Online Supplementary Material

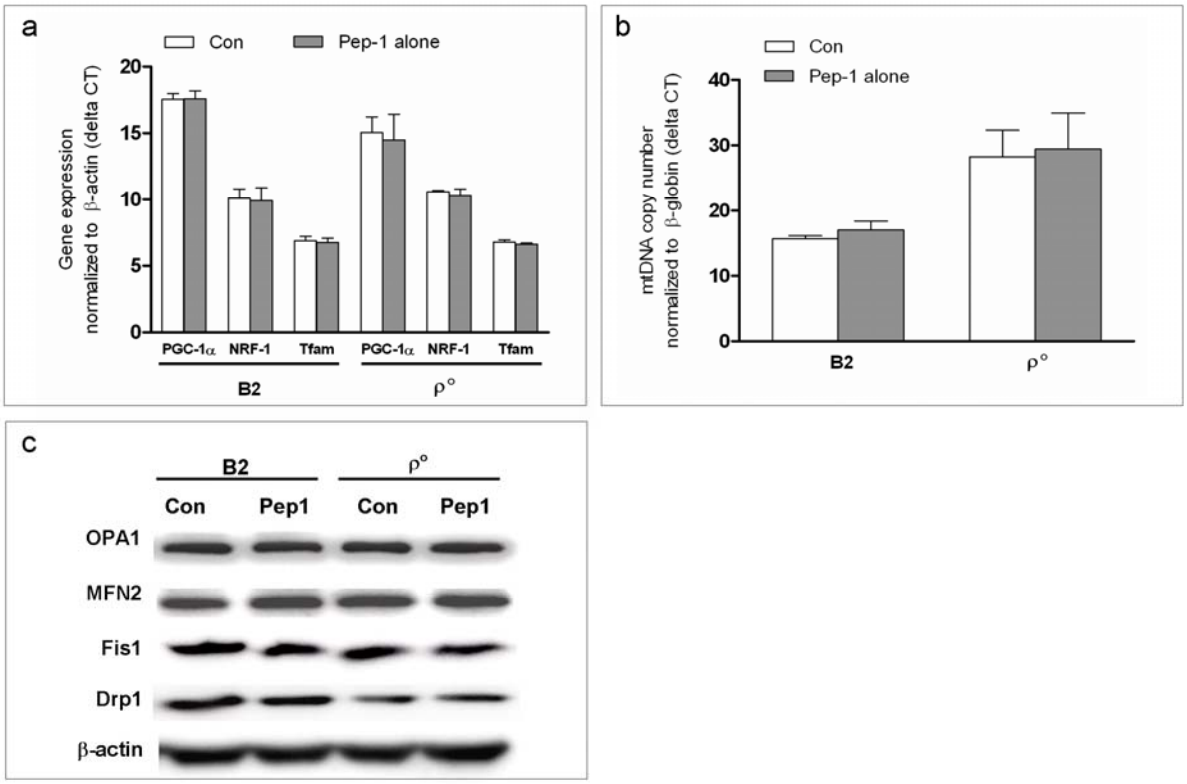

Fig. S. Comprehensive assessment of mitochondrial homeostasis including mitochondrial biogenesis, content and dynamic proteins in cells after 2 days of Pep-1 alone treatment. Mitochondrial biogenesis-related genes of PGC-1α, NRF-1, and Tfam were analyzed by real-time RT-PCT in MERRF cybrid (B2) and ρ° cells (ρ°) with Pep-1 treatment (Pep-1 alone) or not (Control, con) (a). The procedure of Pep-1 alone treatment was following the some condition of Pep-1-mediated mitochondrial delivery. Mitochondrial DNA (mtDNA) copy number was definitely by quantifying the mtDNA ND1 gene copies relative to the invariable β-globin nuclear gene (b). Mitochondrial dynamics-related proteins (fusion proteins, OPA1 and MFN2; Fission proteins, Fis1 and Drp1) were analyzed by western blotting (c). (n=3).

