

# Loss of OPA1 disturbs cellular calcium homeostasis and sensitizes for excitotoxicity

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Optic atrophy 1 (*OPA1*) mutations cause dominant optic atrophy (DOA) with retinal ganglion cell (RGC) and optic nerve degeneration. The mechanism for the selective degeneration of RGCs in DOA remains elusive. To address the mechanism, we reduced *OPA1* protein expression in cell lines and RGCs by RNA interference. *OPA1* loss results in mitochondrial fragmentation, deficiency in oxidative phosphorylation, decreased ATP levels, decreased mitochondrial  $\text{Ca}^{2+}$  retention capacity, reduced mtDNA copy numbers, and sensitization to apoptotic insults. We demonstrate profound cristae depletion and loss of crista junctions in *OPA1* knockdown cells, whereas the remaining crista junctions preserve their normal size. *OPA1*-depleted cells exhibit decreased agonist-evoked mitochondrial  $\text{Ca}^{2+}$  transients and corresponding reduction of  $\text{NAD}^+$  to NADH, but the impairment in NADH oxidation leads to an overall more reduced mitochondrial NADH pool. Although in our model *OPA1* loss in RGCs has no apparent impact on mitochondrial morphology, it decreases buffering of cytosolic  $\text{Ca}^{2+}$  and sensitizes RGCs to excitotoxic injury. Exposure to glutamate triggers delayed calcium deregulation (DCD), often in a reversible manner, indicating partial resistance of RGCs to this injury. However, when *OPA1* is depleted, DCD becomes irreversible. Thus, our data show that whereas *OPA1* is required for mitochondrial fusion, maintenance of crista morphology and oxidative phosphorylation, loss of *OPA1* also results in defective  $\text{Ca}^{2+}$  homeostasis.

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Mutations in optic atrophy 1 (*OPA1*), a dynamin-related large GTPase with mitochondrial fusion function, cause dominant optic atrophy (DOA), the most common hereditary form of optic neuropathy.<sup>1,2</sup> Progressive degeneration of retinal ganglion cells (RGCs) and the optic nerve, the hallmarks of DOA, are associated with several functional and structural alterations of mitochondria (reviewed in Lenaers *et al.*<sup>3</sup>). Whereas the *OPA1* mutation in DOA patients is present from birth in all cells, it remains unknown why only certain cell types, such as RGCs, degenerate<sup>4–8</sup> and what are the mechanisms leading to their loss.

In addition to its role in mitochondrial fusion, *OPA1* governs crista architecture. *OPA1* is anchored in the mitochondrial inner membrane and faces the intermembrane space.<sup>9,10</sup> Electron microscopy (EM) analyses indicate that *OPA1* loss causes abnormal mitochondrial ultrastructure with cristae disorganization.<sup>9–11</sup> Interaction of *OPA1* with membranes can induce a structural remodeling into tubules,<sup>12</sup> a feature characteristic of crista junctions,<sup>13–15</sup> supporting the proposal

that *OPA1* is a key component of these junctions.<sup>16–18</sup> Because crista junctions are normally uniform, a change in their size may indicate alteration in mitochondrial functional status. Yet, disagreements have arisen concerning crista junction remodeling in apoptosis.<sup>17–21</sup>

Emerging evidence suggests that abnormal  $\text{Ca}^{2+}$  homeostasis contributes to the pathogenesis in DOA.<sup>22,23</sup>  $\text{Ca}^{2+}$  enters mitochondria via the  $\text{Ca}^{2+}$  uniporter where it stimulates NADH and ATP production via activation of NADH-producing dehydrogenases including pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and NAD-linked isocitrate dehydrogenase.<sup>24</sup> During neurotransmission, ionotropic glutamate receptors, including the *N*-methyl-D-aspartate (NMDA) receptor, open and cytoplasmic  $\text{Ca}^{2+}$  accumulates in neurons and regulates downstream enzymes and signaling pathways.<sup>25</sup> However, if excessive glutamate is released into the extracellular space, for example, during stroke, epilepsy, or neuronal injury, neurons suffer excitotoxic damage. In cell culture models, a phenomenon known as delayed  $\text{Ca}^{2+}$

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**Abbreviations:** DOA, dominant optic atrophy; DCD, delayed  $\text{Ca}^{2+}$  deregulation; Drp1, dynamin-related protein 1;  $[\text{Ca}^{2+}]_c$ , cytoplasmic  $\text{Ca}^{2+}$  concentration;  $[\text{Ca}^{2+}]_m$ , mitochondrial  $\text{Ca}^{2+}$  concentration;  $\Delta\psi_m$ , mitochondrial membrane potential; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; MPT, mitochondrial permeability transition; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; OMM, outer mitochondrial membrane; RGC, retinal ganglion cell; siRNA, small interfering RNA; TPP<sup>+</sup>, tetraphenylphosphonium

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deregulation (DCD) occurs during acute glutamate exposure.<sup>26,27</sup> DCD is thought to be a consequence of mitochondrial  $\text{Ca}^{2+}$  overload-mediated injury,<sup>28</sup> and in most paradigms leads to neuronal cell death.<sup>29,30</sup> Remarkably, RGCs are known to be resistant to excitotoxicity in contrast to hippocampal neurons.<sup>31</sup>

To investigate the mechanism of RGC degeneration in DOA, OPA1 expression in cell lines and primary RGCs was decreased by RNAi, and ultrastructure, bioenergetics, and  $\text{Ca}^{2+}$  homeostasis of control and RNAi cells were compared. We demonstrate that in RGCs, decreased mitochondrial  $\text{Ca}^{2+}$  buffering and DCD occur in the absence of significant changes in mitochondrial morphology, suggesting a functional role of OPA1 in  $\text{Ca}^{2+}$  signaling.

## Results

**OPA1 loss results in mitochondrial fission and sensitization to apoptosis.** To explore the effects of OPA1 loss on cellular function we transfected cells with OPA1 small interfering RNA (siRNA). Because RGCs constitute only 1% of all cells in the retina and the yields are low after purification, we were forced to use cell lines (HeLa cells) for some experiments. Although control HeLa cells exhibited a typical fused mitochondrial network, ~80% of OPA1 siRNA cells showed dramatic mitochondrial fragmentation (Figure 1a). Effective OPA1 knockdown was confirmed by immunoblotting (Supplementary Figure 1).

Previous studies have reported that cells lacking OPA1 spontaneously release cytochrome *c* from mitochondria and undergo cell death.<sup>9,32</sup> However, subcellular fractionation with immunoblotting (Figure 1b) and confocal microscopy of OPA1 siRNA cells (Figures 1c and d) demonstrated that OPA1 knockdown did not result in spontaneous cytochrome *c* release from mitochondria. Measurements of cell death by three independent methods (Figures 1e–g and Supplementary Figure 2) did not reveal greater than baseline cell death in OPA1 siRNA cells. However, in agreement with an earlier study,<sup>32</sup> OPA1 siRNA cells died faster when challenged with staurosporine (STS), an apoptosis inducer, and exhibited a significantly higher caspase activity after STS treatment than STS-treated control cells (Figures 1e and f). Taken together, these data indicate that OPA1 loss *per se* does not cause cytochrome *c* release from mitochondria or cell death; instead, it conveys an increased susceptibility to apoptosis.

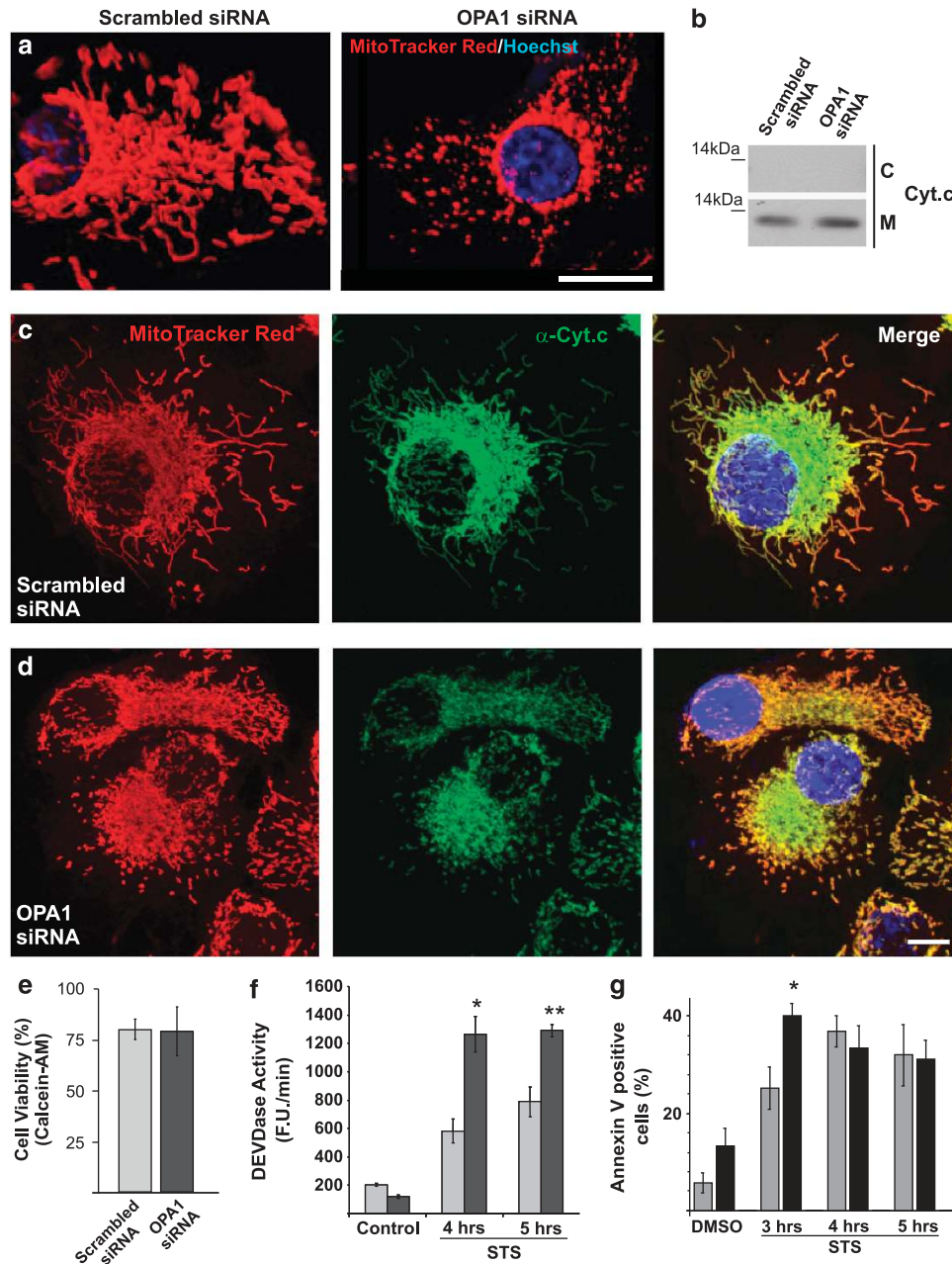
**OPA1 loss leads to cristae depletion.** Conventional 2D EM analyses demonstrate that OPA1 loss causes abnormal mitochondrial ultrastructure.<sup>9–11</sup> However, a detailed 3D picture and quantitative analysis of the mitochondrial ultrastructure in intact, mammalian OPA1-deficient cells are lacking. Only 3D reconstructions of isolated mitochondria from OPA1-null cells were published previously.<sup>17</sup> To gain better insights into the mitochondrial ultrastructure in OPA1 knockdown cells, we performed EM tomography and a high-resolution mitochondrial quantitative analysis. Through tomographic reconstructions from scrambled siRNA-transfected cells, 2-nm thick slices show elongated mitochondria and an intact outer mitochondrial membrane (OMM; Figures 2A and B; Supplementary Movie File 1). The tomographic view

