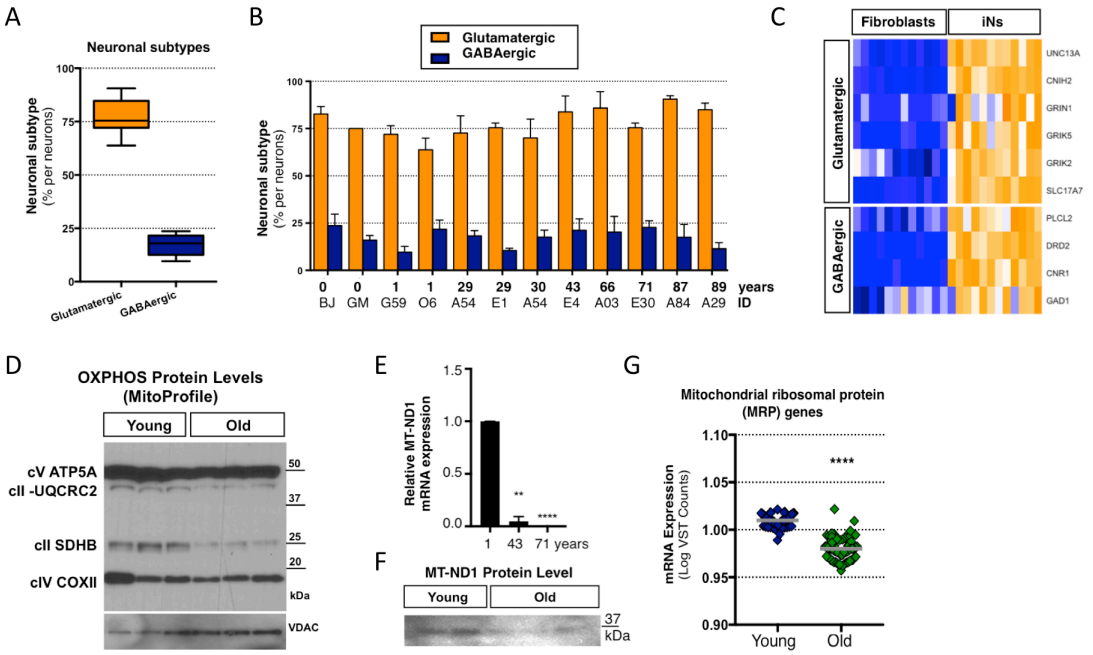
Cell Reports, Volume *23*

Supplemental Information

Mitochondrial Aging Defects Emerge in Directly Reprogrammed Human Neurons due to Their Metabolic Profile

Yongsung Kim, Xinde Zheng, Zoya Ansari, Mark C. Bunnell, Joseph R. Herdy, Larissa Traxler, Hyungjun Lee, Apua C.M. Paquola, Chrysanthi Blithikioti, Manching Ku, Johannes C.M. Schlachetzki, Jürgen Winkler, Frank Edenhofer, Christopher K. Glass, Andres A. Paucar, Baptiste N. Jaeger, Son Pham, Leah Boyer, Benjamin C. Campbell, Tony Hunter, Jerome Mertens, and Fred H. Gage

Figure S1. Neuronal subtype and mitochondrial characterization of aging iNs, Related to Figure 1



(**A**-**B**) Average fractions of glutamatergic (vGlut1/βIII-tubulin-positive) and GABAergic (GABA/Map2ab-positive) iNs across all 12 donors (**A**), and per donor (**B**).
(**C**) Heatmap showing relative expression of the glutamatergic neuron-specific genes Unc-13 homolog A (*UNC13A*), AMPA receptor auxiliary protein 2 (*CNIH2*), NMDA1 (*GRIN1*), GluK5 (*GRIK5*), GluK2 (*GRIK2*) and vGLUT1 (*SLC17A7*), and the GABAergic neuron-specific genes Phospholipase C like 2 (*PLCL2*), Dopamine Receptor D2 (*DRD2*), Cannabinoid receptor 1 (*CNR1*) and Glutamate decarboxylase 1 (*GAD1*). Blue = low expression, orange = high expression; normalized by row)