Result

To explore the effect of vehicle of mitochondria in MERRF cybrid (B2) and ρ° cells (ρ°), cells were individually treated Pep-1 alone following the same procedure and conditions of Pep-1-mediated mitochondrial delivery. The Mitochondrial biogenesis-related genes (PGC-1α, NRF-1, and Tfam), mitochondrial DNA (mtDNA) replication and mitochondrial dynamics-related proteins (fusion proteins, OPA1 and MFN2; Fission proteins, Fis1 and Drp1) were analyzed after 2 days of treatment (Fig. S). The results demonstrated that Pep-1 alone treatment did not affect the expressions of mitochondrial biogenetic genes (a) , mtDNA copy number (b) and mitochondrial dynamic proteins (c) in MERRF cybrid (B2) and ρ° cells (ρ°) , comparing to each untreated cells (Fig. S). It meant that Pep1-mediated mitochondrial delivery in regulation of mitochondrial homeostasis did not dominated by vehicle of mitochondrial delivery, Pep-1.

Materials and methods

DNA extraction and PCR-RFLP analysis

Total DNA was prepared from cells with or without mitochondrial delivery for 2 days using Gentra Puregene Cell Kit (QIAGEN). To detect the A8344G mutation, a segment of mtDNA was amplified by PCR, using the following primers: 8344-F (5`-CCgggggTATACTACggTC-3`),8344-R (5`ggggCATTTCACTgTAAAgAggTgCCgg-3`), standard cycling conditions, and an annealing temperature of 55 °C. Before reaction, 0.3 μl FdNTP was added to increase signal (Perkin-Elmer, Foster City, CA, USA), the products were digested with *Nae* I. The 223-bp PCR product could be cleaved by *Nae* I into 197 fragments and the digested DNA mixture was further analyzed by electrophoresis with a 4% agaros gel.

Confocal microscopy

After application of PMD, mitochondria in the rescued cells were labeled by the addition of a Green fluorescent mitochondrial dye to the cultures (100 nM final concentration; MitoTracker Green; Invitrogen-Molecular Probes, Eugene, OR) and maintaining it for 20 minutes in a CO2 incubator. This dye is concentrated in active mitochondria by a process that is non-dependent on mitochondrial membrane potential. The cultures were subsequently fixed with 0.5% glutaraldehyde (Ted Pella, Redding, CA) in Dulbecco's phosphate-buffered saline (DPBS) for 30 minutes at 4°C. Mitochondrial morphology was observed by an inverted Laser Scanning Microscope LSM510 microscope (Axiovert 100, Carl Zeiss) with the appropriate for the maximum signal for mitochondria-labeled cells. In order to observe the location of delivered mitochondria which labeled with MitoTracker Red CMXRos, the three dimensional (3D) section was analyzed. At the appropriated pinhole settings, for each synaptic site 17-45 sections were collected every 1.2 μm. To describe the distribution of delivered mitochondria around host mitochondria at a given synaptic site in the z-plane, we resampled the volume data from a z-series of confocal images collected in the dual wavelength mode along a specified line. The integration of line scans through the z-axis yielded a longitudinal view of the synaptic site by using the Zeiss LSM510 software.

Mitochondrial membrane potential assay using Rhodamine 123 and JC1

Changes in the mitochondrial membrane potential were measured by flow cytometry using JC-1 (Invitrogen-Molecular Probes) and Rodamine 123. Cells after treatments (1×10^6) were incubated with Rhodamine 123 (13 μM) for 30 min at 37°C prior to completion of the drug treatment. Rhodamine 123 is a cationic dye which localizes in the mitochondria of viable cells. The cells were washed, harvested, resuspended in HBSS and analyzed by flow cytometry. Data was acquired on a flow cytometer using WinMDI 2.9 software for

presentation. Ten thousand cells were analyzed for each sample.