of mitochondria in OPA1 siRNA cells was dramatically different from controls, not surprisingly showing many more round and small mitochondria. Figures 2A and Bc–f show a triplet of mitochondria from OPA1 siRNA cells whose OMMs are in close contact, suggesting recent fission before fixation. As with the control mitochondria, no signs of OMM breaks or matrix swelling were observed, consistent with our observation that cytochrome *c* remains localized within mitochondria and spontaneous cell death does not occur. Mitochondria of OPA1 siRNA cells displayed fewer and smaller cristae (Figures 2Bc–f), with some mitochondria even devoid of cristae (Figures 2Bd and f, and Supplementary Movie File 2). A few mitochondria had cristae oriented parallel to the long axis of the organelle, unusual for HeLa cells.

Additionally, in tomographic volumes, we quantified mitochondrial numbers per cubic micron and mitochondrial volume relative to cellular volume. Although there was no difference in the total mitochondrial volume, mitochondrial number increased twofold in OPA1 siRNA-transfected cells (Figures 2Ca and b). These results confirm that OPA1 loss promotes mitochondrial fission rather than a decrease in mitochondrial density. We also measured cristae surface area compared with that of the OMM surface area and evaluated cristae surface area relative to cell volume (Figures 2Cc and d). The total cristae membrane surface area of OPA1 siRNA-transfected cells was 25% less than that of controls (Figures 2Cc and d).

**OPA1 loss leads to mitochondrial structural heterogeneity, but not widening of crista junctions.** Because of the report of heterogeneity in mitochondrial membrane potential ( $\Delta\psi_m$ ) after OPA1 loss,<sup>33</sup> we investigated possible accompanying structural heterogeneity. Using conventional EM, we found that ~10% of mitochondria were considerably more condensed than the majority (Figures 3a–e). To clarify whether crista junction changes occurred in our OPA1 siRNA samples, we measured junctional sizes in tomographic volumes. In agreement with the previous report,<sup>17</sup> we found that the junctional size remains essentially unaltered with loss of OPA1 (Figures 3f–h;  $10.0 \pm 0.4$  nm for control and  $8.9 \pm 0.5$  nm for OPA1 siRNA crista junctions; mean  $\pm$  S.E.M.). However, the number of junctions was significantly reduced in OPA1 siRNA mitochondria (Figure 3h;  $54 \pm 4$  CJ/S.A. for control and  $21 \pm 6$  CJ/S.A. for OPA1 siRNA; mean  $\pm$  S.E.M.;  $P < .001$ ). Loss of crista junctions is consistent with cristae depletion (Figure 2); this phenomenon was also reported in a previous study using a TOM19-removal mechanism for cristae depletion.<sup>34</sup> Collectively our findings indicate that OPA1 loss results in cristae depletion without dilation of their junctions.

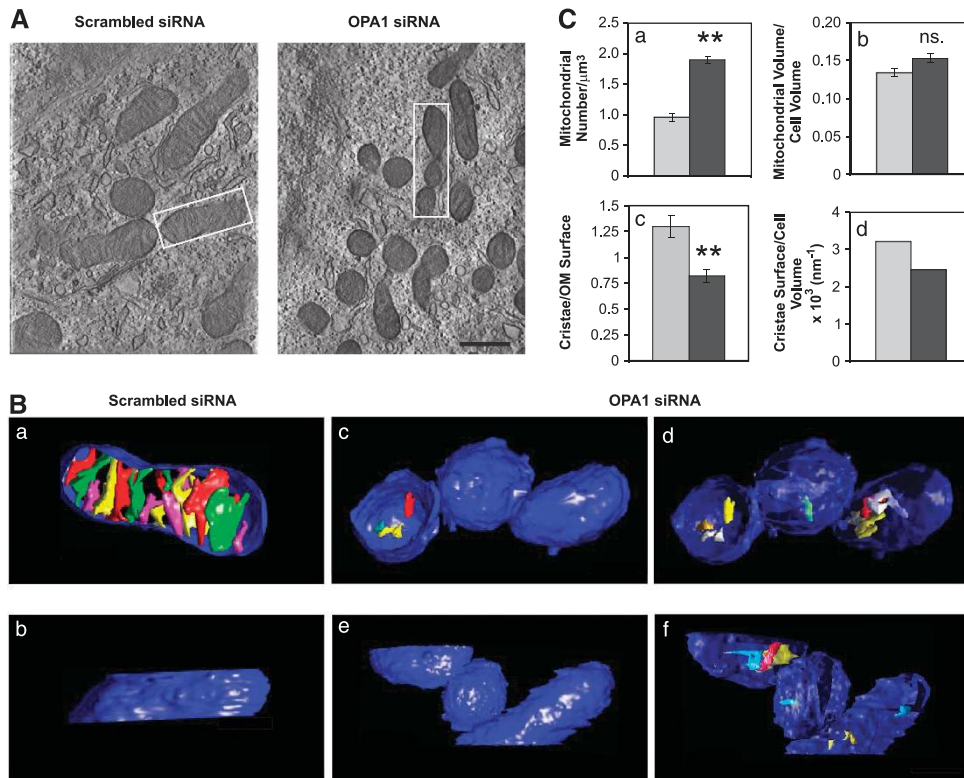
**OPA1 loss results in bioenergetic defects, mtDNA loss, and impaired mitochondrial  $\text{Ca}^{2+}$  buffering.** Because mitochondrial cristae are abnormal in OPA1 siRNA cells, we investigated the impact of these abnormalities on bioenergetics. Respiratory changes induced by OPA1 knockdown were evaluated in digitonin-permeabilized and intact cells. To measure respiration in digitonin-permeabilized cells, mitochondria were supplemented with Complex I-linked substrates, glutamate and malate. The rates of ADP-induced



**Figure 1** OPA1 loss results in mitochondrial fission without loss in cell viability. (a) Mitochondrial structure in scrambled (control) and OPA1 siRNA-transfected HeLa cells at 3 days after transfection. Mitochondria and nuclei were stained with MitoTracker Red and Hoechst 33342 (blue), respectively. Scale bar, 20  $\mu$ m. (b) Immunoblot of cytosolic (C) and mitochondrial fraction (M) of siRNA-transfected HeLa cells probed with anti-cytochrome *c* antibodies. (c and d) Immunocytochemistry and confocal microscopy of fixed control (c) and OPA1 siRNA-transfected (d) HeLa cells labeled with anti-cytochrome *c* antibodies (green) and MitoTracker Red (red). Hoechst 33342 counterstaining (blue) marks nuclei. (e) Cell viability of scrambled (gray bars) and OPA1 siRNA-transfected (black bars) HeLa cells measured using Calcein-AM 3 days after transfection. (f) DEVDase activity and (g) Annexin-V labeling of scrambled (gray bars) and OPA1 siRNA-transfected (black bars) HeLa cells treated with 1  $\mu$ M STS for 3–5 h. The rate of zDEVD-AMC cleavage is expressed in arbitrary fluorescence units per minute (FU/min). For (e–g), data are means  $\pm$  S.E.M. of three experiments (\* and \*\* denote  $P < 0.05$  and  $P < 0.01$  respectively)

(state 3) and maximal (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP)-induced or state 3u) respiration were reduced by  $\sim 20\%$  in OPA1 siRNA cells compared with controls (Figure 4A and Table 1). Comparable inhibition of respiration was observed in intact OPA1 siRNA cells (Table 1). Note that the oligomycin-inhibited (state 4o)

respiration, an indicator of proton leak through the inner mitochondrial membrane, remained unaltered in digitonin-permeabilized and intact OPA1 siRNA cells (Figure 4A). Thus, OPA1 loss does not induce proton leak and mitochondrial uncoupling, but rather causes partial respiratory inhibition.



