*: F 5'-GCATGAGTCTGGACGGAGTA-3', R 5'-GTTTCAGAAGCCCTGTTGGT-3'; *PARK2*: F 5'-AAAGGCCCTGTCAAAGAGT-3', R 5'-ATCATCCCAGCAAGATGGAC-3'; *PINK1*: F 5'-CCAAGTTTGTGTGACCGGC-3', R 5'-CTTCATAACGAGGAACAGCGTCC-3'; *RPLP0*: F 5'-GTGATGTGCAGCTGATCAAGACT-3', R 5'-GATGACCAGCCCAAAGGAGA-3'.

Down regulation of PINK1 and PARK2. siRNA targeting *PINK1* or *PARK2* were purchased from Sigma Aldrich 000010; 000070, scramble (GTCTCCACGCGCAGTACCATTT). Cybrids from adenocarcinoma or rhabdomyosarcoma were plated in 6-well plates and transfected with 75 pmol of each siRNA oligo using Lipofectamine 2000 (Life Technologies-ThermoFisher Scientific, 11668-027). At 24 h or 48 h post-transfection, cells were collected and lysed as described in the 'Biochemical analysis' section.

mtDNA removal. WT or heteroplasmic A549.B2 and RD.Myo cybrids were treated with or without 50 ng/ml EtBr (Sigma-Aldrich, E8751) and with or without 50 μ M CQ. After 22 h, cells were collected and the DNA was extracted, as described previously.²¹ In each sample the mtDNA copy number was quantified by SYBR Green (Life Technologies-ThermoFisher Scientific, 11733-046), quantitative Real Time PCR, using the ABI PRISM 7000 sequence detection system (Applied Biosystem-ThermoFisher Scientific, Foster City, CA, USA). The mtDNA copy number was related to the *AR* (*androgen receptor*) gene. The following primers were used: *AR*: F 5'-CAACTCCTTCAGCAACAGCA-3', R 5'-GGACACCGACACTGCCTTAC-3'; *MT-CO2*: F 5'-CGTCTGAACTATCCTGCCCG-3', R 5'-TGGTAAGGGAGGGATCGTTG-3'.

Mitochondrial genotyping by last-cycle-hot PCR. The amount of heteroplasmy for the m3243G mutation was measured in DNA samples extracted from cells as described

previously.²¹ Briefly, the regions around the mutation site in the *MT-TL1* gene, were PCR amplified using mtDNA-specific primer pairs: F 5'- GTTCGTTTGTTC AACGATT -3'; R 5'- GGTAAGATTACCGTTACCG -3', with addition of [α -³³P]dCTP (Hartmann Analytic, SRF205/09.25) in the last synthesis cycle. Labeled PCR products were then digested with the restriction enzyme, diagnostic for the site polymorphisms created by the mutations (*ApaI*) (New England Biolabs, R0114S), and products of replicate digestions were separated by polyacrylamide gel electrophoresis and phosphoimages were analyzed with Gel-Pro Analyzer software.

Biochemical analysis

Western blotting analysis. Total cell lysates were prepared by maintaining the cells on ice for 30 min with 200 μ l of RIPA buffer (65 mM Tris, 150 mM NaCl, 1% Nonidet P-40/IGEPAL CA-630 (Sigma-Aldrich, I3021), 0.25% Na-DOC (Sigma-Aldrich, D6750), 1 mM EDTA, pH 7.4) and 7 μ l of a cocktail of protease inhibitors (Sigma-Aldrich, P8340)). Mitochondria were extracted as described previously.⁸⁹ Total protein levels were determined using the BCA protein assay kit (ThermoFisher Scientific, 23227). After centrifugation at 14,000 g_{max} for 15 min at 4°C, an equal amount of protein (30 μ g) 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 (Sigma-Aldrich, P1379) for 1 h at room temperature. The membrane was incubated overnight at 4°C in primary antibodies to: anti-LC3B monoclonal (Sigma-Aldrich, L7543; 1:2000); anti-SQSTM1 monoclonal (Sigma-Aldrich, 041M4812; 1:5000); anti-PINK1 monoclonal (Cell Signaling Technology, D8G3; 1:1000); anti-PARK2 monoclonal (Santa Cruz Biotechnology, sc-32282; 1:500); anti-BNIP3 polyclonal (Sigma-Aldrich, B7931; 1:1000), anti-DNM1L monoclonal (BD Biosciences, 611112; 1:1000); anti-p-DNM1L S637 polyclonal (Cell Signaling Technology, 4867S; 1:1000); anti-MFN1 monoclonal

(Merck-Millipore, ABC41; 1:1000); anti-MFN2 monoclonal (Abnova, H00009927-M03; 1:1000); anti-OPA1 monoclonal (BD Biosciences, 612606; 1:1000); anti-FUNDC1 polyclonal (Santa Cruz Biotechnology, sc-133597; 1:1000); anti-BCL2L13 monoclonal (Santa Cruz Biotechnology, sc-390598; 1:1000); anti-ACTB monoclonal (Chemicon International-Millipore, MAB1501; 1:4000); anti-TOMM20 polyclonal (Santa Cruz Biotechnology, sc-11415; 1:1000); anti-ATP5J/ATP5A monoclonal (Abcam, ab110273; 1:10000); anti-GAPDH monoclonal (Mark-Millipore, MAB374; 1:5000); anti-ubiquitin monoclonal (Mark-Millipore, MAB1510; 1:1000). Bound secondary peroxidase conjugated antibodies: anti-mouse (GE Healthcare, NA931B); anti-rabbit (GE Healthcare, NA934B) were visualized using an ECL reagent (GE Healthcare, RPN2106). Densitometric analysis of WB signal was performed using Gel-Pro Analyzer 3 software.