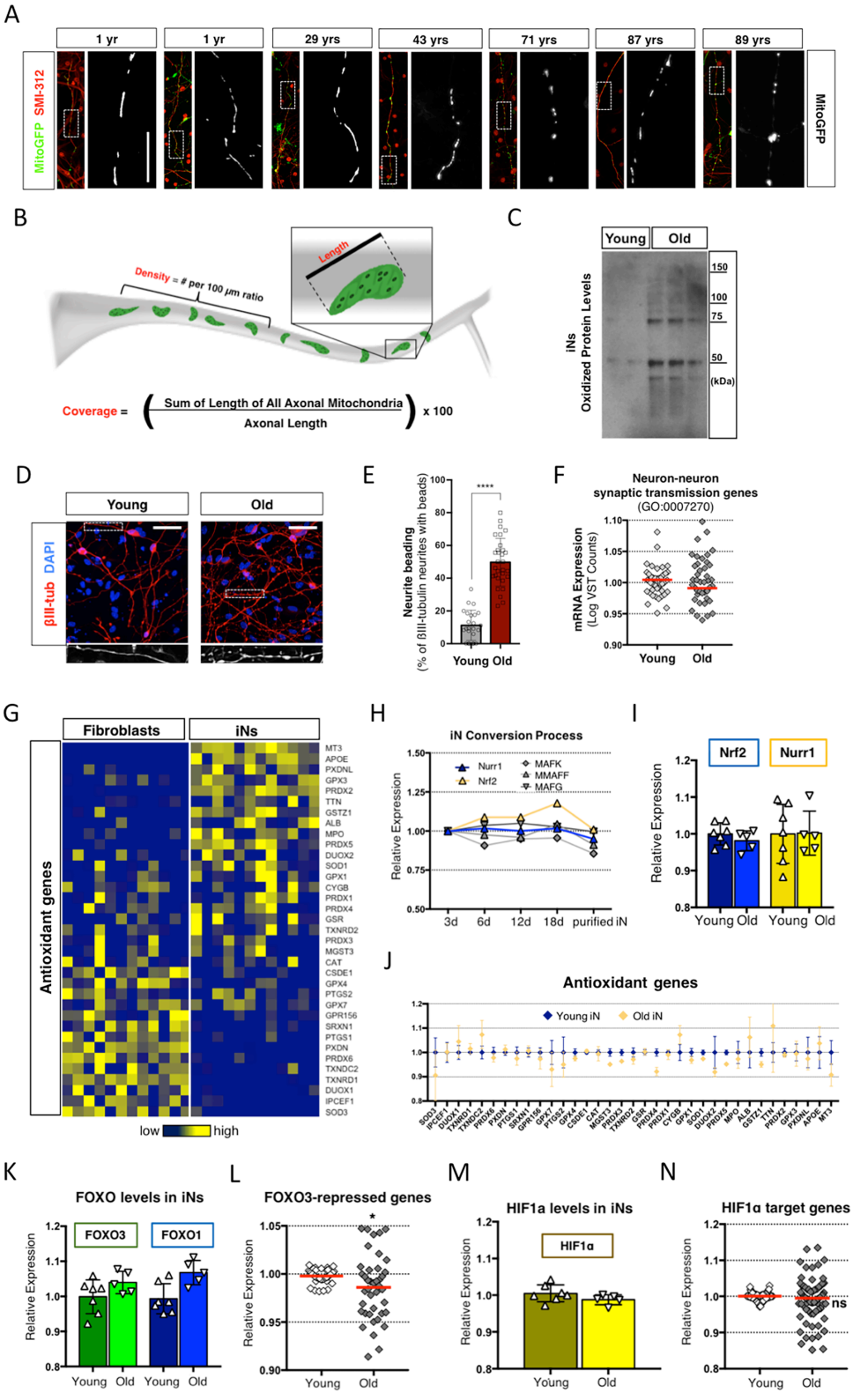
(**D**) Representative MitoProfile blot for protein levels of OXPHOS genes in young and old iNs. The blot shows, from large to small: ATP5A (Complex V, cV), UQCRC2 (Complex III, cIII), SDHB (Complex II, cII), and COXII (Complex IV, cIV, mtDNA-encoded). Quantitative data was normalized to VDAC.

(**E**) Quantitative RT-PCR (qPCR) for the mtDNA-encoded gene MT-ND1 in aging iNs. Expression data were normalized to GAPDH.

(**F**) Western blot for the mtDNA-encoded gene MT-ND1 in aging iNs shows low protein levels that decrease with age.