**Figure 2** OPA1 loss leads to mitochondrial fission and cristae abnormalities. **(A)** Two 2-nm thick slices through EM tomographic volumes of mitochondria in fixed scrambled (control) and OPA1 siRNA-transfected HeLa cells *in situ*. Scale bar, 500 nm. White rectangle marks mitochondria chosen for volume segmentation and surface rendering. **(B)** Surface-rendered volumes of a segmented control mitochondrion and three segmented OPA1 siRNA mitochondria found in close proximity. The OMM is in blue and cristae are in various colors. (a) Top view of control mitochondrion after segmentation of the outer and cristae membranes into separate objects. (b) Side view of the OMM showing no ruptures. (c and d) Top view of a mitochondrial triplet in OPA1 siRNA-transfected HeLa cells. (e and f) View of same triplet from a side orientation. (d and f) Mitochondria are shown with translucent representation of the OMM to visualize the cristae. Scale bar, 200 nm. **(C)** Structural analysis of mitochondria by EM tomography. For mitochondrial number and volume,  $n$  (number of measured mitochondria) = 41 (scrambled siRNA, light gray bars) and 60 (OPA1 siRNA, black bars) (\*\* $P < 0.01$ ). For cristae surface area (per outer membrane surface area) measurements,  $n = 12$  (scrambled siRNA) and 23 (OPA1 siRNA) were used; the same samples were used to determine total cristae surface in the reconstituted volume per cell volume. Data are means  $\pm$  S.E.M. In (d), all of the cristae in the reconstructed volume were summed

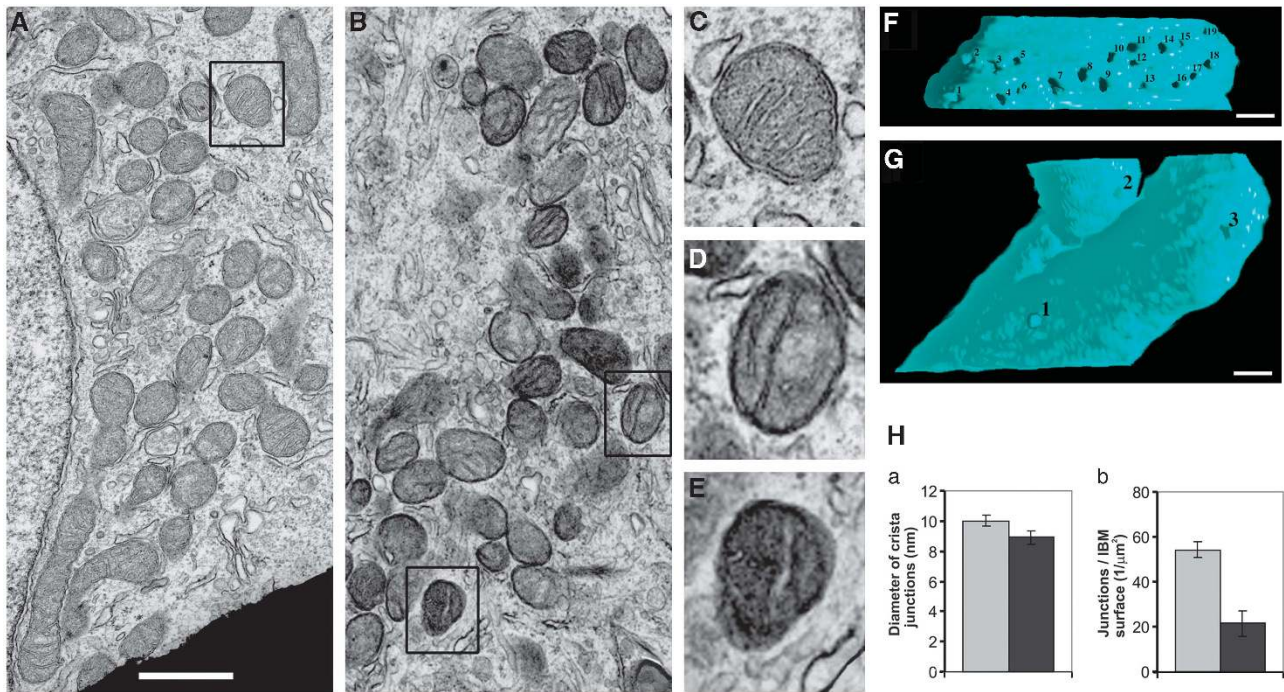
The maximal rate of respiration may be limited by the transport of substrates and/or activity of the tricarboxylic acid cycle, in addition to the activity of the electron transport chain. To directly test Complex I activity coupled with the activity of downstream complexes, we measured rotenone-sensitive NADH-dependent respiration in alamethicin-permeabilized mitochondria. Figure 4B illustrates that the activity of electron transport chain (Complexes I–III–IV) was reduced by  $39 \pm 4\%$  ( $n = 4$ ) in OPA1 siRNA cells. Consistent with the reduced respiratory capacity, OPA1 siRNA cells displayed lower ATP levels (Figure 4C).

Next, we determined the mitochondrial DNA copy number by quantitative PCR. The mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) ratio was significantly decreased in OPA1 siRNA cells (Figure 4D). Thus, mitochondrial crista depletion and the mtDNA copy number decrease may be the causes of partial respiratory inhibition resulting from OPA1 loss.

To dissect further the downstream mechanism of mitochondrial dysfunction in OPA1 siRNA cells, we assessed mitochondrial  $\text{Ca}^{2+}$  retention capacity using digitonin-permeabilized cells. Extramitochondrial  $\text{Ca}^{2+}$  concentration

(Figure 4Ea) was measured simultaneously with measurements of  $\Delta\psi_m$  (Figure 4Eb) and mitochondrial swelling (Figure 4Ec) in a multiparameter chamber. Control and OPA1 knockdown cells were repetitively challenged with small  $\text{Ca}^{2+}$  pulses. At baseline, OPA1 siRNA cells exhibited a  $\Delta\psi_m$  similar to scrambled siRNA cells (Figure 4Eb). Accordingly, the initial rates of  $\text{Ca}^{2+}$  uptake were similar. Repeated  $\text{Ca}^{2+}$  additions caused a gradual decline in  $\Delta\psi_m$  (Figure 4Eb) followed by abrupt depolarization, release of accumulated  $\text{Ca}^{2+}$  (Figure 4Ea), and mitochondrial swelling (Figure 4Ec), all indications of the mitochondrial permeability transition (MPT). Although  $\text{Ca}^{2+}$ -induced MPT occurred in both control and OPA1 siRNA cells, the threshold concentration of  $\text{Ca}^{2+}$  needed to trigger mitochondrial  $\text{Ca}^{2+}$  release was significantly lower in OPA1 siRNA cells (Figure 4E). An  $\sim 25\%$  decrease in  $\text{Ca}^{2+}$  retention capacity was observed (Figure 4F).

**OPA1 loss alters histamine-induced  $\text{Ca}^{2+}$  transients in intact HeLa cells.** Because mitochondrial  $\text{Ca}^{2+}$  retention capacity was reduced in digitonin-permeabilized OPA1 siRNA cells, we tested  $\text{Ca}^{2+}$  homeostasis *in situ* using live



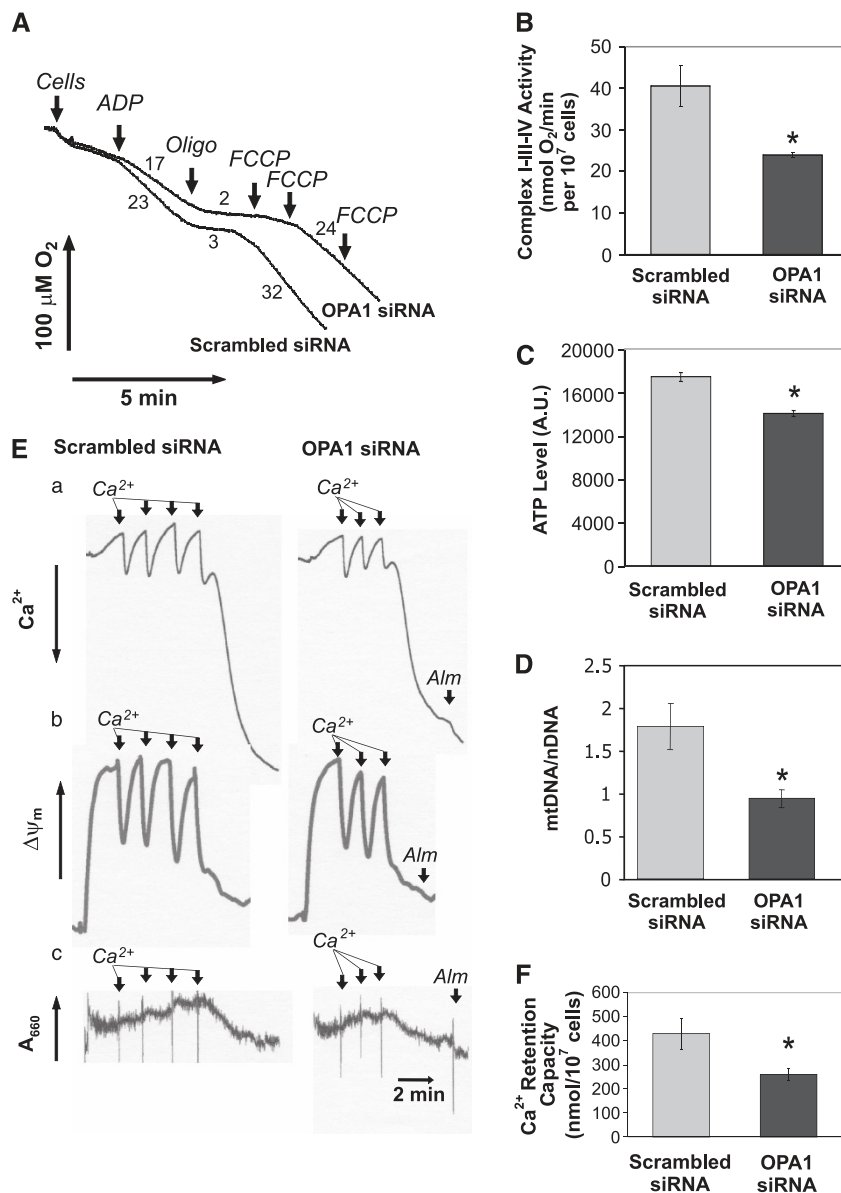
**Figure 3** OPA1 loss leads to mitochondrial structural heterogeneity and depletion of crista junctions, but not their dilation. (A) Electron micrograph showing a field inside a control HeLa cell densely populated with mitochondria having uniform structural features. Scale bar, 500 nm. (B) Electron micrograph showing a field inside an OPA1 siRNA cell densely populated with mitochondria displaying structural heterogeneity in the form of greater matrix condensation in a number of organelles. Same scale as (A). (C) Typical mitochondrial structure in control cells showing well-formed and abundant cristae with light matrix, enlarged from the boxed region in (A). (D) An example of the structure of the majority of mitochondria in OPA1 siRNA cells showing well-formed yet less abundant cristae than in the control, but still having a light matrix, enlarged from the upper boxed region in (C). (E) An example of the structure of the minority of mitochondria in OPA1 siRNA cells again showing less abundant cristae, but having a dark (condensed) matrix, enlarged from the lower boxed region in (B). (F) Inner mitochondrial membrane of the segmented tomographic volume from the scrambled siRNA sample and (G) from the OPA1 siRNA sample. By not displaying the outer membrane, the crista junction openings are easily seen (numbered). In all, 19 crista junctions are in view for the control volume, yet only 3 such junctions are in view for the OPA1 siRNA volume. Scale bar, 50 nm. (H) Crista junction measurements from the volumes. The junctional opening in OPA1 siRNA mitochondria remains roughly circular with a diameter essentially the same as the control junction (a). In all, 52 crista junctions were measured for control (gray bar) and 47 crista junctions for OPA1 siRNA (black bar) mitochondria. For 11 control mitochondria (gray bar) and 26 OPA1 siRNA mitochondria (black bar), the number of crista junctions per mitochondrion was measured and then normalized to each inner boundary membrane surface area (measured from the segmented volume) to determine the density of junctions (b). The crista junction density for the OPA1 siRNA mitochondria was less than half for the control mitochondria;  $P < 0.001$

cell and fluorescence time-lapse microscopy. Mitochondrial and cytosolic  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_m$  and  $[\text{Ca}^{2+}]_c$ , respectively) were assessed with the  $\text{Ca}^{2+}$ -sensitive fluorescent probe Rhod-2-AM using high-pass filtering of recorded fluorescence images.<sup>35</sup>