Citrate synthase activity was assayed by incubating cell samples with 0.02% Triton X-100, and monitoring the reaction by measuring the rate of free coenzyme A release spectrophotometrically according to Sgarbi et al.⁹¹

Mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\psi_m$) changes were evaluated by measuring rhodamine 123 fluorescence quenching^{91, 92} under the following conditions: 2×10^6 cells were added to 0.5 ml buffer (250 mM sucrose, 10 mM HEPES, 100 AM K-EGTA, 2 mM $MgCl_2$, 4 mM KH_2PO_4 , pH 7.4) containing an ADP regenerating system (10 mM glucose and 2.5 U Hexokinase (Sigma-Aldrich, H5000)) and permeabilized with 20 μ g/ml of digitonin (Sigma-Aldrich, D5628). Before rhodamine 123 (Molecular Probes ThermoFisher Scientific, R302) (50 nM) addition, samples were incubated with 1.8 mM malonate (Sigma-Aldrich, M4795), and 0.1 mM ADP (Sigma-Aldrich, A6646). Finally, mitochondria were energized by 10 mM:10 mM glutamate (Sigma-Aldrich, G1251):malate (Sigma-Aldrich, 7554-12-3) in the presence or absence of oligomycin (Sigma-Aldrich, 75351) (0.2 μ M) to detect

membrane potential changes associated with state 4 and state 3 respiratory conditions, respectively.

Mitochondrial ATP synthesis rate and cellular ATP content assay. The oligomycin-sensitive ATP synthase activity in permeabilized cells was determined according as described.^{91, 92} Essentially, cybrids (2×10^6 cells/ml) were incubated for 15 min with 60 mg/ml digitonin in a Tris-HCl buffer, pH 7.4. Complex I-driven ATP synthesis was induced by adding 10 mM:10 mM glutamate:malate (+ 0.6 mM malonate (Sigma-Aldrich, M4795)) and 0.5 mM ADP (Sigma-Aldrich, A6646) to the sample. 2 to 3 min later the reaction was stopped by adding dimethylsulphoxide (Sigma-Aldrich, D4540). Newly synthesized ATP and intracellular ATP were measured by bioluminescence using a luciferin–luciferase system (ATP bioluminescent assay kit CLS II; Roche, 11699695001) according to the manufacturer's instructions. The amount of ATP measured was referred to sample cell number.

Flow cytometry analysis.

Mitochondrial depolarization was evaluated by following the loss of TMRM (tetramethylrhodamine methyl ester; Molecular Probes Thermofisher Scientific, T668) staining in a nonquenching mode,⁵⁸ by using FACS analyser (FACS Canto Analyser, Becton-Dickinson, Franklin Lakes, NJ, USA), as described.⁹³ Cells were preincubated with 20 nM TMRM and 1.6 μ M cyclosporine H (Enzo Life Sciences, ALX-380-286), to inhibit the nonspecific cell extrusion of the probe by P-glycoprotein, for 30 min. Cells were washed with PBS, trypsinized, centrifuged and resuspended in 300 μ l of PBS. After first acquisition at $t = 0$, 1.6 μ M oligomycin was added and loss of TMRM fluorescence was monitored over time at $t = 10, 20, 30, 45, 60$ and 90 min. Physical parameters were used to gate the singly dispersed cells.

Imaging.

A549.B2 and RD.Myo cybrids harboring 0%, 70% and 80% m3243G mutant mtDNA were: i) transiently transfected with mtRFP⁹⁴ and GFP-LC3B⁹⁵ using Lipofectamine 2000. 24 h after transfection, the cells were fixed in 4% formaldehyde (Carlo Erba Reagents, 415661) and observed with a confocal microscope (LEICA TCS SP5, Wetzlar, Germany); ii) untransfected and analyze with anti-LC3B and anti-ATP5J/ATP5A antibodies. For immunohistochemical analysis, the cells were fixed in 4% formaldehyde, treated with 0.5% Triton X-100 for 5 min and with blocking solution (10% FBS in PBS) for 30 min, then were sequentially incubated at 4°C in separate overnight sections with primary polyclonal anti-LC3B (Cell Signaling Technology, 2775; 1:200) and monoclonal ATP5J/ATP5A (Abcam, ab110273, 1:200) in 10% FBS+PBS. The appropriate secondary fluorescent antibody (Alexa Fluor 488 (Invitrogen ThermoFisher Scientific, A11008) for anti-LC3 and Alexa Fluor 647, (Invitrogen ThermoFisher Scientific, A21235) for anti-ATP5J/ATP5A) was used for 1 h at room temperature at the end of the respective section. In the z-axis stacks acquired, each image was separated by 0.5 µm along the z-axis. Using the Fiji program, the number of objects, in voxel, was analyzed in each green and red slide of the Z-stack. The autophagosome is a globular organelle with a diameter of approximately 1 µm. Only the voxels with that dimension or greater were considered.⁹⁶ Colocalization: once obtained all the voxels with the right dimension, the colocalization was analyzed using the following formula:

$\sqrt{(x1 - x2)^2 + (y1 - y2)^2 + (z1 - z2)^2}$. A red-green colocalized voxel was considered only when the green voxel was bigger than the red one.