(**G**) Relative expression of mitochondrial ribosomal proteins (MRPs) in in young and old iNs. VST normalized counts normalized to young; dots indicate mean ± SEM; significance values calculated by Wilcoxon test.

Figure S2. Neurite and neuronal morphologies of young and old iNs, and Antioxidant-, FOXO-, and HIF1a-related gene expression in aging iNs Related to Figure 2



(**A**) Images of mitochondria expressing hSyn::MitoEGFP (green) with axon maker SMI-312 (red) in young and old iNs. Right panels indicate magnified images from boxed regions in the left panels. Scale bar: 20 μm

(**B**) Schematic drawing of iN axonal mitochondrial morphology indexes: length, density and coverage.

(**C**) Representative OxyBlot for oxidized proteins in young and old iNs.

(**D**) Representative immunofluorescence images of young and old iNs stained for βIII-tubulin and Dapi. Magnified examples show severe neurite beading in old, but not in young iNs.

(**E**) Quantification of neurite beading (% of βIII-tubulin neurites that show beading) in young and old iNs. Bars indicate mean ± SD; symbols represent data from individual images.

(**F**) Relative expression of Neuron-neuron synaptic transmission genes (GO:0007270) in in young and old iNs. VST normalized counts normalized to young; dots indicate mean ± SEM; significance values calculated by Wilcoxon test.

Significance values in this figure: ****$p < 0.001$.

(**G**) Heatmap showing relative expression of antioxidant genes in fibroblasts and iNs (n=12 each). Genes were sorted by FoldChange in expression from iN-enriched (top) to fibroblast-enriched (bottom) genes.

(**H**) Expression of the antioxidant regulators Nurr1 and Nrf2, an its co-factors, over the time course of iN conversion in bulk cultures, and in purified iNs at day 18.

(**I**) Relative expression of Nrf2 and Nurr1 in young and old iNs (n = 12 donors, triangles depict individuals). Bars indicate mean ± SD.

(**J**) Relative expression of antioxidant genes in young and old iNs (n = 12 donors). Symbols indicate mean ± SEM.

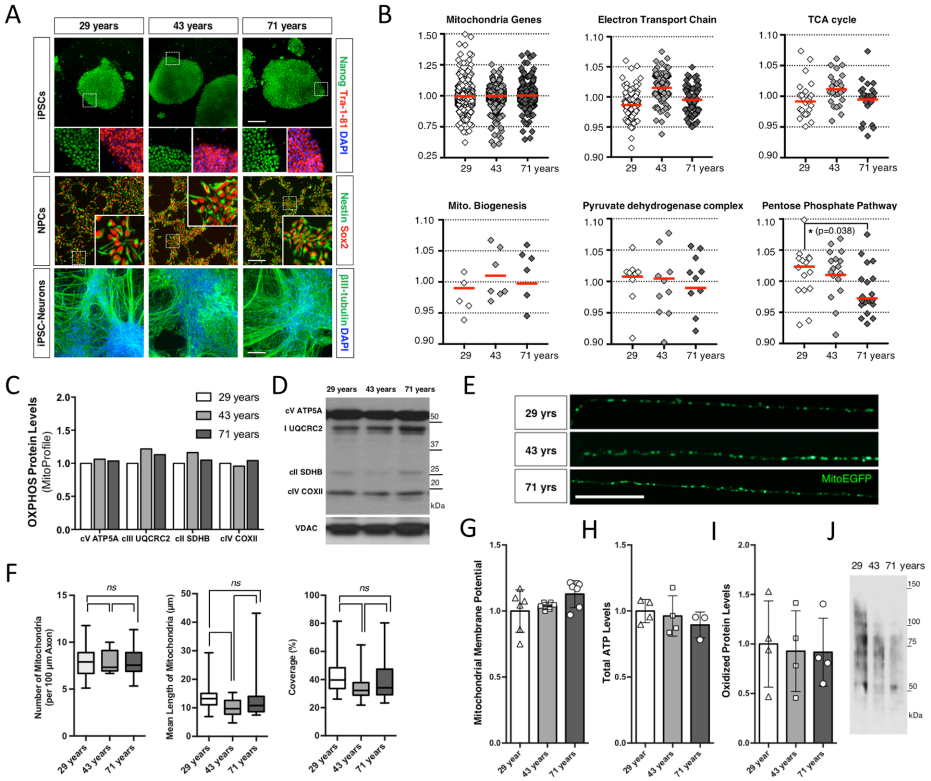
(**K**) Relative expression of FOXO3 and FOXO1 in young and old iNs (n = 12 donors, triangles depict individuals). Bars indicate mean ± SD.