To elicit intracellular  $\text{Ca}^{2+}$  loads, cells were repeatedly challenged with short pulses of histamine, which induces inositol 1,4,5-triphosphate ( $\text{InsP}_3$ )-mediated release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (ER). We first assessed  $[\text{Ca}^{2+}]_m$  in control and OPA1 siRNA cells. The amplitude of the  $[\text{Ca}^{2+}]_m$  transient caused by the first histamine pulse was significantly decreased in OPA1 siRNA cells compared with controls (Figures 5Aa and b). However,  $[\text{Ca}^{2+}]_m$  transients in response to all subsequent histamine stimuli were not significantly different in either amplitude or decay kinetics between both cells (Figures 5Ab and c). In contrast, histamine-triggered  $[\text{Ca}^{2+}]_c$  transients were significantly larger in OPA1 siRNA cells than in controls. This difference was also more pronounced for the first histamine pulse (Figures 5Ba and b). Additionally, the decay rate of  $[\text{Ca}^{2+}]_c$  transients was significantly higher in OPA1 siRNA cells than in

controls (Figure 5Bb and c). In summary, these data support that OPA1 siRNA cells exhibit an impaired mitochondrial  $\text{Ca}^{2+}$  uptake and reduced buffering of cytosolic  $\text{Ca}^{2+}$  (Figure 4).

**OPA1 loss leads to augmented reduction of the NAD(H) pool.**  $\text{Ca}^{2+}$  activates NADH-producing dehydrogenases in the mitochondrial matrix, thereby increasing the NADH/NAD<sup>+</sup> ratio and rate of ATP synthesis.<sup>24</sup> NADH and NADPH exhibit indistinguishable autofluorescence, but under our experimental conditions, changes in NAD(P)H autofluorescence intensity are expected to reflect changes in the reduction state of the mitochondrial NAD(H) pool. This is because the pool size of NADP(H) is a fraction of the NAD(H) pool size, and is almost completely reduced under physiological conditions. To evaluate the  $\text{Ca}^{2+}$ -dependent bioenergetic status *in situ*, we measured the NAD(P)H autofluorescence in OPA1 siRNA cells.  $[\text{Ca}^{2+}]$  transients were evoked by exposure to repetitive histamine pulses. Each histamine application triggered a transient increase in fluorescence reflecting increased reduction of NAD<sup>+</sup> to NADH (Figure 6Aa). Consistent with the  $[\text{Ca}^{2+}]_m$  transients



**Figure 4** OPA1 loss leads to mitochondrial respiration inhibition, ATP drop, and decrease in mtDNA copy number, and compromises mitochondrial Ca<sup>2+</sup> retention capacity. **(A)** Mitochondrial respiration was measured in digitonin-permeabilized HeLa cells. Arrows indicate additions of cells ( $8 \times 10^6$ ), 200  $\mu$ M ADP, 1.25  $\mu$ g/ml oligomycin, and 100 nM FCCP. Oxygen consumption rates are expressed in nmol of O<sub>2</sub> per min per 10<sup>7</sup> cells. **(B)** Complex I–III–IV enzyme activity is expressed as mean  $\pm$  S.E.M. of four independent experiments. \*Significance at  $P < 0.01$  by Student's *t*-test. **(C)** ATP levels in scrambled and OPA1 siRNA-transfected HeLa cells expressed as arbitrary luminescence units (A.U.). Data were collected from replicate wells ( $n = 6$ ) and plotted as means  $\pm$  S.E.M.; \*Significance at  $P < 0.01$  by Student's *t*-test. Data shown are representative of eight experiments. **(D)** MitDNA to nDNA ratio of scrambled and OPA1 siRNA-transfected cells ( $n = 6$ ) measured by Q-PCR and plotted as mean  $\pm$  S.E.M. \*Significance at  $P < 0.01$  by Mann–Whitney test. **(E)** Mitochondrial Ca<sup>2+</sup> retention capacity was determined in digitonin-permeabilized HeLa cells. Sequential Ca<sup>2+</sup> additions cause release of accumulated Ca<sup>2+</sup> from mitochondria (a),  $\Delta\psi_m$  decline (b), and mitochondrial swelling (as indicated by a decrease in A<sub>660</sub>) (c), all attributable to MPT induction. Alamethicin (Alm) was added to induce maximal swelling. Arrows indicate timing of additions of 50  $\mu$ M Ca<sup>2+</sup> and 20  $\mu$ g/ml Alm. Data are representative of four experiments. **(F)** Mitochondrial Ca<sup>2+</sup> retention capacity in scrambled and OPA1 siRNA cells. Data are means  $\pm$  S.E.M. of four independent experiments. \*Significance at  $P < 0.05$  by Student's *t*-test

shown in Figure 5, the relative peak amplitude triggered by the first application of histamine was diminished in OPA1 siRNA compared with control cells (Figure 6B). The decay time of NAD(P)H signals was shorter (Figure 6C) in OPA1 siRNA cells than in controls, which is consistent with the lower Ca<sup>2+</sup> uptake capacity (Figures 5Aa and b). To measure the NADH/NAD<sup>+</sup> ratio after a series of histamine

stimuli, autofluorescence was calibrated by first oxidizing mitochondrial NADH to NAD<sup>+</sup> by uncoupling respiration using FCCP. Following FCCP treatment, Complex I was inhibited by rotenone and the substrate  $\beta$ -hydroxy-butyrate was added allowing maximal reduction of NAD<sup>+</sup> to NADH (Figure 6A). In control cells, the reduction level of NAD(H) pool returned to baseline following the histamine series

**Table 1** Effects of OPA1 knockdown on mitochondrial respiration in different metabolic states

| Metabolic state           |                                 | Respiration rates, O <sub>2</sub> nmol/min per 10 <sup>7</sup> cells |                                |  |
|---------------------------|---------------------------------|--|--------------------------------|--|
|                           |                                 | Control<br>(scrambled siRNA)   | OPA1 knockdown<br>(OPA1 siRNA) | Inhibition of respiration by<br>OPA1 knockdown: % inhibition |
| Permeabilized cells (n=6) | V <sub>3</sub> <sup>*</sup>     | 16.7 ± 1.6   | 13.4 ± 1.0                     | 19.1 ± 2.2   |
|                           | V <sub>4</sub>                  | 3.3 ± 0.2  | 3.1 ± 0.3                      | 6.4 ± 7.9  |
|                           | V <sub>3u</sub> <sup>*</sup>    | 20.2 ± 2.5   | 17.3 ± 1.9                     | 14.0 ± 4.5   |
| Intact cells (n=8)        | V <sub>basal</sub> <sup>*</sup> | 17.2 ± 1.9   | 13.0 ± 1.2                     | 21.0 ± 5   |
|                           | V <sub>4</sub>                  | 8.8 ± 6.3  | 6.3 ± 0.5                      | 15.7 ± 12.1  |
|                           | V <sub>3u</sub> <sup>*</sup>    | 25.0 ± 2.0   | 18.3 ± 1.6                     | 26.0 ± 4   |

Data are means ± S.E.M.; n, number of experiments. To determine the percent inhibition, respiration rates for each experiment were pairwise normalized to corresponding rates in the control (defined as 100%)

<sup>\*</sup>Significant at *P* < 0.05 by paired Student's *t*-test

(Figure 6D). In contrast, OPA1 siRNA cells exhibited a higher NADH/NAD<sup>+</sup> ratio (Figure 6D). An accumulation of NADH after a histamine-evoked [Ca<sup>2+</sup>]<sub>c</sub> rise in OPA1 siRNA cells is consistent with a defective respiratory capacity, and this outweighs the diminished NADH production caused by decreased Ca<sup>2+</sup> uptake.

**OPA1 loss in RGCs has no impact on mitochondrial morphology, but triggers glutamate-induced DCD.** To test the effects of OPA1 loss on Ca<sup>2+</sup> homeostasis in a pathophysiological setting relevant to optic neuropathies, we purified RGCs by immunopanning.<sup>36</sup> Because RGC transfection efficiency was low, we labeled the siRNA with fluorescein (FAM) and determined the [Ca<sup>2+</sup>]<sub>c</sub> only of FAM-positive RGCs. Scrambled and OPA1 siRNA-transfected cells were stained similarly with MitoTracker Red (Figure 7 and Supplementary Figure 3) that indicated no substantial mitochondrial depolarization (as was also observed in HeLa cells; Figures 1A and 4E). In contrast to HeLa cells, scrambled and OPA1 siRNA-transfected RGCs exhibited similar, short mitochondria (Figure 7a). Neither mitochondrial length nor the longitudinal fraction of RGC processes occupied by mitochondria was significantly affected by OPA1 knockdown (Figure 7a and Supplementary Figure 3). To test whether OPA1 loss in RGCs has an impact on Ca<sup>2+</sup> homeostasis, we measured [Ca<sup>2+</sup>]<sub>c</sub> using a low-affinity (K<sub>d</sub> = 2.9 μM) fluorescent Ca<sup>2+</sup> dye, Fura-FF, which is a sensitive indicator of both physiological and pathological Ca<sup>2+</sup> levels.