Statistical analysis.

Data were expressed as mean \pm SD or mean \pm SE. Statistical analysis of group differences was examined by using the Student t test. Values of $P < 0.05$ were considered significant.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

There were no potential conflicts of interest to be disclosed

REFERENCES

1. Benard G, Karbowski M. Mitochondrial fusion and division: Regulation and role in cell viability. *Seminars in cell & developmental biology* 2009; 20:365-74.
2. Baker BM, Haynes CM. Mitochondrial protein quality control during biogenesis and aging. *Trends in biochemical sciences* 2011; 36:254-61.
3. Ni HM, Williams JA, Ding WX. Mitochondrial dynamics and mitochondrial quality control. *Redox biology* 2015; 4:6-13.
4. Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal* 2008; 27:433-46.
5. Novak I. Mitophagy: a complex mechanism of mitochondrial removal. *Antioxidants & redox signaling* 2012; 17:794-802.
6. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nature reviews Molecular cell biology* 2011; 12:9-14.
7. Chan NC, Salazar AM, Pham AH, Sweredoski MJ, Kolawa NJ, Graham RL, et al. Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Human molecular genetics* 2011; 20:1726-37.
8. Okatsu K, Saisho K, Shimanuki M, Nakada K, Shitara H, Sou YS, et al. p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria. *Genes to cells : devoted to molecular & cellular mechanisms* 2010; 15:887-900.
9. Wong YC, Holzbaur EL. Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation. *Proceedings of the National Academy of Sciences of the United States of America* 2014; 111:E4439-48.
10. Johansen T, Lamark. Selective autophagy mediated by autophagic adapter proteins. *Autophagy* 2011, 7:279-96
11. Mouli PK, Twig G, Shirihai OS. Frequency and selectivity of mitochondrial fusion are key to its quality maintenance function. *Biophysical journal* 2009; 96:3509-18.
12. DiMauro S, Schon EA, Carelli V, Hirano M. The clinical maze of mitochondrial neurology. *Nature reviews Neurology* 2013; 9:429-44.
13. Goto Y, Nonaka I, Horai S. A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature* 1990; 348:651-3.
14. El-Hattab AW, Adesina AM, Jones J, Scaglia F. MELAS syndrome: Clinical manifestations, pathogenesis, and treatment options. *Molecular genetics and metabolism* 2015; 116:2-12.
15. Attardi G, Yoneda M, Chomyn A. Complementation and segregation behavior of disease-causing mitochondrial DNA mutations in cellular model systems. *Biochimica et biophysica acta* 1995; 1271:241-8.
16. Jeppesen TD, Schwartz M, Olsen DB, Vissing J. Oxidative capacity correlates with muscle mutation load in mitochondrial myopathy. *Annals of neurology* 2003; 54:86-92.
17. Chinnery PF, Zwijnenburg PJ, Walker M, Howell N, Taylor RW, Lightowlers RN, et al. Nonrandom tissue distribution of mutant mtDNA. *American journal of medical genetics* 1999; 85:498-501.
18. Frederiksen AL, Andersen PH, Kyvik KO, Jeppesen TD, Vissing J, Schwartz M. Tissue specific distribution of the 3243A->G mtDNA mutation. *Journal of medical genetics* 2006; 43:671-7.
19. Samuels DC, Li C, Li B, Song Z, Torstenson E, Boyd Clay H, et al. Recurrent tissue-specific mtDNA mutations are common in humans. *PLoS genetics* 2013; 9:e1003929.
20. Naue J, Horer S, Sanger T, Strobl C, Hatzler-Grubwieser P, Parson W, et al. Evidence for frequent and tissue-specific sequence heteroplasmy in human mitochondrial DNA. *Mitochondrion* 2015; 20:82-94.