(**L**) Expression of 41 previously validated FOXO3 target genes that become repressed as a result of FOXO3 activity (Delpuech et al. 2007; symbols mark individual genes). Significance value: *$p < 0.05$.

(**M**) Relative expression of HIF1α in young and old iNs (n = 12 donors, triangles depict individuals). Bars indicate mean ± SD.

(**N**) Relative expression of HIF1α target genes in young and old iNs (n = 12 donors). Bars indicate mean ± SD. Symbols mark individual genes. Significance value: *$p < 0.05$.

Figure S3. Rejuvenated neurons from young- and old-derived iPSCs, Related to Figure 3



(**A**) Representative images of immunofluorescent characterization of young (29 years), mid (43 years) and old-derived (71 years) iPSCs for Nanog and Tra-1-81, iPSC-derived neural progenitor cells (NPCs) for Nestin and Sox2, and iPSC-Neurons for ßIII-tubulin. Scale bars = 100 µm.

(**B**) Expression of functional gene groups in young, mid and old-derived iPSC-Neurons. Logarithmic VST normalized counts; bars indicate mean values; dots represent genes; the non-parametric Wilcoxon test was used to determine if significance differences exist. Significance values: *p < 0.05.

(**C-D**) Relative protein levels of OXPHOS proteins in young and old iNs assessed by quantitative MitoProfile Western blot (n = 3 individuals). No significance detected by t-test.

(**E**) Representative images of MitoGFP-labeled mitochondria in neurites of young, mid and old-derived iPSC-Neurons. Scale bar = 100 µm.

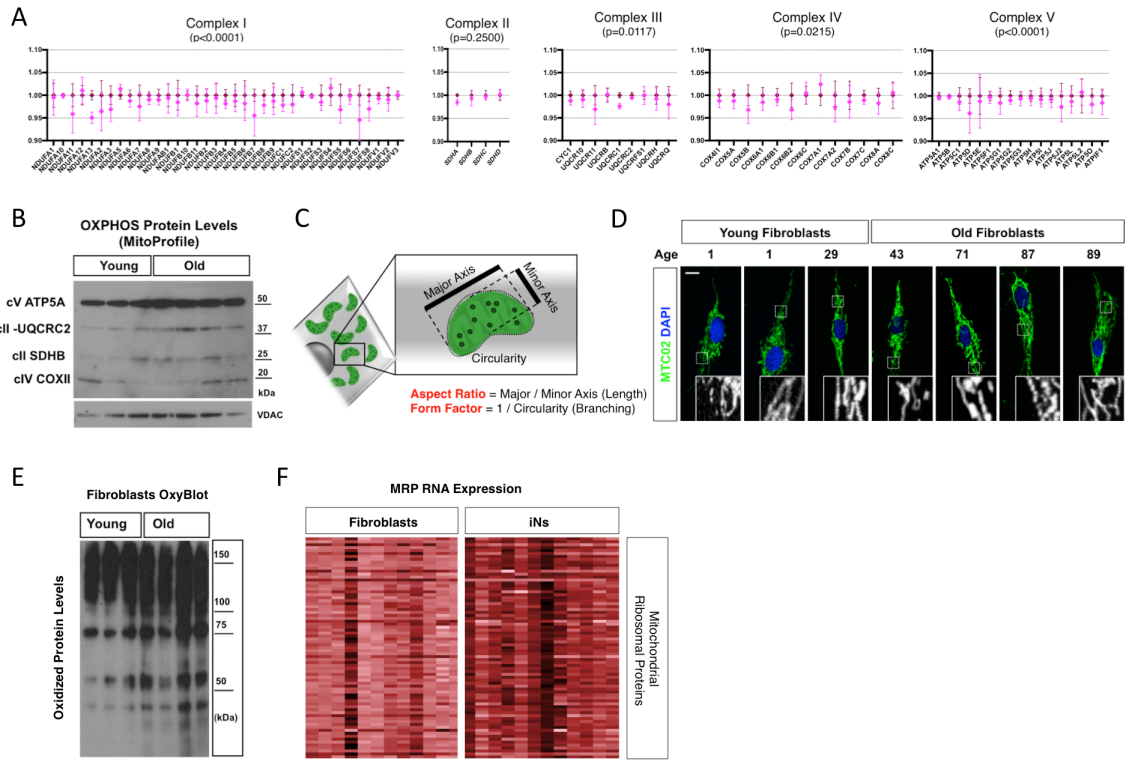
(**F**) Quantification of axonal mitochondrial morphologies for densities, mean lengths and coverage in iPSC-Neurons (6 experimental replicates per individual). Box plots show 25th to 75th percentiles, bars indicate medians, and whiskers show ranges. Significance values calculated by unpaired t-tests.