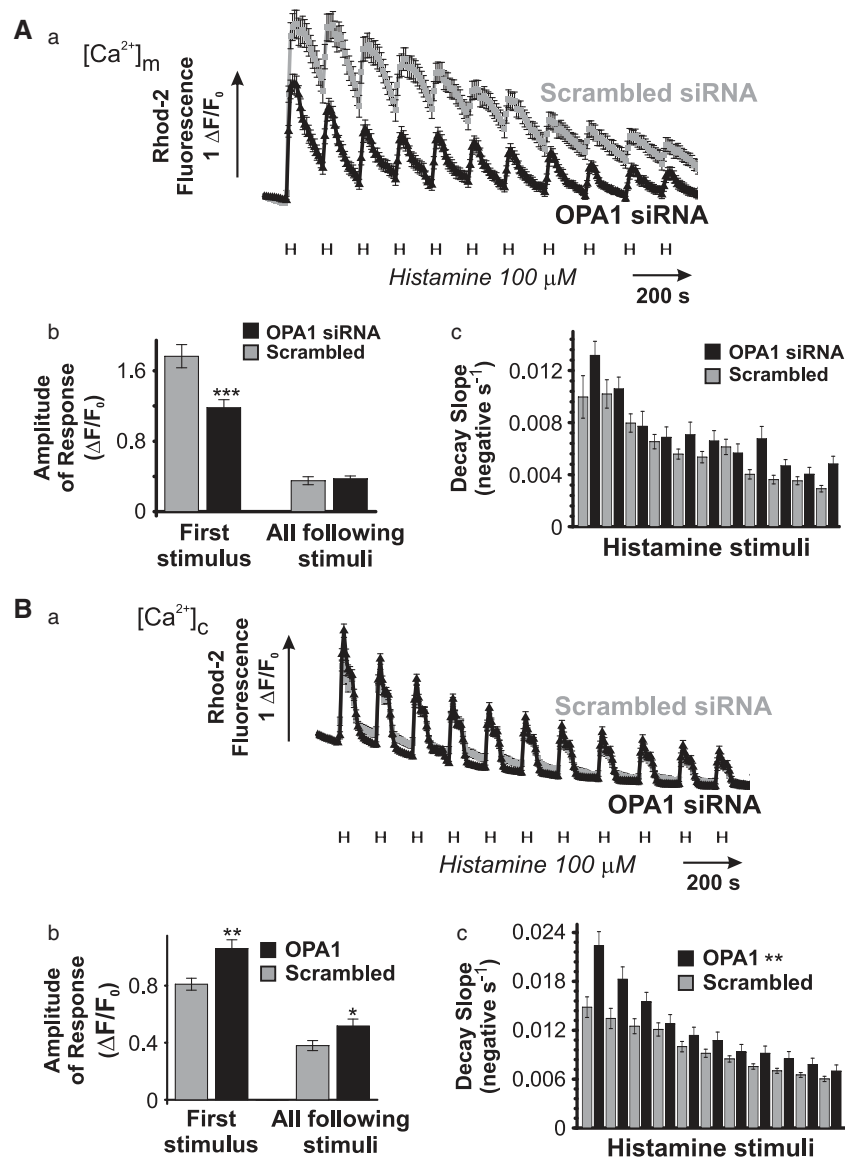
We employed two experimental paradigms to evoke Ca<sup>2+</sup> overload in RGCs. In the first paradigm, repetitive pulses of high extracellular [K<sup>+</sup>] were used to induce plasma membrane depolarization and Ca<sup>2+</sup> entry into the cytoplasm via voltage-dependent Ca<sup>2+</sup> channels. The resulting cytoplasmic [Ca<sup>2+</sup>]<sub>c</sub> transients were increased in OPA1 siRNA cells compared with scrambled siRNA controls, especially after repetitive stimulations; this difference was observed in a subset of OPA1 siRNA-transfected RGCs (5 out of 22; Figure 7b) but never in control RGCs. Because Ca<sup>2+</sup> extrusion rates depend on the actual [Ca<sup>2+</sup>]<sub>c</sub>, we evaluated recovery of [Ca<sup>2+</sup>]<sub>c</sub> after washout of the high [K<sup>+</sup>] medium by measuring the decay rate uniformly when [Ca<sup>2+</sup>]<sub>c</sub> was ~ 0.75 μM. The recovery after the peak was initially faster in OPA1 siRNA-transfected RGCs compared with controls (see

higher extrusion rates in Figure 7b). The faster drop of [Ca<sup>2+</sup>]<sub>c</sub> after washout of high [K<sup>+</sup>] medium is consistent with decreased buffering of intracellular Ca<sup>2+</sup>. This could be because of decreased mitochondrial Ca<sup>2+</sup> uptake, or decreased release of mitochondrially accumulated Ca<sup>2+</sup> during the recovery phase of the [Ca<sup>2+</sup>]<sub>c</sub> transient. The Ca<sup>2+</sup> extrusion slowed significantly after the third challenge in OPA1 RNAi, but not in control RGCs, indicating a progressive, stimulation-evoked deterioration of Ca<sup>2+</sup> homeostasis.

In the second paradigm, sustained levels of glutamate were applied to induce Ca<sup>2+</sup> entry via glutamate receptors. This challenge caused an immediate small transient increase of [Ca<sup>2+</sup>]<sub>c</sub>, followed by a plateau and a secondary high-magnitude [Ca<sup>2+</sup>]<sub>c</sub> rise known as DCD (Figure 7c). A significant proportion of control RGCs (6 out of 18) exhibited spontaneous reversibility of DCD, whereas in all OPA1 siRNA-transfected cells the secondary rise of [Ca<sup>2+</sup>]<sub>c</sub> was sustained and irreversible for the duration of the recording (Figure 7c). Thus, OPA1 knockdown in RGCs conferred increased sensitivity to glutamate-mediated excitotoxicity, which may provide a mechanistic explanation for their degeneration in DOA.

## Discussion

OPA1 is a key mediator of mitochondrial fusion, but it is currently unclear whether defects in OPA1-mediated fusion function are solely responsible for RGC degeneration in DOA.<sup>8</sup> Whereas HeLa cells exhibit a fused mitochondrial network that fragments into multiple round mitochondria after OPA1 loss, we report here that RGCs have much shorter mitochondria at baseline and undergo no significant change in size and shape upon OPA1 depletion. Previous studies reported mitochondrial fragmentation in OPA1 knockdown RGCs.<sup>23,37</sup> However, morphological changes become apparent at longer post-transfection times (1 to 2 weeks), but not at 3 days after transfection.<sup>37</sup> Morphological changes coincided with induction of apoptosis and membrane depolarization.<sup>23</sup> All our experiments were performed no later than 3 days after transfection. Notably, despite a lack of mitochondrial shape transformation (Figure 7 and Supplementary Figure 3), we observed a significant change in Ca<sup>2+</sup> handling. Thus, we propose that in RGCs, impaired Ca<sup>2+</sup> homeostasis and DCD represent an early consequence of OPA1 loss, whereas other



**Figure 5** Effect of OPA1 knockdown on histamine-induced mitochondrial and cytoplasmic  $[Ca^{2+}]$  transients. Measurements of mitochondrial (A) and cytoplasmic (B)  $[Ca^{2+}]$ . siRNA-transfected HeLa cells were loaded with  $2 \mu M$  Rhod-2-AM and stimulated by repeated application and washout of  $100 \mu M$  histamine for 20 s, as indicated. (a) Scrambled siRNA and OPA1 siRNA-cells are represented by gray and black traces, respectively. Data points are means  $\pm$  S.E.M. of individual cells;  $n = 37$  and  $n = 41$  in 6 and 5 separate experiments for scrambled and OPA1 siRNA, respectively. (b) Relative peak amplitude of the initial  $[Ca^{2+}]$  transient and the average of the relative peak amplitudes of all following transients. Bars show means  $\pm$  S.E.M. of cells corresponding to (a). \*\*\*, \*\*, and \* significance at  $P < 0.001$ ,  $P < 0.01$ , and  $P < 0.05$ , respectively, by Student's *t*-test. (c) Decay slopes of sequential  $[Ca^{2+}]$  transients (from left to right) approximated by linear regression of the trace immediately following the peak; in a 60-s span for mitochondria (Ac) and in a 20-s span for cytosol (Bc). Bars show means  $\pm$  S.E.M. of cells corresponding to (a). \*\*Significance at  $P < 0.01$  by two-way ANOVA comparing OPA1 (black) with scrambled (gray) siRNA categories

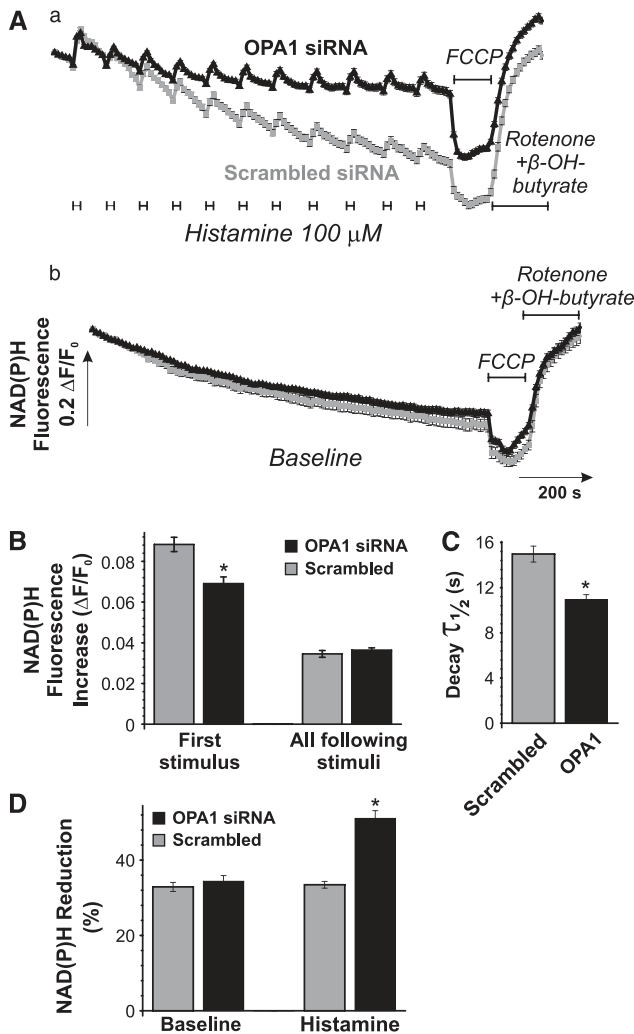
defects associated with fusion deficiency are possibly secondary or delayed effects.