21. Malena A, Loro E, Di Re M, Holt IJ, Vergani L. Inhibition of mitochondrial fission favours mutant over wild-type mitochondrial DNA. *Human molecular genetics* 2009; 18:3407-16.
22. Vergani L, Malena A, Sabatelli P, Loro E, Cavallini L, Magalhaes P, et al. Cultured muscle cells display defects of mitochondrial myopathy ameliorated by anti-oxidants. *Brain : a journal of neurology* 2007; 130:2715-24.
23. Dunbar DR, Moonie PA, Jacobs HT, Holt IJ. Different cellular backgrounds confer a marked advantage to either mutant or wild-type mitochondrial genomes. *Proceedings of the National Academy of Sciences of the United States of America* 1995; 92:6562-6.
24. Holt IJ, Dunbar DR, Jacobs HT. Behaviour of a population of partially duplicated mitochondrial DNA molecules in cell culture: segregation, maintenance and recombination dependent upon nuclear background. *Human molecular genetics* 1997; 6:1251-60.
25. Chinnery PF. Modulating heteroplasmy. *Trends in genetics : TIG* 2002; 18:173-6.
26. Hill BG, Benavides GA, Lancaster JR, Jr., Ballinger S, Dell'Italia L, Jianhua Z, et al. Integration of cellular bioenergetics with mitochondrial quality control and autophagy. *Biological chemistry* 2012; 393:1485-512.
27. Sgarbi G, Matarrese P, Pinti M, Lanzarini C, Ascione B, Gibellini L, et al. Mitochondria hyperfusion and elevated autophagic activity are key mechanisms for cellular bioenergetic preservation in centenarians. *Aging* 2014; 6:296-310.
28. Baracca A, Sgarbi G, Padula A, Solaini G. Glucose plays a main role in human fibroblasts adaptation to hypoxia. *The international journal of biochemistry & cell biology* 2013; 45:1356-65.
29. Mathupala SP, Ko YH, Pedersen PL. Hexokinase-2 bound to mitochondria: cancer's stygian link to the "Warburg Effect" and a pivotal target for effective therapy. *Seminars in cancer biology* 2009; 19:17-24.
30. Guha M, Srinivasan S, Biswas G, Avadhani NG. Activation of a novel calcineurin-mediated insulin-like growth factor-1 receptor pathway, altered metabolism, and tumor cell invasion in cells subjected to mitochondrial respiratory stress. *The Journal of biological chemistry* 2007; 282:14536-46.
31. Zu XL, Guppy M. Cancer metabolism: facts, fantasy, and fiction. *Biochemical and biophysical research communications* 2004; 313:459-65.
32. O'Riordan TC, Zhdanov AV, Ponomarev GV, Papkovsky DB. Analysis of intracellular oxygen and metabolic responses of mammalian cells by time-resolved fluorometry. *Analytical chemistry* 2007; 79:9414-9.
33. D'Erchia AM, Atlante A, Gadaleta G, Pavesi G, Chiara M, De Virgilio C, et al. Tissue-specific mtDNA abundance from exome data and its correlation with mitochondrial transcription, mass and respiratory activity. *Mitochondrion* 2015; 20:13-21.
34. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* 2011; 147:728-41.
35. Wang K, Klionsky DJ. Mitochondria removal by autophagy. *Autophagy* 2011; 7:297-300.
36. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo-Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2016; 12: 1-222.
37. Springer w, Kahle PJ. Regulation of PINK1-Parkin-mediated mitophagy. *Autophagy* 2011; 7: 266-78.
38. Jin SM, Youle RJ. PINK1- and Parkin-mediated mitophagy at a glance. *Journal of cell science* 2012; 125:795-9.
39. Becker D, Richter J, Tocilescu MA, Przedborski S, Voos W. Pink1 kinase and its membrane potential ($\Delta\psi$)-dependent cleavage product both localize to outer

mitochondrial membrane by unique targeting mode. *The Journal of biological chemistry* 2012; 287:22969-87.

40. Wei H, Liu L, Chen Q. Selective removal of mitochondria via mitophagy: distinct pathways for different mitochondrial stresses. *Biochimica et biophysica acta* 2015; 1853:2784-90.

41. Murakawa T, Yamaguchi O, Hashimoto A, Hikoso S, Takeda T, Oka T, et al. Bcl-2-like protein 13 is a mammalian Atg32 homologue that mediates mitophagy and mitochondrial fragmentation. *Nature communications* 2015; 6:7527.

42. Pontarin G, Ferraro P, Bee L, Reichard P, Bianchi V. Mammalian ribonucleotide reductase subunit p53R2 is required for mitochondrial DNA replication and DNA repair in quiescent cells. *Proceedings of the National Academy of Sciences of the United States of America* 2012; 109:13302-7.

43. Desjardins P, Frost E, Morais R. Ethidium bromide-induced loss of mitochondrial DNA from primary chicken embryo fibroblasts. *Molecular and cellular biology* 1985; 5:1163-9.

44. King MP, Attardi G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science (New York, NY)* 1989; 246:500-3.

45. Vergani L, Prescott AR, Holt IJ. Rhabdomyosarcoma rho(0) cells: isolation and characterization of a mitochondrial DNA depleted cell line with 'muscle-like' properties. *Neuromuscular disorders : NMD* 2000; 10:454-9.

46. Palmer CS, Osellame LD, Stojanovski D, Ryan MT. The regulation of mitochondrial morphology: intricate mechanisms and dynamic machinery. *Cellular signalling* 2011; 23:1534-45.

47. Detmer SA, Chan DC. Functions and dysfunctions of mitochondrial dynamics. *Nature reviews Molecular cell biology* 2007; 8:870-9.

48. Meeusen S, DeVay R, Block J, Cassidy-Stone A, Wayson S, McCaffery JM, et al. Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. *Cell* 2006; 127:383-95.

49. Song Z, Ghochani M, McCaffery JM, Frey TG, Chan DC. Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. *Molecular biology of the cell* 2009; 20:3525-32.

50. Loson OC, Song Z, Chen H, Chan DC. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. *Molecular biology of the cell* 2013; 24:659-67.

51. Galloway CA, Lee H, Yoon Y. Mitochondrial morphology-emerging role in bioenergetics. *Free radical biology & medicine* 2012; 53:2218-28.

52. Cereghetti GM, Stangherlin A, Martins de Brito O, Chang CR, Blackstone C, Bernardi P, et al. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. *Proceedings of the National Academy of Sciences of the United States of America* 2008; 105:15803-8.

53. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS biology* 2010; 8:e1000298.

54. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *The Journal of cell biology* 2010; 191:933-42.

55. Irwin WA, Bergamin N, Sabatelli P, Reggiani C, Megighian A, Merlini L, et al. Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. *Nature genetics* 2003; 35:367-71.

56. Solaini G, Sgarbi G, Lenaz G, Baracca A. Evaluating mitochondrial membrane potential in cells. *Bioscience reports* 2007; 27:11-21.