(**G**) Quantification of the MMP in iPSC-Neurons (6 replicates per individual). Bar graph shows means ± SD, triangles indicate individual measurements. Significance values calculated by Mann-Whitney test.

(**H**) ATP levels in iPSC-Neurons (4 replicates per individual). Bar graph shows means ± SD, triangles indicate individual measurements. Significance values calculated by Mann-Whitney test.

(**I-J**) Quantification and representative image of oxidized protein levels (OxyBlot) in iPSC-Neurons.

Figure S4. Mitochondrial features in aging fibroblasts, Related to Figure 3



(**A**) Relative expression of ETC complex I, II, III, IV and V in young and old fibroblasts. VST normalized counts normalized to young; dots indicate mean ± SEM; significance values for each complex calculated by Wilcoxon test.

(**B**) Representative MitoProfile blot for protein levels of indicated OXPHOS genes in young and old iNs. Quantitative data was normalized to VDAC.

(**C**) Schematic diagram of fibroblast mitochondrial morphology indexes: aspect ratio and form factor.

(**D**) Images of mitochondria with mitochondrial marker (anti-MTC02 antibody, green) and DAPI (blue) in young and old fibroblasts. The bottom panels indicate magnified images of MTC02 from boxed regions. Scale bar: 20 μm.

(**E**) Representative OxyBlot for oxidized proteins in young and old fibroblasts.

(**F**) Heatmap showing relative expression of mitochondrial ribosomal protein (MRP) genes in 12 fibroblast and 12 iN cultures. Darker color indicates higher expression. See also Table S3.

Table S1. Human fibroblasts used in this study, Related to Supplemental Experimental Procedure

Age [yr]	ID	Full Name	Sex	Health	Fibroblast Source	Fibroblast RNAseq Passage
0	BJ	BJ CRL-2522	m	healthy	ATCC	p13
0	GM	GM22159	m	healthy	Coriell	p16
1	O6	AG08498	m	healthy	Coriell	p16
1	G59	GM05659	m	healthy	Coriell	p16
29	A54	AG04054	m	healthy	Coriell	p5
29	E1	ERF1	f	healthy	Erlangen	p10
30	A53	AG13153	m	healthy	Coriell	p5
43	E4	UKERf1JF-X-001	m	healthy	Erlangen	p12
66	A03	AG07803	m	healthy	Coriell	p14
71	E30	UKERfO3H-X-	m	healthy	Erlangen	p11
87	A84	AG10884	m	healthy	Coriell	p10
89	A29	AG13129	m	healthy	Coriell	p9

Table S2. Antibodies, Related to Supplemental Experimental Procedure

Antigen and species	Manufacturer	ICC dilution	WB dilution
MTC02 ms	Abcam	1:500	
SDHB ms	Abcam		1:1,000
UQCRC2 ms	Abcam		1:1,000
COXII ms	Abcam		1:1,000
ATP5A ms	Abcam		1:1,000
VDAC rb	Cell Signaling Technology		1:1,000
SMI-312 ms	Abcam	1:200	
TuJ rb	Millipore	1:3000	
vGlut rb	Synaptic Systems	1:1000	
GABA rb	Sigma Aldrich	1:300	
Map2ab chick	Sigma Aldrich	1:500	
MTC02	Thermo Fisher Scientific	1:100	
Nanog gt	R&D Systems	1:200	
Tra-1-81 ms	Millipore	1:200	
Sox2 rb	Cell Signaling	1:250	
Nestin ms	Millipore	1:200	