**Bioenergetic defects.** The trigger for RGC loss in DOA likely has several components, not least of which is a bioenergetic crisis, and may also involve altered mitochondrial network dynamics, defective mtDNA maintenance, and disrupted axonal transport.<sup>8</sup> We found partial inhibition of respiration and decreased enzyme activity of electron transport chain complexes, but  $\Delta\psi_m$  remained sufficiently high to support energy-dependent  $Ca^{2+}$  uptake (Figure 4). In

contrast, loss of mitochondrial fusion resulted in almost complete inhibition of respiration in mouse embryonic fibroblasts,<sup>33</sup> highlighting the variability of findings using different cellular models.

Because respiration inhibition quantitatively correlates with crista surface area reduction (25%; Figure 2), the partial loss of cristae observed here may be responsible for the bioenergetic deficiency seen in OPA1 siRNA cells and the moderate ATP decrease observed. Respiratory inhibition is consistent with observed excessive reduction of pyridine nucleotides (Figure 6), and this effect outweighs the





**Figure 6** OPA1 knockdown leads to more reduced mitochondrial pyridine nucleotide pool. (A) Histamine-stimulated autofluorescence (a) and baseline NAD(P)H autofluorescence (b). Scrambled siRNA and OPA1 siRNA-treated HeLa cells are represented by gray and black traces, respectively. Data points show means  $\pm$  S.E.M. of  $n = 44$  and  $n = 49$  cells in 3 separate experiments for scrambled and OPA1 siRNA, respectively, for baseline traces;  $n = 122$  and  $n = 103$  in separate experiments for scrambled and OPA1 siRNA, respectively, for histamine-treated cells. To determine the redox status of the mitochondrial NADH/NAD<sup>+</sup> pool at the end of each experiment, mitochondrial NADH was fully oxidized to NAD<sup>+</sup> with 1  $\mu$ M FCCP (0% reduction) followed by inhibition of complex I with rotenone (1  $\mu$ M) plus substrate ( $\beta$ -hydroxy-butyrate; 10 mM) to fully reduce NAD<sup>+</sup> to NADH (100% reduction). (B) Bar graph shows the peak amplitude of the initial transient and the average of the relative peak amplitudes of all subsequent transients of NAD(P)H autofluorescence upon histamine stimuli. (C) Bar graph shows average decay half-time of sequential autofluorescence transients approximated by exponential regression of the 60 s span of the trace immediately following the peak. (D) Analysis of mitochondrial redox status after histamine stimuli in experiments shown in (A). Bar graph shows the resulting NADH redox status expressed as percentage. Gray bars, scrambled siRNA; black bars, OPA1 siRNA. (B–D) Data are means  $\pm$  S.E.M. of all cells corresponding to (A). \*Significant difference between OPA1 and scrambled siRNA transfection at  $P < 0.05$  by Student's *t*-test

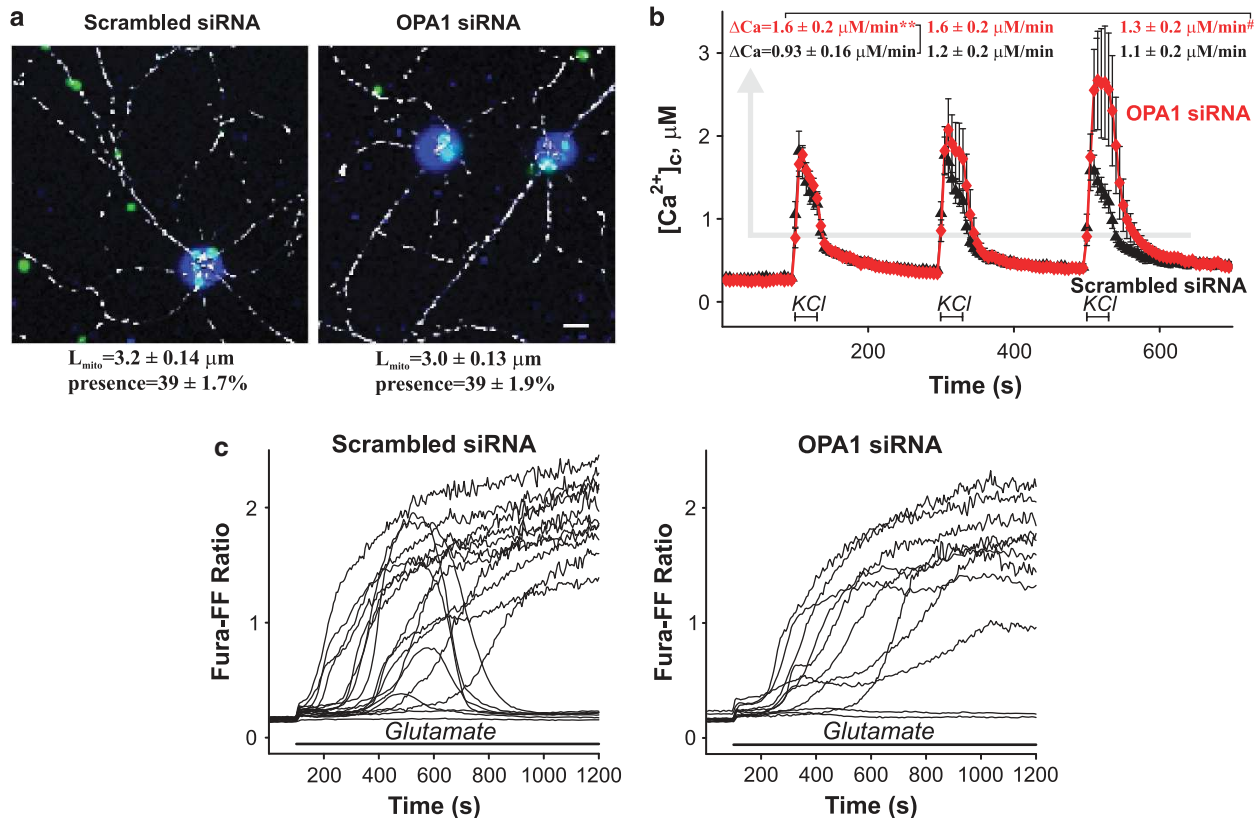
decreased activation of dehydrogenases by the reduced Ca<sup>2+</sup> transients. Postexercise phosphocreatine resynthesis, a measure of mitochondrial ATP generation, is delayed in

patients with OPA1 mutations,<sup>38</sup> lending further support for OPA1 influencing bioenergetics. Reduced enzymatic activity of the respiratory chain reported here is also consistent with recent finding of Complex IV deficiency in OPA1-null pancreatic  $\beta$ -cells.<sup>39</sup> However, in this model, cells lacking OPA1 maintained normal copy numbers of mtDNA,<sup>39</sup> whereas we observed mtDNA loss (Figure 4). Our data are in line with the observation that mtDNA content is lower in DOA patients.<sup>31</sup>

**Ultrastructure.** Several studies have demonstrated that loss of OPA1 (or its yeast ortholog Mgm1) leads to aberrant mitochondrial ultrastructure.<sup>17,40–45</sup> We observed a number of structural abnormalities consisting of rounded mitochondria with smaller and fewer cristae (not unlike what Amutha *et al.*<sup>40</sup> reported for Mgm1 deletion mutants) and a small fraction with condensed matrix or having cristae oriented parallel to the long axis of the organelles. At variance from an earlier study<sup>6</sup> showing a wide range of abnormal membrane structures in OPA1 siRNA mitochondria, including curved or ring-shaped cristae, unusual cristae shapes were not observed in our model (Figure 2). The variability of reported ultrastructural changes might be due to the different time points of knockdown.

We found that partial cristae depletion is associated with reduction in the number of cristae junction. Crista junction geometry has been proposed to control molecular diffusion inside mitochondria<sup>46,47</sup> as well as the release of cytochrome *c* in apoptosis.<sup>18,20</sup> However, in contrast to observations in pancreatic  $\beta$ -cells of OPA1 KO mice,<sup>39</sup> we did not observe dilation of crista junctions. It should be remarked that the crista junction dilation found in the pancreatic  $\beta$ -cells of OPA1 KO mice could be due to the increased matrix condensation observed in the study.<sup>39</sup>

**Impaired mitochondrial Ca<sup>2+</sup> handling.** The clinical manifestation of OPA1 mutations is often highly variable between siblings.<sup>48</sup> Experimental knockdown of OPA1 performed by different tools and laboratories resulted in highly variable phenotypes (see above). Ca<sup>2+</sup> handling is not an exception from this. Here we report diminished agonist-evoked mitochondrial Ca<sup>2+</sup> signaling consistent with attenuation of activation of matrix dehydrogenases in OPA1 knockdown cells. In contrast, Fulop *et al.*<sup>22</sup> reported increased histamine-evoked mitochondrial Ca<sup>2+</sup> transients in OPA1 knockdown cells, despite reduced  $\Delta\psi_m$ , and a hastened kinetics of Ca<sup>2+</sup> uptake in the absence of  $\Delta\psi_m$ . We believe that this discrepancy can be explained by mitochondrial swelling, as they reported a high level of circularity of OPA1 knockdown mitochondria, an indicator of swelling. Mitochondrial Ca<sup>2+</sup> fluxes may be controlled by crista junction geometry.<sup>46,47</sup> Dilation of crista junctions may occur by mechanical forces during overt matrix condensation,<sup>39</sup> or mitochondrial swelling. When mitochondria swell, crista unfolding might lead to breakdown of the diffusion barrier and increased mitochondrial Ca<sup>2+</sup> uptake. In our study, OPA1 knockdown in HeLa cells did not lead to swelling and we observed only small number of condensed mitochondria (Figure 3). The impact of crista and matrix remodeling on mitochondrial Ca<sup>2+</sup> transport warrants further investigation.



