

Supplemental Information

Human Dopaminergic Neurons Lacking PINK1

Exhibit Disrupted Dopamine Metabolism

Related to Vitamin B6 Co-Factors

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Transparent Methods

Ethics statement and data protection

All procedures were in accordance and approved by the ethical board at The University of Tübingen and according to the international standards defined in the declaration of Helsinki. Human samples were obtained with consent and prior ethical approval at The University of Tübingen and the Hertie Institute for Clinical Brain Research Biobank number 146/2009B01. Medical Faculty of the University of Tübingen (<https://www.medizin.uni-tuebingen.de/de/medizinische-fakultaet/ethikkommission>).

RAW sequences of RNA and peptides will not be made freely available according to EU and German data protection laws to protect the identity of the healthy donor used as an isogenic control in this study. See data availability below.

Generation of human induced pluripotent stem cells (hiPSCs) and *PINK1* knockout

hiPSCs were cultured in self-made E8 media on Vitronectin (VTN-N, Gibco) coated cell culture dishes. iPSCs from a healthy individual that were previously characterized (Reinhardt et al., 2013) were transfected with a TALEN and a homologous construct for *PINK1* exon1 using an Amaxa Nucleofector II with the Stem cell Nucleofection Kit (both from Lonza). The transfected iPSCs were plated on VTN-N –coated 10cm dishes in E8 medium containing 10 μ M ROCK inhibitor Y27632. Homologous recombined iPSC colonies were selected with 250 μ g/ml G418 (Biochrome) or 10 μ g/ml-1 Blasticidin (InvivoGen) in the second round of TALEN

transfection and re-plated in 12-well plates. Resistant iPSC colonies were characterized by sequencing, qRT-PCR and Western blot to confirm successful homozygous gene knockout. TALENs were designed with the online tool TALE Effector Nucleotide Targeter 2.0 (Cornell University) and generated using a cloning protocol adapted from (Cermak et al., 2011). The following RVD sequences were used for the TALEN monomers: HD HD NI NH NH NG NH NI NH HD NH NH NH NH HD and NI NH HD NG HD HD NH NG HD HD NG HD HD NH HD. Colony-PCR after the first TALEN reaction was conducted with the primers pCR8_F1 (5'-TTGATGCCTGGCAGTTCCT-3') and pCR8_R1 (5'-CGAACCGAACAGGCTTATGT-3'). After the second Golden Gate reaction the colony-PCR was performed with the primers TAL_F1 (5'-TTGGCGTCGGCAAACAGTGG-3') and TAL_R2 (5'-GGCGACGAGGTGGTCGTTGG-3').

Generation of *PINK1* Q126P hiPSCs and subsequent gene correction

Human fibroblasts were derived from a skin biopsy of a female PD patient with a *PINK1* Q126P mutation in Tübingen of which the family was previously described (Prestel et al., 2008). Human samples were obtained with consent and prior ethical approval at The University of Tübingen and the Hertie Institute for Clinical Brain Research Biobank. The *PINK1* Q126P 15167 hiPSC line was generated using the CoMIP 4in1 method. Reprogramming and characterization (pluripotency, stability of karyotype, and differentiation potential) was performed in the iPSC Core Facility of Helmholtz Zentrum München. *PINK1* Q126P hiPSCs were gene corrected using pCAG-Cas9 vector (Addgene plasmid 84918) and a donor vector carrying a cassette

flanked by a *PINK1* 5'homology arm containing the g.155732C>A and a 3' homology arm. The hiPSCs and the gene corrected line were kindly provided through collaboration with Daniela M. Vogt Weisenhorn and Wolfgang Wurst (Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Developmental Genetics, Munich-Neuherberg, Germany) as part of a BMBF MitoPD project.