57. Nicholls DG, Ward MW. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends in neurosciences* 2000; 23:166-74.

58. Solaini G, Harris DA, Lenaz G, Sgarbi G, Baracca A. The study of the pathogenic mechanism of mitochondrial diseases provides information on basic bioenergetics. *Biochimica et biophysica acta* 2008; 1777:941-5.
59. Sunada Y, Saito F, Matsumura K, Shimizu T. Differential expression of the parkin gene in the human brain and peripheral leukocytes. *Neuroscience letters* 1998; 254:180-2.
60. Wani WY, Boyer-Guittaut M, Dodson M, Chatham J, Darley-Usmar V, Zhang J. Regulation of autophagy by protein post-translational modification. *Laboratory investigation; a journal of technical methods and pathology* 2015; 95:14-25.
61. Mai S, Klinkenberg M, Auburger G, Bereiter-Hahn J, Jendrach M. Decreased expression of Drp1 and Fis1 mediates mitochondrial elongation in senescent cells and enhances resistance to oxidative stress through PINK1. *Journal of cell science* 2010; 123:917-26.
62. Suen DF, Narendra DP, Tanaka A, Manfredi G, Youle RJ. Parkin overexpression selects against a deleterious mtDNA mutation in heteroplasmic cybrid cells. *Proceedings of the National Academy of Sciences of the United States of America* 2010; 107:11835-40.
63. Dai Y, Zheng K, Clark J, Swerdlow RH, Pulst SM, Sutton JP, et al. Rapamycin drives selection against a pathogenic heteroplasmic mitochondrial DNA mutation. *Human molecular genetics* 2014; 23:637-47.
64. Hamalainen RH, Manninen T, Koivumaki H, Kislin M, Otonkoski T, Suomalainen A. Tissue- and cell-type-specific manifestations of heteroplasmic mtDNA 3243A>G mutation in human induced pluripotent stem cell-derived disease model. *Proceedings of the National Academy of Sciences of the United States of America* 2013; 110:E3622-30.
65. Valenci I, Yonai L, Bar-Yaacov D, Mishmar D, Ben-Zvi A. Parkin modulates heteroplasmy of truncated mtDNA in *Caenorhabditis elegans*. *Mitochondrion* 2015; 20:64-70.
66. Gilkerson RW, De Vries RL, Lebot P, Wikstrom JD, Torgyeke E, Shirihai OS, et al. Mitochondrial autophagy in cells with mtDNA mutations results from synergistic loss of transmembrane potential and mTORC1 inhibition. *Human molecular genetics* 2012; 21:978-90.
67. Moran M, Delmiro A, Blazquez A, Ugalde C, Arenas J, Martin MA. Bulk autophagy, but not mitophagy, is increased in cellular model of mitochondrial disease. *Biochimica et biophysica acta* 2014; 1842:1059-70.
68. Charan RA, LaVoie MJ. Pathologic and therapeutic implications for the cell biology of parkin. *Molecular and cellular neurosciences* 2015; 66:62-71.
69. Sauvanet C, Duvezin-Caubet S, di Rago JP, Rojo M. Energetic requirements and bioenergetic modulation of mitochondrial morphology and dynamics. *Seminars in cell & developmental biology* 2010; 21:558-65.
70. Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, Saavedra E. Energy metabolism in tumor cells. *The FEBS journal* 2007; 274:1393-418.
71. Westermann B. Bioenergetic role of mitochondrial fusion and fission. *Biochimica et biophysica acta* 2012; 1817:1833-8.
72. Kageyama Y, Hoshijima M, Seo K, Bedja D, Sysa-Shah P, Andrabi SA, et al. Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. *The EMBO journal* 2014; 33:2798-813.
73. Nakada K, Sato A, Hayashi J. Mitochondrial functional complementation in mitochondrial DNA-based diseases. *The international journal of biochemistry & cell biology* 2009; 41:1907-13.
74. Chen H, Vermulst M, Wang YE, Chomyn A, Prolla TA, McCaffery JM, et al. Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell* 2010; 141:280-9.
75. Skulachev VP. Mitochondrial filaments and clusters as intracellular power-transmitting cables. *Trends in biochemical sciences* 2001; 26:23-9.