**Figure 7** Effect of OPA1 knockdown on intracellular [Ca<sup>2+</sup>]<sub>i</sub> transients in RGCs. RGCs transfected with FAM-conjugated scrambled or OPA1 siRNA were labeled with Fura-FF plus MitoTracker Red and imaged by low-light-level fluorescence microscopy before time-lapse Ca<sup>2+</sup> imaging. **(a)** Mitochondria labeled with MitoTracker Red are visualized in grayscale; the blue color shows the soma marked by Fura-FF fluorescence; and green indicates fluorescence of the siRNA conjugate FAM. Scale bar, 10  $\mu\text{m}$ . Mitochondrial length ( $L_{\text{mito}}$ ) and the longitudinal fraction of RGC processes occupied by mitochondria (presence) was measured by image segmentation and skeletonization in FAM-positive cells only ( $n = 11$  and  $n = 14$  view fields for scrambled and OPA1 siRNA). See also Supplementary Figure 3. **(b)** Cytoplasmic [Ca<sup>2+</sup>]<sub>i</sub> transients induced by consecutive depolarization of the plasma membrane with 50 mM KCl for 30 s, as indicated. Scrambled siRNA and OPA1 siRNA RGCs are represented by black and red traces, respectively. Data points are means  $\pm$  S.E.M. of individual FAM-positive cells; for scrambled siRNA,  $n = 15$  in 4 experiments and for OPA1 siRNA,  $n = 22$  in 4 experiments. Ca<sup>2+</sup> extrusion rates ( $\Delta\text{Ca}$ ) were determined by temporal differentiation of the traces in the recovery phase of the K<sup>+</sup>-evoked [Ca<sup>2+</sup>]<sub>i</sub> transients, where [Ca<sup>2+</sup>]<sub>i</sub> was  $\sim 0.75 \mu\text{M}$  in average.  $\Delta\text{Ca}$  was significantly different between OPA1 and scrambled siRNA treatments;  $P < 0.01$ , two-way ANOVA comparing the siRNA treatments as categories. \*\*Significance at  $P < 0.01$  by Welch-test; #Significance at  $P < 0.05$  by paired *t*-test. **(c)** DCD in RGCs. Fura-FF ratio is shown without calibration because of the presence of close to saturation values. Bar below traces indicates timing of perfusion of 100  $\mu\text{M}$  glutamate plus 10  $\mu\text{M}$  glycine with no Mg<sup>2+</sup>. Each trace corresponds to an individual FAM-positive cell from three independent cultures of OPA1 ( $n = 11$ ) or scrambled siRNA ( $n = 18$ )-treated RGCs. Of the 18 scrambled siRNA RGCs, 6 showed reversible secondary rise of [Ca<sup>2+</sup>]<sub>i</sub> compared with 0 of 11 OPA1 siRNA RGCs,  $P < 0.05$  by one-sided Fisher's exact test

Dayanithi *et al.*<sup>23</sup> reported CCCP-triggered increase in intracellular Ca<sup>2+</sup> levels after high [K<sup>+</sup>] stimulation in OPA1 RNAi RGCs. However, in this study extracellular Ca<sup>2+</sup> was not removed from the medium, and therefore it is likely that the observed elevation of [Ca<sup>2+</sup>]<sub>i</sub> reflects Ca<sup>2+</sup> entry from the extracellular space through voltage-dependent channels upon deenergization by CCCP rather than mitochondrial Ca<sup>2+</sup> release.

Our data in permeabilized cells clearly demonstrate that OPA1 knockdown reduces mitochondrial Ca<sup>2+</sup> retention capacity (Figure 4E) and are consistent with impaired Ca<sup>2+</sup> buffering capacity observed *in situ* (Figure 5). Interestingly, a similar reduction in Ca<sup>2+</sup> retention capacity was observed in dynamin-related protein 1 (Drp1)- and Fis1-expressing cells.<sup>49</sup> In contrast to the active, electrophoretic Ca<sup>2+</sup> uptake addressed in our experiments, OPA1 knockdown resulted in increased rates of passive Ca<sup>2+</sup> entry into mitochondria after

depolarizing and swelling mitochondria in permeabilized HeLa cells.<sup>22</sup>

**Impaired Ca<sup>2+</sup> homeostasis and neurodegeneration.** Excessive glutamate exposure evokes irreversible, high elevation of [Ca<sup>2+</sup>]<sub>i</sub> and the inability of neurons to return to normal Ca<sup>2+</sup> homeostasis, ultimately resulting in their demise.<sup>25,50</sup> We report here that glutamate exposure results in irreversible DCD in OPA1 siRNA RGCs, whereas a significant fraction of control RGCs recovered and exhibited reversible DCD, indicating some level of resistance to excitotoxic damage, as was observed earlier.<sup>31</sup> Interestingly, the NMDA receptor inhibitor memantine is able to protect RGCs in glaucomatous retinas and prevent release of OPA1 from mitochondria.<sup>51</sup> Using cerebellar granule neurons, Jahani-Asl *et al.*<sup>52</sup> showed that NMDA receptor overactivation triggers downstream Calpain activation, loss of OPA1

oligomers, deformation of crista junctions, and disruption of the respiratory chain complexes. Importantly, calpain activation is secondary to DCD.<sup>30</sup> Therefore, loss of OPA1 might exert its deleterious effects under excitotoxic conditions by downstream activation of the cell death mediator  $\text{Ca}^{2+}$ -dependent calpain.

DCD is controlled by two factors: initial mitochondrial  $\text{Ca}^{2+}$  load upon glutamate exposure<sup>53</sup> and bioenergetic capacity of mitochondria.<sup>54</sup> Based on our data, mitochondrial  $\text{Ca}^{2+}$  load is likely to be reduced in OPA1-deficient RGCs, and therefore the aggravation of DCD that was observed is possibly due to the decreased respiratory capacity. Along this line, DOA patients with higher respiratory complex activities have been reported to maintain visual acuity for a longer period of time,<sup>48</sup> again suggesting that the bioenergetic compromise may be at the root of RGC damage. Because RGC mitochondria do not show any abnormalities in protein expression, the focus should turn to RGC idiosyncrasies in terms of mitochondrial energy metabolism and environmental stress factors.<sup>3</sup> Our work suggests that inhibitors of excitotoxic injuries should be considered for therapeutic intervention in DOA.

## Materials and Methods

Unless indicated otherwise, chemicals were of analytical or molecular biology grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Thermo-Fisher Scientific (Waltham, MA, USA).

**Cell culture.** HeLa cells were purchased from American Tissue Culture Company (ATCC; Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; HyClone-Thermo-Fisher, Logan, UT, USA) containing 4.5 g/l glucose, 2 mM L-glutamine, 10% fetal bovine serum (FBS) (Tissue Culture Biologicals, Los Alamitos, CA, USA), containing 100 units/ml penicillin and 1000  $\mu\text{g}/\text{ml}$  streptomycin (Invitrogen, Carlsbad, CA, USA). RGCs were isolated from postnatal rats by immunopanning<sup>36</sup> and used at 7 days *in vitro*.

**RNA interference.** Scrambled I duplex (control) and OPA1 siRNA, the latter corresponding to nucleotides 1810–1833 of the OPA1 open reading frame (GenBank accession number AB011139) were from Dharmacon (Lafayette, CO, USA). siRNA (100–200 nM) was transfected into HeLa or RGCs with Oligofectamine (Invitrogen). To verify siRNA transfer in RGCs, siRNA was labeled with FAM (Ambion, Austin, TX, USA). Experiments were performed on day 3 after transfection.

**Subcellular fractionation and immunoblotting.** Subcellular fractionation and immunoblotting were done as described previously.<sup>55</sup> The primary antibody for cytochrome *c* was mouse monoclonal anti-cytochrome *c* (1:1000; clone 7H8.2.84; BD Biosciences Pharmingen, Franklin Lakes, NJ, USA). The OPA1 antibody (1:1000) was a gift from Dr. Kubo. Blots were probed with secondary HRP-conjugated anti-mouse antibody (1:2000; Amersham Biosciences, Piscataway, NJ, USA) for 2 h and immunocomplexes were visualized using a chemiluminescence detection kit (ECL) (Amersham Biosciences).

**Fluorescence microscopy.** HeLa cells were grown on 35 mm MatTek glass bottom dishes and mitochondria were labeled with 100 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) for 20 min in culture medium. Cells were fixed with 0.5% glutaraldehyde (Ted Pella, Redding, CA, USA) in ice-cold PBS (HyClone-Thermo-Fisher) for 30 min and autofluorescence was quenched by 1% sodium borohydride (Sigma-Aldrich) for 30 min at room temperature. Nuclei were stained with Hoechst 33342 (0.1  $\mu\text{g}/\text{ml}$ ; Molecular Probes). The 3D images were acquired using a Zeiss Axiovert 100M fluorescence microscope (Oberkochen, Germany) equipped with a Sencicam CCD camera controlled by Slidebook software (Intelligent Imaging Innovation, Denver, CO, USA). The z-series were deconvolved using the Slidebook constrained iterative algorithm and volumes were surface-rendered using Volocity 2 (Improvision, Coventry, UK).

**Electron microscopy.** HeLa cells were fixed as described.<sup>42</sup> Roughly 80-nm thick sections were cut with a Reichert (Cambridge, England, UK) ultramicrotome and poststained 10 min in uranyl acetate and 2 min in Sato lead. Sections were examined using a JEOL (Peabody, MA, USA) 1200FX electron microscope operated at 80 kV. Images were recorded on film at 6000 magnification. The negatives were digitized at 1200 d.p.i. using a Nikon (Melville, NY, USA) CoolScan system, giving an image size of 2689  $\times$  4006 pixel array and a pixel resolution of 4.2 nm.

**EM tomography.** Sections from *in situ* embedded monolayers of scrambled or OPA1 siRNA-transfected HeLa cells were cut at a thickness of 400–500 nm. EM tomography was performed as described.<sup>56</sup> Measurements of structural features were made within segmented volumes by the programs Synuarea and Synuvolume (National Center for Microscopy and Imaging Research, San Diego, CA, USA). Numbers of mitochondria were counted in the volume and normalized by dividing by the cytoplasmic volume reconstructed.