Derivation of NPCs from iPSCs and differentiation into mature, human, mid-brain specific dopaminergic neurons (hDANs)

Mature hDANs were generated from iPSCs via neural progenitor cells (NPC) intermediates using a protocol adapted from (Reinhardt et al., 2013a). The healthy control line (K7.1) has been previously fully characterized and described (Reinhardt et al., 2013a). Two distinct PINK1 KO iPSC clones devoid of PINK1 transcript were used in this study (PINK1 KO1-Δ8.9 and PINK1 KO2-Δ40.7). Briefly, iPSCs were maintained in E8 medium. For the generation of embryoid bodies (EBs), iPSCs were cultured in '50:50 base medium' (one to one mixture of DMEM Hams F12 (#FG4815 Biochrome/Millipore): Neurobasal® medium (#21103-049 Gibco/Thermo Scientific), 1X Penicillin/Streptomycin (Biochrome/Millipore), 1X GlutaMAX supplement Thermo Scientific), 1X B27 supplement (Gibco/Thermo Scientific) and 1X N2 supplement (Gibco/Thermo)) plus the addition of 10μM SB431542 (Sigma, SB), 1μM dorsomorphin, 3μM CHIR99021 (CHIR, Axon) and 0.5μM pumorphamine (PMA, Alexis) on uncoated 6-well cell culture plates. EBs were then transferred to Matrigel (Corning)-coated 6-well plates in NPC maintenance media ('50:50 base medium' plus the addition of 150μM Ascorbic Acid (AA, Sigma), 3μM CHIR, 0.5μM PMA).

After several passages NPCs were cultivated in NPC priming medium ('50:50 base medium' plus the addition of 150 μ M AA and 3 μ M CHIR99021). Differentiation of confluent NPCs was initiated by cultivation in a patterning medium for seven days ('50:50 base medium' plus the addition of 10ng/mL FGF8 (Peprotech), 1 μ M PMA, 200 μ M AA, 20ng/mL BDNF (Peprotech). The differentiating neurons were matured in maturation media ('50:50 base medium' plus the addition of 10ng/mL BDNF, 10ng/mL GDNF (Peprotech), 1ng/mL TGF β -III (Peprotech), 200 μ M AA, 500 μ M dbcAMP (Appllichem) and 10 μ M DAPT (Sigma).

Removal of antioxidant supplements and apoptosis inhibitors

hDANs require N2 and B27 supplements (GIBCO) and ascorbic acid in their culture media. We also routinely use apoptosis inhibitors for splitting and plating NPCs and hDANs, Prior to all experiments, the maturation medium was replaced 24h before the experiment with 'N2 medium' (using 50:50 base media without the B27 supplement or ascorbic acid) to reduce excessive amounts of antioxidants in the media that could quench phenotypes. Also, no apoptosis inhibitors such as APOI/ROCK inhibitor were used after the final plating of the hDANs throughout maturation.

Immunofluorescence staining

Mature hDANs were cultivated on Matrigel-coated glass coverslips and fixed with 4% (w/v) paraformaldehyde (PFA, Sigma) in phosphate buffered saline (PBS) for 15 min at room temperature (RT). Permeabilization with ice-cold, neat methanol for 5 min at