76. Hyvarinen AK, Pohjoismaki JL, Reyes A, Wanrooij S, Yasukawa T, Karhunen PJ, et al. The mitochondrial transcription termination factor mTERF modulates replication pausing in human mitochondrial DNA. *Nucleic acids research* 2007; 35:6458-74.
77. Shutt T, Geoffrion M, Milne R, McBride HM. The intracellular redox state is a core determinant of mitochondrial fusion. *EMBO reports* 2012; 13:909-15.
78. Mailloux RJ, Jin X, Willmore WG. Redox regulation of mitochondrial function with emphasis on cysteine oxidation reactions. *Redox biology* 2014; 2:123-39.
79. Norton M, Ng AC, Baird S, Dumoulin A, Shutt T, Mah N, et al. ROMO1 is an essential redox-dependent regulator of mitochondrial dynamics. *Science signaling* 2014; 7:ra10.
80. Willems PH, Rossignol R, Dieteren CE, Murphy MP, Koopman WJ. Redox Homeostasis and Mitochondrial Dynamics. *Cell metabolism* 2015; 22:207-18.
81. Vergani L, Floreani M, Russell A, Ceccon M, Napoli E, Cabrelle A, et al. Antioxidant defences and homeostasis of reactive oxygen species in different human mitochondrial DNA-depleted cell lines. *European journal of biochemistry / FEBS* 2004; 271:3646-56.
82. Kuznetsov AV, Hermann M, Saks V, Hengster P, Margreiter R. The cell-type specificity of mitochondrial dynamics. *The international journal of biochemistry & cell biology* 2009; 41:1928-39.
83. Campesi I, Straface E, Occhioni S, Montella A, Franconi F. Protein oxidation seems to be linked to constitutive autophagy: a sex study. *Life sciences* 2013; 93:145-52.
84. Oliván S, Calvo AC, Manzano R, Zaragoza P, Osta R. Sex differences in constitutive autophagy. *BioMed research international* 2014; 2014:652817.
85. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *International journal of cancer Journal international du cancer* 1976; 17:62-70.
86. Tonin PN, Scrable H, Shimada H, Cavenee WK. Muscle-specific gene expression in rhabdomyosarcomas and stages of human fetal skeletal muscle development. *Cancer research* 1991; 51:5100-6.
87. Fan TW, Kucia M, Jankowski K, Higashi RM, Ratajczak J, Ratajczak MZ, et al. Rhabdomyosarcoma cells show an energy producing anabolic metabolic phenotype compared with primary myocytes. *Molecular cancer* 2008; 7:79.
88. Vergani L, Martinuzzi A, Carelli V, Cortelli P, Montagna P, Schievano G, et al. MtDNA mutations associated with Leber's hereditary optic neuropathy: studies on cytoplasmic hybrid (cybrid) cells. *Biochemical and biophysical research communications* 1995; 210:880-8.
89. Fernandez-Vizarra E, Ferrin G, Perez-Martos A, Fernandez-Silva P, Zeviani M, Enriquez JA. Isolation of mitochondria for biogenetical studies: An update. *Mitochondrion* 2010; 10:253-62.
90. Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, et al. Autophagy is required to maintain muscle mass. *Cell metabolism* 2009; 10:507-15.
91. Sgarbi G, Baracca A, Lenaz G, Valentino LM, Carelli V, Solaini G. Inefficient coupling between proton transport and ATP synthesis may be the pathogenic mechanism for NARP and Leigh syndrome resulting from the T8993G mutation in mtDNA. *The Biochemical journal* 2006; 395:493-500.
92. Baracca A, Sgarbi G, Solaini G, Lenaz G. Rhodamine 123 as a probe of mitochondrial membrane potential: evaluation of proton flux through F(0) during ATP synthesis. *Biochimica et biophysica acta* 2003; 1606:137-46.
93. Pantic B, Trevisan E, Citta A, Rigobello MP, Marin O, Bernardi P, et al. Myotonic dystrophy protein kinase (DMPK) prevents ROS-induced cell death by assembling a hexokinase II-Src complex on the mitochondrial surface. *Cell death & disease* 2013; 4:e858.
94. de Brito OM, Scorrano L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* 2008; 456:605-10.

95. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, et al. FoxO3 controls autophagy in skeletal muscle in vivo. *Cell metabolism* 2007; 6:458-71.
96. Komatsu M, Ichimura Y. Selective autophagy regulates various cellular functions. *Genes to cells : devoted to molecular & cellular mechanisms* 2010; 15:923-33.

LEGEND TO THE FIGURES

Figure 1. Bioenergetic parameters in A549.B2 and RD.Myo cells with different m3243G mutant mtDNA. **(A)** Mitochondrial ATP synthesis rate fuelled by glutamate-malate in permeabilized A549.B2 and RD.Myo cells. Values normalized for CS (citrate synthase) activity, expressed as mean \pm SD. **(B)** Mitochondrial mass express as CS activity. Values, normalized for number of cells, expressed as mean \pm SD. **(C)** ATP level in total cellular lysate from A549.B2 and RD.Myo cells. Values, normalized for number of cells, expressed as mean \pm SD. **(D and F)** HK2 protein level in total lysate and **(E and G)** in isolated mitochondria from A549.B2 and RD.Myo cells. In **F** HK2 blots at low (3 sec) and high (10 sec) exposure to visualize HK2 in A549.B2 cells. Values, normalized for ACTB (actin beta) (total lysate) and TOMM20 (translocase of outer mitochondrial membrane 20) (isolated mitochondria), expressed in a.u., (arbitrary units) as mean \pm SD. **(H and I)** Mitochondrial membrane potential in A549.B2 **(H)** and RD.Myo **(I)** cells in the presence of ADP (state 3) and in the absence of ADP (state 4), expressed as mean \pm SD of fluorescent quencing (F.Q). All the data are obtained from 3 different experiments. Significance by the Student t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. LC3-II and SQSTM1/p62 protein levels in total lysate and in isolated mitochondria of A549.B2 and RD.Myo cells with increased m3243G mutant mtDNA. LC3-II **(A)** and SQSTM1/p62 **(B)** were tested by WB analysis in total cellular lysate of A549.B2 **(C)** and RD.Myo **(D)** cells. In isolated mitochondria of the same cells mtLC3-II **(E)**, and mtSQSTM1/p62 **(F)** tested by WB analysis **(G)**. In mtLC3 blot **(G)** at low exposure (5 sec) only LC3-II band was present; an high exposure (60 sec) visualized both the LC3-I and LC3-II bands. Autophagic flux, expressed as ratio +/- chloroquine (CQ) (50 μ M) untreated and treated cells for 6 h. Values, normalized for ACTB (total lysate) and TOMM20 (isolated mitochondria), expressed as mean \pm SE. Data are representative of 3 or more independent

experiments. In A549.B2 correlation between percentage of mutant load and level of mtLC3-II (**H**), mtSQSTM1/p62 (**M**); in RD.Myo correlation between mutant load and level of mtLC3-II (**L**), mtSQSTM1/p62 (**N**). Significance by the Student t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. a.u., arbitrary units.