**Q-PCR.** To quantify mtDNA, real-time PCR was performed as described<sup>57</sup> using a Mx3000P real time PCR system (Stratagene, La Jolla, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Total cellular DNA was purified with a PUREGENE Cell & Tissue kit (Gentra Systems, Minneapolis, MN, USA) 3 days after siRNA transfection. Serially diluted total cellular DNA from untransfected cells was used as a template for two standard DNA curves, one for amplification with nDNA primers and the second one with mtDNA primers. The relative quantity of amplified DNA was determined from respective standard curves for each sample, starting with 10 ng for nDNA and 0.1 ng for mtDNA and the mtDNA/nDNA ratio was calculated.

**ATP measurements and cell viability.** HeLa cells were plated on black 96-well plates (Costar, Corning, Corning, NY, USA). ATP levels were determined using a CellTiter-Glo luminescence assay kit (Promega, Madison, WI, USA). Luminescence was measured in a PolarStar plate reader (BMG, Berlin, Germany). Each data set was collected from replicate wells ( $n = 6$ ).

For cell viability measurements, cells were loaded with 2  $\mu\text{M}$  Calcein-AM (Molecular Probes) in PBS. Generation of fluorescent Calcein was followed for 20 min at 490 nm excitation and 540 nm emission using a PolarStar plate reader. Cell viability of scrambled or OPA1 siRNA-transfected cells was expressed as the percentage of the rate of Calcein-AM hydrolysis measured from untransfected control cells (defined as 100%). Each data set was collected from replicate wells ( $n = 6$ –8). Caspase activity was measured using the fluorogenic substrate z-DEVD-AMC (Molecular Probes). Apoptotic cell death was measured by Annexin-V-FITC staining as described previously.<sup>42</sup>

**Cytochrome *c* immunocytochemistry and confocal microscopy.** HeLa cells were grown on MatTek dishes and fixed with 3.7% formaldehyde in KCl-based PIPES-KOH, pH 7.2, buffer containing 5% sucrose, for 15 min at 37°C and permeabilized with 1% Triton in PBS for 20 min at 37°C. Non-specific binding was blocked in 2% BSA, 0.05% Tween-20, and 0.1%  $\text{Na}_2\text{S}_2\text{O}_3$  for 40 min at 37°C. Samples were incubated with anti-cytochrome *c* antibodies in blocking solution (1:200, subclone 6H2B4, BD Pharmingen, San Diego, CA, USA) overnight at 4°C. Samples were incubated with secondary Alexa488-conjugated antibodies (1:200, Molecular Probes) for 2 h at room temperature. Confocal imaging was carried out using an Olympus (Tokyo, Japan) Fluoview-1000 laser scanning confocal microscope. Image stacks of 800  $\times$  800 pixels, 20 planes (0.09  $\times$  0.09  $\times$  0.5  $\text{mm}^3$  voxel size) were recorded and projected over maximum intensity. For excitation of Hoechst 33342, Alexa-488, and MitoTracker Red, the 405, 488, and 543 nm laser lines of a diode, an Ar-ion, and a He-Ne laser, respectively, were used. Emission was detected through a DM405/488/543 dichromic mirror and the spectral detector of the microscope at 420–480, 500–530, and > 560 nm, respectively.

**Respiration measurements.** Oxygen consumption was measured using a Clark-type electrode (Oxygraph, Hansatech Instruments, King's Lynn, UK). HeLa cells were trypsinized, centrifuged at 500  $\times g$ , and resuspended in the culture medium. For measurements in intact cells, 8  $\times 10^6$  cells were pelleted at 200  $\times g$ , 2 min and resuspended in 1 ml DMEM. After recording basal respiration for 2 to 3 min, oligomycin (1.25  $\mu\text{g}/\text{ml}$ ) was added followed by sequential additions of FCCP (100 nM). Respiration rates were normalized for equal numbers of live cells, evaluated by Trypan Blue exclusion.

For measurements in permeabilized cells, 8  $\times 10^6$  cells were resuspended in 1 ml buffer containing 250 mM sucrose, 10 mM HEPES/KOH, pH 7.4, 2 mM

$\text{KH}_2\text{PO}_4$ , centrifuged at  $200 \times g$ , 2 min, and resuspended in 1 ml of respiration buffer containing 125 mM KCl, 20 mM HEPES/KOH, pH 7.4, 2 mM  $\text{KH}_2\text{PO}_4$ , and 1 mM  $\text{MgCl}_2$ . Then, 5 mM glutamate and 5 mM malate were added as respiratory substrates. The plasma membrane was permeabilized by addition of titrated amounts of digitonin (0.012%). ADP (200  $\mu\text{M}$ ), oligomycin (1.25  $\mu\text{g}/\text{ml}$ ), and FCCP (200 nM) were added, as noted.

**Measurements of mitochondrial  $\text{Ca}^{2+}$  retention capacity,  $\Delta\psi_m$ , and mitochondrial swelling.** A custom-made chamber was used to simultaneously measure  $[\text{Ca}^{2+}]_m$ ,  $\Delta\psi_m$ , and optical density at 660 nm as described previously.<sup>58</sup> To measure the  $\text{Ca}^{2+}$  retention capacity,  $8 \times 10^6$  siRNA-transfected cells were permeabilized with digitonin (0.012%) in 1 ml of sucrose-based buffer and pelleted at  $200 \times g$ . Cells were resuspended in the respiration buffer plus 2  $\mu\text{M}$  tetraphenylphosphonium ( $\text{TPP}^+$ ) and without  $\text{MgCl}_2$  and pulsed with successive  $\text{Ca}^{2+}$  (50  $\mu\text{M}$ ) additions.  $\text{Ca}^{2+}$  retention capacity was defined as the amount of  $\text{Ca}^{2+}$  required to induce  $\Delta\psi_m$  loss and  $\text{Ca}^{2+}$  release.

**Measurements of Complex I–III–IV enzyme activity.** An assay of mitochondrial enzyme activity using a pore-forming peptide, alamethicin, has been described.<sup>59</sup> Briefly,  $8 \times 10^6$  cells were permeabilized with 0.012% digitonin and mitochondria were permeabilized with 30  $\mu\text{g}/\text{ml}$  alamethicin in respiration buffer (see above) containing 2  $\mu\text{M}$  cytochrome c. Oxygen consumption induced by 2 mM NADH was measured for 2 to 3 min followed by rotenone (2  $\mu\text{M}$ ) addition. The rate of rotenone-insensitive respiration was subtracted from the total rate of NADH-dependent respiration.

**Fluorescence time-lapse imaging for  $\text{Ca}^{2+}$  and NADH.** For simultaneous measurements of  $[\text{Ca}^{2+}]_m$  and  $[\text{Ca}^{2+}]_c$ , cells were loaded with 2  $\mu\text{M}$  Rhod-2-AM (Molecular Probes) in the presence of 15 mM malate for 15 min at 37°C. To measure  $[\text{Ca}^{2+}]_c$  in RGCs, neurons were loaded with 1  $\mu\text{M}$  Fura-FF-AM (Molecular Probes) for 10 min at 37°C. To visualize mitochondria, 2 nM MitoTracker Red was included. High-speed, live-cell imaging was performed in perfusion buffer containing 140 mM NaCl, 3.9 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{Na}_2\text{SO}_4$ , 0.4 mM  $\text{Na}_2\text{HPO}_4$ , 5.6 mM  $\text{D-glucose}$ , and 20 mM HEPES, pH 7.4. Cells on coverslips were placed in a perfusion chamber on the stage of an Olympus IX81 inverted microscope. Fluorescence was excited by a DG-4 (Sutter Instruments, Novato, CA, USA) Xenon-arc illumination unit through 340/10 nm and 380/10 nm bandpass filters for Fura-FF, a 492/18 nm filter for FAM, a 525/30 nm filter for Rhod2-AM, and a 572/25 nm filter for MitoTracker Red. Fluorescence was detected by an ultra-sensitive cooled CCD camera (Photometrics Cascade 512B; Roper Scientific, Vista, CA, USA) through emission filters: 510/40 nm for Fura-FF, 535/20 nm for FAM, 600/45 nm for Rhod-2-AM, and 630/60 nm for MitoTracker Red. Data acquisition was controlled by Metafluor 6.2 (Molecular Devices, Silicon Valley, CA, USA).

For selective assessment of  $[\text{Ca}^{2+}]_m$ , high-resolution images were taken (512  $\times$  512 16-bit 0.26  $\mu\text{m}/\text{pixel}$ ; at 5 s intervals) and fluorescence originating from mitochondria was separated by high-pass filtering of fluorescent images, whereas  $[\text{Ca}^{2+}]_c$  was determined over the nucleus<sup>35</sup> using Metafluor Analyst (Molecular Devices).

For RGCs, MitoTracker Red and FAM fluorescence was measured once at the start of the experiment (512  $\times$  512 16-bit 0.8  $\mu\text{m}/\text{pixel}$ ). Length and presence of mitochondria in processes of RGCs were determined by high-pass filtering and segmentation of images using locally adaptive techniques in Image Analyst MKII. Finally, the skeletal length of mitochondria and processes were calculated for FAM-positive cells. Mitochondrial length is given as mean skeletal length. Mitochondrial presence is defined as total mitochondrial length per total neurite length (see Supplementary Figure 3B). During time-lapse experiments, Fura-FF signals were recorded at  $2 \times 2$  binning (256  $\times$  256; 16-bit; 1.6  $\mu\text{m}/\text{pixel}$ ; 5 s/ratio frame). Fura-FF ( $K_d = 2.9 \mu\text{M}$ ) ratios were calibrated to  $[\text{Ca}^{2+}]_m$  by exposing each sample to zero and high (10 mM)  $[\text{Ca}^{2+}]_m$  at the end of the experiment with a procedure previously described.<sup>30</sup> The  $\text{Ca}^{2+}$  calibration and analysis of  $\text{Ca}^{2+}$  extrusion rates were performed in Mathematica 5.2 (Wolfram Research, Oxfordshire, England, UK).

NADH autofluorescence in HeLa cells was measured using 340/10 nm excitation and 465/30 nm emission filters. Images were taken at 256  $\times$  256 pixels; 16-bit; 0.8  $\mu\text{m}/\text{pixel}$ , at 10 s intervals. Decay slope and half-life of fluorescence transients in histamine-stimulated cells were determined by standard functions in Mathematica 5.2. The percentage of NAD(H) reduction was expressed from the NAD(P)H autofluorescence intensity before application of FCCP scaled between the intensities measured in the presence of FCCP (0%) and rotenone (100%).

## Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)