-20°C. After washing with 0.01% (v/v) Tween in PBS (PBS-T), the fixed hDANs were blocked with 5% (v/v) normal goat serum in PBS-T for 1h at RT. Afterwards the cells were washed again and incubated overnight at 4°C with the primary antibody in 2.5% (v/v) serum in PBS-T. On the following day the cells were incubated with the secondary fluorescent antibody (#A32721, #A11070, #A21449, #A11010 all from Molecular probes/Thermo Scientific) for 2h at RT in darkness and nuclei stained with DAPI (Sigma). Coverslips were mounted in mounting medium (Dako) on glass slides. Immunofluorescence was imaged using an AxioVert fluorescence microscope (Zeiss). MAP2 (AbCam ab5392) (n=diff5), TH (AbCam ab112) (n=diff5), FOXA2 (Millipore AB4125) (nDiff 3), DAT (Millipore MAB369) (nDiff3). Statistics Figure 1B: Blinded IF images were counted manually (IF positive cell as a percent of total cells in a field of view). All values were listed in Graphpad Prism in columns (nDiff=4). The number of % IF positive values from each field of view are as follows; CTRL MAP2 (68), PINK1 KO clones 1 and 2, MAP2 (98), CTRL FOXA2 (31), PINK1 KO clones 1 and 2 FOXA2 (49), CTRL TH (68), PINK1 KO clones 1 and 2, TH (89), CTRL DAT (37) and PINK1 KO clones 1 and 2, DAT (49). Ten outliers were removed by Graphpad Prism (all from CTRL TH). Significance test; All data sets was tested for normal Gaussian distribution using Graphpad Prism (D'Agostino & Pearson test). FOXA2 data was normally distributed and the Unpaired t-test used (ns = not significant). For the remaining non-normally distributed data, the non-parametric Mann Whitney U-test was used. **** = $p < 0.0001$. Levene's test of unequal variance was performed in Excel followed by single ANOVA (TH marker CTRL v PINK1 KO $p0.0206$).

Quantitative Reverse Transcription PCR (qRT-PCR)

RNA was isolated from mature hDANs (and NPCs) using a RNeasy Mini Kit (QIAGEN), including the on-column DNA digestion step. A one-step qRT-PCR was performed on 0.1-1 μ g RNA (equalized to the same input amount) with the QuantiTect SYBR Green RT-PCR Kit (QIAGEN) on a LightCycler®480 (Roche). The relative expression levels were calculated with the $2^{-\Delta\Delta C_t}$ method, based on a biological reference and housekeeping genes (GAPDH and HMBS) for normalization. Statistics

Figure 1C: All $\Delta\Delta C_t$ gene of interest/GAPDH were normalised to the CTRL in each case. All values were listed in Graphpad Prism in columns (nDiff=4, except vGlut nDiff = 3). The number of values; CTRL (12), PINK1 KO clones 1 and 2 vGLUT (3), PINK1 KO clones 1 and 2 TH (12), PINK1 KO clones 1 and 2 MAO-A, (11), PINK1 KO clones 1 and 2, MAO-B (10), PINK1 KO clones 1 and 2, DAT (6), PINK1 KO clones 1 and 2, SYP (6), PINK1 KO clones 1 and 2 THP2 (5) and PINK1 KO clones 1 and 2, MAP2 (6). One outlier was removed by Graphpad Prism (from THP2).

Significance test; All data sets were tested for normal Gaussian distribution using Graphpad Prism (D'Agostino & Pearson test). TH (**=p0.0063), MAO-A (ns = not significant) and MAO-B (ns = not significant) data were normally distributed and the unpaired T-test used. For the remaining data normality could not be assigned due to low n, the non-parametric Mann Whitney U-test was used. vGlut (**=p0.0022), TPH2 (**= p<0.0002), MAP2 (**=p0.0043). Statistics Figure 4C and 4D: All $\Delta\Delta C_t$ gene of interest/GAPDH were listed in Graphpad Prism in columns. One column was made for both PINK1 KO clones. The data was not normalized to the control in each PINK1 line (except for PINK1 Q456X where there are 3 patients, the $\Delta\Delta C_t$ data is normalized to each corresponding gene corrected control line). No outliers were removed by Graphpad Prism. Significance test; assuming normal distribution with