Thirty minutes prior to cytometric analysis, JC-1 was added to 1 ml of cells to a final concentration of 10 μm and incubated at 37°C, 5 % CO_2 for 30 minutes. At the designated time, 1×10^4 cells were examined for each sample on a FL-1 (530 nm) versus FL-2 (585 nm) dot plot on a flow cytometic. JC-1 has dual emission depending on the state of the mitochondrial membrane potential. JC-1 forms aggregates (JC1 aggreates) in cells with a high FL-2 fluorescence indicating a normal mitochondrial membrane potential. Loss of the mitochondrial membrane potential results in a reduction in FL-2 fluorescence with a concurrent gain in FL-1 fluorescence as the dye shifts from an aggregate to monomeric state (JC 1 monomers). Therefore, retention of the dye in the cell can be monitored through the increase in FL-1 fluorescence. The data were converted to density plots using WinMDI 2.9 software for presentation. Cell with the co-expression of high JC1 monomers and low (JC1 aggreates) indicated as the lower right quadrant of the plot represented mitochondrial membrane potential depolarization.

Oxygen consumption

In prior to measure of dissolved oxygen during the bioprocess of rescued cells by using a NeoFox Oxgen sensor (Ocean Optics), cell medium was replaced with restrictive medium that consisted of same medium with low glucose and lacking sodium pyruvate and uridine for overnight. Oxgen consumption (%) was evaluated by monitoring cells (more than 1×10^6 cells) suspended in 200 μl same medium lacking glucose, sodium pyruvate and uridine before and after the administration of 4.5% glucose for 1 hr at 37 °C. The data were presented as the net oxygen consumption rate (percentage difference of before and after glucose-induced values for 1 hr) normalized the number of available cells (oxygen consumed %/ mins/ 10^6 cells).

Intracellular ATP content

Intracellular ATP level was measured by the Bioluminescent Somatic Cell Assay Kit (Sigma-Aldrich). An aliquot of 50 μl of viable cell suspension was mixed with 150 μl of Somatic Cell Releasing Reagent to release the intracellular ATP. Half of the mixture was then transferred into an OptiPlate™ white opaque 96-well microplates (Perkin-Elmer) containing 100 μl of ATP Assay Mix, and the luminescence intensity was measured by the FLUOstar OPTIMA multidetection microplate reader. ATP levels were then determined using a standard curve of ATP dilutions and were present by normalizing cell number.

Lactate production rate

Lactate production rate was measured by a Lactate Reagent kit (Trinity Biotech plc., Bray, Ireland). Cells in six-well plates were replenished with fresh medium and incubated for 8 hours. An aliquot of 10 μl of medium was then transferred to a 96-well plate to mix with the Lactate Reagent, and the absorbance at 540 nm was measured by the FLUOstar OPTIMA microplate reader. The concentration of extracellular labctate was determined using a standard curve of Lactate dilutions and presented by normalizing total cell number and divided by the time of incubation.

Calcein AM/PI double staining

Cell viability was assessed by retention of calcein and propidium iodide (PI) uptake using a Nikon TE 300 inverted fluorescence microscope. Cells were seeded at 24 well plates (8×10^3 cells/per well) and cultivated with 2ml same medium lacking glucose alone for 4 days without exchange the medium. Cultured cells were rinsed carefully with PBS and incubated with 1 μg/ml calcein-AM and 10 μg/ml PI in PBS at 37°C for 35 min. For cell counting, cells were rinsed with PBS and visualized using a Nikon 10x objective lens. Calcein fluorescence was visualized using FITC filters [excitation (Ex) 488 nm; emission (Em) 515 nm], and PI

fluorescence was visualized using Texas Red filters (Ex 536 nm; Em 645 nm). Images were collected using a Princeton Instruments MicroMax CCD camera. The cell viability was expressed as the ratio of calcein-positive cells to the sum of calcein-positive and PI-positive cells.

Western Blot Analysis

An aliquot of 25 μg of proteins was separated on a 12% SDS-polyacrylamide gel electrophoresis gel and blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). Nonspecific bindings were blocked by 5% skim milk in Tris-buffered saline tween 20 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4), and the membrane was blotted overnight at 4°C with indicated primary antibodies of Cytochrome *c* (1:100 dilution, BD Biosciences, Pharmingen), Procaspase-3 (1:100 dilution, Santa Cruz Biotechnology), OPA1 (1:500 dilution, BD Biosciences, Pharmingen), MFN2 (1:500 dilution, Sigma-Aldrich), Fis1 (1:500 dilution, Axxora, Switzerland), Drp1 (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (1:1000 dilution, Chemicon). After incubation with a horseradish peroxidase-conjugated secondary antibody, the protein intensity was determined by an enhanced chemiluminescence reagent (Immobilon Western, Millipore, Billerica, USA).