Conjugate and species detected	Manufacturer	ICC dilution	WB dilution
Alexa488 rb ms	Thermo Fisher Scientific Scientific	250	
Alexa647 ms-IgG chicken	Thermo Fisher Scientific Scientific	250	
Alexa555 rb ms	Thermo Fisher Scientific Scientific	250	
HRP rb ms	Jackson		5,000

Supplemental Experimental Procedures

Fibroblasts culture and direct conversion into iNs. Primary human dermal fibroblasts from donors between 0 and 89 years of age were obtained from the Coriell Institute Cell Repository, the University Hospital in Erlangen and ATCC (Table S1). Protocols were previously approved by the Salk Institute Institutional Review Board and informed consent was obtained from all subjects. Fibroblasts were cultured in DMEM containing 15% tetracycline-free fetal bovine serum and 0.1% NEAA (Thermo Fisher Scientific), transduced with lentiviral particles for EtO and XTP-Ngn2:2A:Ascl1 (N2A) and expanded in the presence of G418 (200 µg/ml; Thermo Fisher Scientific) and puromycin (1 µg/ml; Sigma Aldrich) as 'iN-ready' fibroblast cell lines. Following at least 3 passages after viral transduction, 'iN-ready' fibroblasts were trypsinized and pooled into high densities (30.000 – 50.000 cells per cm^2; appx. a 2:1 - 3:1 split from a confluent culture) and, after 24h, the medium was changed to neuron conversion (NC) medium based on DMEM:F12/Neurobasal (1:1) for 3 weeks. NC contains the following supplements: N2 supplement, B27 supplement (both 1x; Thermo Fisher Scientific), doxycycline (2 µg/ml, Sigma Aldrich), Laminin (1 µg/ml, Thermo Fisher Scientific Scientific), dibutyryl cyclic-AMP (500 µg/ml, Sigma Aldrich), human recombinant Noggin (150 ng/ml; Preprotech), LDN-193189 (5 µM; Fisher Scientific Co) and A83-1 (5 µM; Santa Cruz Biotechnology Inc.), CHIR99021 (3 µM, LC Laboratories), Forskolin (5 µM, LC Laboratories) and SB-431542 (10 µM; Cayman Chemicals). Medium was changed every third day. For further maturation up to 6 weeks, iNs were switched to BrainPhys (STEMCELL Technologies)-based neural maturation media (NM) containing N2, B27, GDNF, BDNF (both 20 ng/ml, R&D), dibutyryl cyclic-AMP (500 µg/ml, Sigma Aldrich), doxycycline (2 µg/ml, Sigma-Aldrich) and laminin (1 µg/ml, Thermo Fisher Scientific Scientific). For maturation on astrocytes for morphological analysis, iNs were carefully trysinized during week 4 and replated on a feeder layer of mouse astrocytes and cultured in NM media containing 1% KOSR (Thermo Fisher Scientific).

Mitochondrial morphological analysis in iNs and fibroblasts. Following conversion for 3 weeks, iNs were transduced with a lentiviral vector encoding MitoEGFP under the human synapsin promoter (hSyn) and subsequently cultured on a layer of mouse astrocytes for another 3 weeks (6 weeks in total). The fibroblasts were plated on a µ-slide 8-well slides (Ibidi, Germany). The cells were fixed in 4% paraformaldehyde and 4% sucrose and then permeabilized with 0.05% Triton-X100 in Tris-Cl buffer solution (TBS). The cells were then blocked in TBS containing 3% bovine serum albumin (BSA) for 1 h, followed by incubation with primary antibody overnight at 4°C. After 4 time washes with TBS, cells were incubated with secondary antibodies for 1 h at room temperature. Following 3 time washes with TBS, cells were incubated with DAPI (0.1 µg/ml, Sigma) for 15 min, followed by 3 time washes with TBS to remove DAPI. Fluorescent signals were detected using a LSM780 confocal microscopy (Carl Zeiss). The primary antibodies used were mouse anti-MTC02 antibody (1:500, abcam, ab3298) and mouse anti-pan axonal neurofilament antibody (1:200, Abcam, SMI-312, ab24574). The GFP signal (MitoEGFP) and green fluorescence (MTC02) were used to analyze the mitochondrial number, size, coverage, aspect ratio, and form factor using the Particle Analysis tool in ImageJ software (National Institute of Health).