unequal standard deviation, the student's t test with Welch correction was performed. PINK1 exon 1-2 (****= $p<0.0001$ for PINK1 KO v CTRL and PINK1 Q456X v GC CTRL), PNPO (****= $p<0.0001$ for PINK1 v CTRL, **= $p=0.0027$ for PINK1 Q456X v GC CTRL), DDC (**= $p=0.0010$ for PINK1 Q126P v GC CTRL), TPH1 (**= $p=0.0035$ for Q126P v GC CTRL). All t test comparisons were made for all other CTRL and PINK1 lines individually shown for each gene and were not statistically significant. Then all the $\Delta\Delta C_t$ values for each gene were listed in two columns in Graphpad Prism (all CTRL/WT v all PINK1 KO/mutation) and each data set for each gene was tested for Gaussian distribution using the D'Agostino & Pearson test. The student's t test with Welch correction; PINK1 (****= $p<0.0001$), DDC (ns= $p=0.0773$), TPH1 (ns= $p=0.1055$), TPH2 (ns= $p=0.7855$), PDXK (ns= $p=0.8046$), DNAJC12 (ns= $p=0.8663$). The non-parametric Mann Whitney U-test; PNPO (*= $p=0.0192$), TH (ns = $p=0.53$). nDiff=3. Levene's variance test was performed in Excel followed by single ANOVA (TH expression GC v Q126P, $p=0.0000137$).

Primer sequences

DNAJC12 (FW:TCACCCAGACAAGCATCCTGA, RV:

TTACCTCTGACAACCCAGTGC); **TPH1** (FW: AACCCATGCTTGCAGAGAGT, RV:

GCCACAGGACGGATGGAAAA); **TPH2** (FW: GTGGATGTGGCCATGGGTTA, RV:

TGGAGAGCTCCCGGAATACA), **PDXK** (FW: GGGATTTGAGATTGACGCGG, RV:

GGGACGTACATCGAGCCTTC); **DDC** (FW:GAGCCAGACACGTTTGAGGA, RV:

TAGGCGAAGAAGTAGGGGCT); **PNPO** (hFW: AGTCGAAAAGGAAAAGAGCTG;

hRV: GGCGGGAGTGGAAGTAG),

(msFW:CTGAACCGTCAGGTGCGTGTGGAAGGC,

msRV:AAGGTGCAAGTCTCTCATACACCCAGTCT); **TH** (FW: TGTCTGAGGAGCCTGAGATTCG; RV: GCTTGTCTTGGCGTCACTG); **PINK1** (exon 1 FW: GGGTCGAGCGCTGCTGCTGCGCTT; exon 2 FW: TCCGGGGGCCCCTGCCTTCC; exon 4 RV: TTGCTTGGGACCTCTCTTGG); **vGLUT1** (FW: GAGTGGCAGTACGTGTTCT; RV: TCCATTTCGCTGTCGTCCT); **MAO-A** (FW: GCCCTGTGGTTCTTGTGGTATGT; RV: TGCTCCTCACACCAGTTCTTCTC); **MAO-B** (FW: ACTCGTGTGCCTTTGGGTTCAG; RV: TGCTCCTCACACCAGTTCTTCTC); **DAT** (FW: CAAAAGCTGCTTTCCATGGCACACT; RV: CGGCTCCCACCGAGCATTACACT); **SYP** (FW: CAGGGTGGGGCTTAGAATGG; RV: GTGTGTGTGGTGGGGTGCTT); **MAP2** (FW: CCGTGTGGACCATGGGGCTG; RV: GTCGTCGGGGTGATGCCACG); **CP** (FW:CTTAACAGCACCTGGAAGTG; RV:TTGTGAAGGAGGCATCTGTG); **GCLC** (FW:TGAGCTGGGAGGAAACCAAG; RV: AACATGTATTCCACCTCATCGC); **STEAP3** (FW:TTCAGCTTCGTTTCAGTCCTC, RV:AGGCAGGTAGAACTTGTAGC); **SLC7A11** (FW:CTTTCAAGGTGCCACTGTTC, RV: GATAATACGCAGGGACTCCA); **Mitoferrin2** (FW: CCATCGACTGCGTCAAGACC; RV: CAAAATAAAGGGCGTGGGCA).