The active caspase-3/-7 activity

Quantification of Caspase-3/-7 activation was assessed by using carboxyfluorescein (FAM) FLICA (fluorochrome-labeled inhibitor of caspase) apoptosis detection kit (Immunochemistry Technologies, Bloomington, MN, USA) according to the manufacturer's protocol. The assay was based on fluorescein-labeled inhibitors, FAM-DEVD-FMK, that bind specifically to the active forms of caspase-3 and caspase-7 (excitation wavelength, 490 nm; emission wavelength, 520 nm). These inhibitors were nontoxic and cell permeable. The

10 μl of 30× FLICA solution were added to cells (about 1×10^6/300 μl culture medium), incubated at 37°C and 5% CO_2 for 1 h, washed twice, and resuspended in wash buffer. The caspase-3/-7 activity was presented by the calculation of fluorescence-positive cells detected with a flow cytometry (Becton Dickinson) compared with each non-staining cell. The 1×10^4 cells for each sample to be analyzed using the WinMDI 2.9 software.

RNA extraction and quantitative real-time RT-PCR analysis

Total RNA from cells was isolated using the TRIzol reagent (Invitrogen). Total RNA was further purified with NucleoSpin® RNA II Kit (Macherey-Nagel, Düren, Germany) and reverse-transcribed using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Indianapolis, USA). The expressions of mRNA were determined by quantitative analysis of real-time RT–PCR using SYBR Green PCR Master Mix (Roche Applied Science) and an ABI Prism 7300 system (Applied Biosystems). The primers used for RT–PCR were described as: PGC-1α (Forward : 5`-GGAgAggCAgAggCAgAAgg-3` and Reverse: 5`-AAgCATCACAggTATAACggTAgg-3`), NRF-1 (Forward: 5`-CCgTggCTgATggAgAggTggAAC-3` and Reverse: 5`-CTgATgCTTgCgTCgTCTggATgg -3`), Tfam (Forward : 5`-ggAgTTgTgTATTgCCAggAg-3` and Reverse: 5`-CTTCggAgAAACgCCATCg-3`) nd β-actin (Forward: 5`-ATCgTgCgTgACATTAAgAgAAg-3` and Reverse: 5`-AggAAggAAggCTggAAgAgTg-3`). PCR was performed as follows: 1 cycle of hot start at 95°C for 10 min and 40 cycles of 30 seconds denaturation at 95°C, annealing and extension at 60°C for 90 seconds. The mRNA expressions normalized to β-actin respectively were presented as relative expression levels.

Copy number of mitochondrial DNA

An aliquot of 50 ng of DNA was subjected to quantitative PCR using LightCycler-FastStar DNA Master SYBR Green I kit (Roche Applied Sciences). DNA

fragments of NADH dehydrogenase subunit 1 (ND1) gene (mtDNA-encoded) and β-actin gene (nuclear DNA-encoded, served as internal control) were amplified with specific primer pairs (ND1: Forward: 5`-AACATACCCATggCCAACCT -3` and Reverse: 5`-AgCgAAgggTTgTAgTAgCCC-3`; β-actin: Forward: 5`-ATCgTgCgTgACATTAAgAgAAg-3` and Reverse: 5`-AggAAggAAggCTggAAgAgTg-3`), respectively. The relative mtDNA copy number was measured by normalization of the crossing points in quantitative PCR curves between ND1 and β-actin genes using the RelQuant software (Roche Applied Sciences). PCR was performed as follows: 1 cycle of hot start at 95°C for 10 min and 36 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C, and 30 seconds extension at 72°C.

Mitochondrial mass assay

Cells were incubated in fresh medium with 2.5 μM nonyl acridine orange (NAO; Molecular Probes) for 10 min at 37°C in the dark and harvested in a solution containing 5 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES buffer (pH 7.4). The fluorescence intensity of 1×10^4 cells was recorded on a flow cytometer (Beckman Coulter) with the excitation wavelength at 488 nm and emission wavelength at 535 nm.