Figure 3. PINK, PARK2 and BNIP3 protein levels in isolated mitochondria of A549.B2 and RD.Myo cells with increased m3243G mutant mtDNA. mtPINK (**A and I**), mtPARK2 (**B and L**), and mtBNIP3 (**C and M**) were tested by WB analysis. Mitophagic flux, expressed as ratio +/- between CQ (50 μ M) untreated and treated cells for 6 h. Values, normalized for TOMM20, expressed as mean \pm SE. In A549.B2 correlation between mutant load and level of mtPINK1 (**D**), of mtPARK2 (**F**), of mtBNIP3 (**G**). In RD.Myo correlation between mutation and level of mtPINK1 (**E**), of mtBNIP3 (**H**). Gene expression profiles of housekeeping genes *RPLP0* and *B2M* (**N**); of *PINK1* (**O**); of *PARK2* (**P**) and of *BNIP3* (**Q**) in A549.B2 and RD.Myo cells, quantified by RT-PCR. Data expressed as mean \pm SE. In (**N**) the RNA level of *RPLP0* and *B2M* was quantified in 10^7 cells. Data are obtained from 3 or more independent experiments. Significance by the Student t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. a.u., arbitrary units.

Figure 4. MtDNA removal, mitophagy and mitochondrial morphology in A549.B2 and RD.Myo cells with increased m3243G mutant mtDNA. mtDNA copy number in 0% and heteroplasmic mutant A549.B2 (**A**) and RD.Myo (**B**) cells treated for 22 h with ethidium bromide (EtBr) in the presence or absence of CQ. The ratio of mtDNA values between +/- CQ (50 μ M) treated and untreated cells represented the mitophagic mtDNA removal. Data obtained from 3 independent experiments. Representative images of A549.B2 (**C**) and RD.Myo (**D**) cells with 0%, 70% and 80% mutant mtDNA transfected with GFP-LC3B (green) and mtRFP (red) target to mitochondria in the absence or presence of 50 μ M CQ. Zoom: magnification of white insert

area. Scale bar: 10 μm . **(E)** Quantification of mitophagy as LC3-mitochondrial colocalization (yellow), express as mean \pm SE of puncta : cell scored in over 50 cells from multiple field for each category. **(F)** Representative images of mitochondrial network in A549.B2 and RD.Myo cells, untreated with CQ. Mitochondria visualized in cells transfected with mtRFP and analyzed at confocal microscopy. Scale bar: 10 μm . **(G)** Percentage of cell population with fragmented, medium and elongated mitochondria in A549.B2 with 35%, 70% and 80% mutant mtDNA and in RD.Myo cells with 0%, 70% and 80% mutant mtDNA. Over 100 cells were scored for each category. Values expressed as the mean \pm SD. Significance by the Student t test : *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. a.u., arbitrary units.

Figure 5. DNM1L, MFN1, MFN2, and OPA1 protein levels in isolated mitochondria of A549.B2 and RD.Myo cells with increased m3243G mutant mtDNA. Mitochondrial DNM1L **(A, C)**, MFN1 **(E)**; MFN2 **(F)**, and OPA1 **(G)** tested by WB analysis **(H)**. Values from isolated mitochondria of cells untreated with CQ, normalized to TOMM20 and expressed as the mean \pm SE. Ratio of phospho DNM1L:total cytosolic DNM1L in total cell lysate **(B, D)**, expressed as the mean \pm SE. Data are obtained from 3 or more independent experiments. Significance by the Student t test : *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. a.u., arbitrary units.

Figure 6. Mitochondrial depolarization in response to oligomycin in A549.B2 and RD.Myo cells with increased m3243G mutant mtDNA. Cells were loaded with TMRM (20 nM) for 30 min at 37°C and were analyzed by flow cytometry after 90 min of oligomycin (1.6 μM) treatment. Percentage of depolarization of A549.B2 **(A)** and RD.Myo **(B)** cybrids. Total depolarization of all the cells by addition of the protonophore FCCP (5 μM). **(C)** Positive correlation between mutant load and depolarized cells in A549.B2 but not in RD.Myo cells. **(D)** Positive correlation between mutant load and ratio +/- CQ of percentage of depolarization

in A549.B2 but not in RD.Myo. Values expressed as the mean \pm SD. Data obtained from 3 independent experiments. Significance by the Student t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure S1. Mutant mtDNA load and growth rate in A549.B2 and RD.Myo cells. **(A and B)** DNA was harvested at intervals over a period of 12 to 96 months and the proportion of mutant mtDNA estimated by last-cycle hot-PCR (see Materials and Methods). During the current study the average level of mutant mtDNA varied from 70 to 73% in 70% mutant A549.B2 cells and from 78 to 82% in 80% mutant A549.B2 cells; from 78 to 79% and from 88 to 89% in 70% and 80% mutant RD.Myo cells respectively. **(C)** Growth rate of WT and heteroplasmic A549.B2 and RD.Myo cells expressed as duplication time in hours, as described in the Materials and Methods section.