Autophagy and mitophagy induction

Autophagic flux was induced in mature hDANs using Valinomycin (Val, 1μM, 24h) or NH₄CL (20mM, 4h) and Leupeptin (Leu, 200μM, 4h) nDiff=3. Mitophagy was induced in hDANs using; 10μM CCCP for 2h, 4h, 6h, (6h+ 10μM MG132) and 24h or 1μM Valinomycin for 24h nDiff=3.

SDS-PAGE and Western blotting

All cell lysates were prepared in RIPA buffer (Sodium chloride 150mM, Tris-HCL 50mM, Sodium dodecyl sulfate 0.10% (w/v), Sodium deoxycholate 0.50% (w/v), Triton-X-100 1% (v/v)) containing 1X concentration of phosphatase inhibitor cocktail (Complete, Roche) and phosphatase inhibitor cocktail (PhosSTOP, Roche). Briefly, the lysis buffer was added directly to washed cells in dishes or washed cell pellets and kept at 4 °C. Needles were used to further homogenize the lysates (9 passes 20G, 9 passes 27G) and incubated 30 minutes on ice. Insoluble nuclear material was removed after centrifugation at 14,000 rpm for 10 minutes. Proteins were electrophoresed on self-made acrylamide gels or pre-cast Bis-Tris gels (Thermo Scientific) and transferred to nitrocellulose membranes using the iBlot device (Thermo Scientific), with the exception of very large target proteins or heavily lipidated proteins, in which case wet blotting with PVDF membranes was used. Total protein stain Ponceau (Applichem) was used to assess transfer and loading and the PageRuler plus pre-stained protein ladder (Thermo Scientific) for kDa range. Antibodies against PINK1 (Novus), Mitofusin1 (MFN1, AbCam), TOM70 (Santa Cruz), OPA1 (BD Biosciences), Parkin (Cell Signalling Technology), *m*Aconitase (BD Biosciences), β -Actin (Sigma Aldrich), GAPDH (Invitrogen, Thermo Scientific) Tom20 (Santa Cruz Biotechnology), LC3I/II (Novus), PGC1 α (AbCam), MIRO-1 (Sigma Aldrich), OPA1 (Novus Biologicals), TH (Millipore), Vinculin (Sigma-Aldrich), MAO-A (In-house produced monoclonal antibody from Ellen Billett, Nottingham Trent University, UK), NECAB2 (a kind gift from Axel Methner, Johannes Gutenberg University Mainz / Germany) and Mitobiogenesis antibody (containing SDHA, GAPDH and MTCO1, AbCam) were used. Secondary antibodies were purchased

from GE Healthcare (HRP-conjugated) and from LiCOR (α -rabbit and mouse Alexa FluorTM680, α -rabbit and mouse Alexa FluorTM800). Fluorescence detection and analysis were performed using a LI-CORE blot scanner and Image StudioTM Lite software. Densitometry from Western blot was performed using the Image J 1.41o software (Wayne Rasband; National Institutes of Health, USA). Representative Western blots are shown. For calculating mitochondrial turnover statistics Figure S2D/E. Densitometry data was normalized and no outliers removed. Assuming normal distribution, the student's t test was assigned and ns=not significant, $*=p<0.05$. nDiff=3. Levenes test of variance was also performed in Microsoft Excel followed by single ANOVA (Untreated mitobiogenesis, $p>0.050$, valinomycin treated mitobiogenesis $p0.030$).

Complex I Dipstick Assay

Active Complex I was pulled down from mature hDAN homogenates using the Complex I Dipstick Assay from AbCam (ab109720) according to the manufacturer's instructions. The dipsticks were immediately scanned and the band densitometry quantified using the Image J 1.41o software (Wayne Rasband; National Institutes of Health, USA). Statistics Figure 1F and G: All densitometries derived from scanning the dipstick (nDiff=3) were listed in Graphpad Prism in columns. There were three values for each data set and therefore the test for Gaussian distribution could not be performed. Assuming non-normality, the Mann Whitney U-test was performed (Figure 1G, $*=p0.023$, Figure 1F, ns =not significant). Assuming normality, the t test was performed (Figure 1F, $*=<0.05$). 1-way ANOVA with the Kruskal Wallis test was

performed assuming a possible mixture of distributions (Figure 1F, p0.036 and Figure 1G, p0.0003).