Whole transcriptome mRNA sequencing and analysis. Following 3 weeks of iN conversion, iNs were detached and stained for PSA-NCAM (1:100; PE- or APC-coupled from BD Biosciences) for 45 min at 4°C, resuspended in sorting buffer containing EDTA and DNAse and filtered using a 40-µm cell strainer. The PSA-NCAM-positive population was sorted directly into Trizol-LS and RNA was isolated according to the manufacturer's instructions and digested with TURBO DNAse (Thermo Fisher Scientific). RNA from fibroblasts was directly extracted from confluent cultures. RNA integrity (RIN) numbers were assessed using the Agilent TapeStation before library preparation. RNA-Seq libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit according to the manufacturer's instructions (Illumina). Libraries were sequenced single-end 50 bp using the Illumina HiSeq 2500 platform. Read trimming was performed using TrimGalore, read mapping was performed using STAR, raw counts were generated using HTseq and variance stabilizing transformation normalization (vst) and differential expression analysis was performed in DEseq2. Filtering for 1,158 mitochondrial genes was performed using the Human MitoCarta 2.0 atlas from the Broad Institute (Table S3).

Western blotting. Cell lysates were prepared in Lysis buffer A (20mM Tris pH 7.5, 100mM NaCl, 1mM EDTA, 2mM EGTA, 50 mM β -glycerophosphate, 50 mM NaF, 1 mM sodium vanadate, 2 mM dithiothreitol, proteinase inhibitor cocktail (Roche) and 1% Triton X-100) and subjected to Western blot according to the standard procedures. The primary antibodies used were mouse anti-Total OXPHOS human WB antibody (1:1000, Abcam, ab110411) and rabbit monoclonal anti-VDAC antibody (1:1000, Cell Signaling, D73D12, #4661). All relevant information of the antibodies used in this study including citation, clone number and antibody validation profile can be found at the manufacturer's website (Table S2). OxyBlot (Abcam) detection of oxidized proteins was used as recommended in the manufacturer's manual.

ATP analysis. For measurement of cellular ATP content, the cells were lysed directly on plates with passive lysis buffer (Promega) by 2 freeze-and-thaw cycles (-20°C). The ATP content was quantified by Cell Titer-Glo Luminescent Cell Viability/ATP Assay kit (Promega) using Victor X Lite Multilabel Plate Reader (PerkinElmer) and normalized by protein content measured by a Pierce BCA protein assay kit (Thermo Fisher Scientific Scientific).

Immunocytochemistry. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA, 20 min, RT) and blocked with 5% horse serum in PBS and 0.1% TritonX-100. Primary antibodies (Table S2) were incubated overnight at 4°C, washed for 5 min with DAPI solution, incubated with secondary antibodies (Table S2) for 45 min at room temperature (RT) and mounted in PVA-DABCO (Sigma Aldrich) or Shandon Immu-Mount mounting solution (fisher scientific). For confocal imaging, cells were grown on ibidi μ-slides. Antibodies are listed in Supplemental Table S2.

Quantitative RT PCR. RNA was isolated using Trizol and subsequently reversely transcribed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). qPCR was performed using SYBRgreen Real-Time Mastermix (BioRad) and fold changes of mRNA levels were calculated using the delta-delta-Ct method using Microsoft Excel. The following primer sequences were used: GAPDH (fw: TGACAACTTTGGTATCGTGGA, rv: CCAGTAGAGGCAGGGATGAT), MT-ND1 (fw: ATGGCCAACCTCCTACTCCT, rv: GCGGTGATGTAGAGGGTGAT),

iPSC reprogramming and neuronal differentiation.
Fibroblasts were transduced with retroviruses for the four Yamanaka factors and resulting iPSC lines were cultured in STEM-TeSR (Salk Stem Cell Core) on BME matrix-coated (Trevigen) dishes. Following neural differentiation into neural progenitor cells (previously described in Boyer L et al., Current Protocols in Stem Cell Biology 2012), neuronal differentiation was induced by growth factor withdrawal. For RNAseq, PSA-NCAM-positive (Miltenyi) and CD29-low (BD) neurons were isolated by FACS.

Statistical analysis. Statistical values for RNA-Seq data were corrected for false discovery rates (FDR) using the Benjamini-Hochberg method implemented in R. Statistical tests of quantitative data were calculated using GraphPad Prism 7 software with the method indicated for each figure. Significance evaluation are marked as *$p<0.05$; **$p<0.01$; ***$p<0.005$ and ****$p<0.001$.