Figure S2. PINK1, PARK2, BNIP3, LC3-II, and SQSTM1/p62 protein amount in isolated mitochondria from A549.B2 and RD.Myo cells. Representative WB analysis of the indicated proteins in isolated mitochondria from A549.B2 **(A)** and RD.Myo **(B)** cells in the presence or absence of CQ. mtLC3 blot was at low exposure and visualized only LC3-II band, as shown in **Fig 2G**. Data are representative of 3 or more independent experiments.

Figure S3. Positive correlation between percentage of mutation and autophagic flux of mitophagic proteins from isolated mitochondria of A549.B2. Mitophagic flux of mtLC3-II **(A)**, mtSQSTM1/p62 **(B)**, mtPINK1 **(C)**, mtPARK2 **(D)**, mtBNIP3 **(E)**, in A549.B2 isolated mitochondria positively correlates with mutant load. Autophagic flux is expressed as ratio of the values obtained in the presence and in the absence of CQ. The correlation is visualized as straight line and formula.

Figure S4. Knockdown of PINK1 and PARK2 protein level in A549.B2 and RD.Myo cybrids after *PINK1* and *PARK2* siRNA transfection. PINK1 (**A and C**) and PARK2 (**B and D**) protein levels were tested by WB analysis in total lysate of 80% mutant A549.B2 and 0% RD.Myo cells, transfected for 24 h with scramble or with vectors producing shRNAs specific for *PINK1* and *PARK2*. Total cellular lysates and isolated mitochondria (mito)(used as internal marker) were immunoblotted together and probed for PINK1, PARK2, ACTB and ATP5J (ATP synthase, H⁺ transporting, mitochondrial Fo complex subunit F6). (**C**) siRNA determined a reduction of PINK1 protein level, normalized for ACTB, at 21% and 47% in A549.B2 and RD.Myo, respectively. (**D**) Similarly the protein amount of PARK2 was reduced at 35% in A549.B2, whereas PARK2 bands at around 50 kDa were not detected in RD.Myo cells. The experiment confirmed the presence of PINK1 variants with differences in molecular mass in A549.B2 vs RD.Myo cells. PINK1 (**C**) and PARK2 (**D**) protein amount, expressed as percentage of the value in cells treated with scramble siRNA (100%). Data are representative of 3 independent experiments, expressed as mean \pm SE. a.u., arbitrary units.

Figure S5. BCL2L13 and FUNDC1 protein levels in isolated mitochondria of A549.B2 cells with increased m3243G mutant mtDNA. (**A**) Isolated mitochondria of A549.B2 cells, treated with or without CQ, were immunoblotted against BCL2L13, FUNDC1 and ATP5J as protein load. (**B and C**) Values, normalized for ATP5J, expressed as mean \pm SE. Significance by the Student t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of 2 independent experiments. a.u., arbitrary units.

Figure S6. Quantification of mitophagy as endogenous LC3-mitochondrial colocalization in A549.B2 and RD.Myo cells and absence of GFP-LC3 aggregates. (**A**) Representative images of

A549.B2 and RD.Myo cells, with 0% and with 80% mutant mtDNA, immunostained with anti-LC3B (green) and anti-ATP5J (red) in the presence of 50 μ M CQ. **(B)** Quantification of mitophagy as endogenous LC3-mitochondrial colocalization (yellow), expressed as mean \pm SE of puncta: cell scored in over 50 cells from multiple fields for each category. The colocalization analysis of endogenous LC3 confirmed the higher mitophagic flux present in A549.B2 and not in RD.Myo. Scale bar: 5 μ m. **(C)** Detergent-soluble (Supernatant) and -insoluble (Pellet) fractions of GFP-LC3B, transfected in 0% and 80% A549.B2 cells, were immunoblotted against ubiquitin, LC3B and ACTB, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as loading control, as described in the Materials and Method section. GFP-LC3B was present in the soluble fraction, together with GAPDH and ACTB, whereas, as expected, ubiquitinated protein aggregates were observed in the insoluble-pellet fraction. Significance by the Student t test: *** $P < 0.001$. Data are representative of 2 independent experiments.

Figure S7. Mitochondrial depolarization in response to oligomycin in A549.B2 and RD.Myo cells with increased m3243G mutant mtDNA. **(A)** Mitochondrial depolarization, expressed as percentage of TMRM-negative cells in A549.B2 and RD.Myo cells, in the absence (Basal) and in the presence of CQ and of the protonophore FCCP. **(B)** Representative flow cytometry dot plots of A549.B2 and RD.Myo cells. **(C)** Positive correlation between mutant load and depolarized cells was present in CQ-treated A549.B2 but not in RD.Myo cells. Significance by the Student t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of at least 3 independent experiments.

Figure S8. Purity of mitochondrial enrichment. **(A)** Mitochondrial enrichment evaluated as ratio of cytosolic GAPDH and mitochondrial TOMM20 markers in total lysate (TL) and mitochondrial fraction from A549.B2 and RD.Myo cells by WB analysis.⁸⁹ **(B)** Representative

WB of total lysate and mitochondrial fractions, expressed as CS activity, blotted against GAPDH and TOMM20. Data are representative of 3 independent experiments. a.u., arbitrary units.