Transmission Electron Microscopy

Mature hDANs were seeded on Matrigel (Corning) coated glass coverslips and cultivated for three days prior to treatment with N2 medium with and without 1 μ M Valinomycin for 24 hours. After washing and fixation with 2.5% glutaraldehyde (Science Services, Munich, Germany) in cacodylate buffer (pH7.4; Merck-Millipore, Darmstadt, Germany) overnight at 4°C, cells were washed with cacodylate buffer, post fixed in 1% osmiumtetroxide, dehydrated and embedded in epoxide resin (Araldite, Serva, Heidelberg, Germany) as described previously (Wolburg-Buchholz et al., 2009). Ultrathin sections were performed using a Reichert Ultracut ultramicrotome (Leica, Bensheim, Germany) and were analyzed in an EM 10 electron microscope (Zeiss, Oberkochen, Germany). Images were taken by a digital camera (Tröndle, Germany). Qualitative data, representative images are shown, nDiff=3. The high quality images are available on request.

Mitochondrial Morphology (BacMam Mitogreen)

NPCs and mature hDANs were plated on Matrigel covered glass coverslips, treated with CellLight™ Mitochondria-GFP, BacMam 2.0 (Invitrogen) according to manufacturer's instructions for 24 hours in N2 medium. Mitochondria of transfected neurons were imaged using an LSM-510 confocal microscope (Zeiss). Z-stack images were analysed with ImageJ (Fiji, Rasband, W.S., ImageJ, U. S. National

Institutes of Health, Bethesda, Maryland, USA). Statistics Figure 2B: Blinded Z stack IF images were analyzed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>). All values for mitochondrial area were listed in Excel per cell for each field of view (nDiff=4). The data was normalised to the isogenic control for hDANs and for NPCs. No outliers were removed and distribution analysis (D'Agostino & Pearson test) was not possible because nDiff=4. Significance test; Non-normal distribution was assumed and the non-parametric Mann Whitney U-test was used. Ns = not significant, *=p<0.05, **=p<0.005. Statistics Figure S2A: Blinded IF images were analyzed using particle analysis with IMARIS software and ImageJ (Fiji) across z stacks.

Mathematical calculations for mitochondrial circularity, aspect ratio (length) and form factor (degree of branching) was performed using ImageJ (Fiji), as follows; briefly, images were converted to 8-bit greyscale, → 'despeckle', → 'convolve', → 'subtract background', → 'create binary' and 'adjust threshold' (the threshold must remain the same for all images). 'Set measurements', check Area, Perimeter and Fit ellipse. 'Analyze particles'; Smallest particle set to 1 pixel, → 'Show outlines'. Data for each mitochondria is listed and raw data copied in Excel. Mean values for each measurement per image is used. Form factor (or degree of branching is the perimeter squared, divided by ($4\pi \times \text{area}$)). The data was set in columns in Graphpad Prism in columns (nDiff=4). No outliers were removed and distribution analysis (D'Agostino & Pearson test) was not possible because n=Diff4. Significance test; Non-normal distribution was assumed and the non-parametric Mann Whitney U-test was used. ns = not significant.

Calcium imaging

Mature hDANs were seeded on Matrigel-coated glass coverslips. Fluo-4 Direct Calcium Reagent (Invitrogen) was added to the cells and incubated for one hour at 37°C and 5% CO₂. After washing the cells with growth medium, the 24mm coverslips were transferred into self-made imaging chambers and Fluo-4 reagent diluted in growth medium was added. 3 mM EGTA was added per well 10 minutes prior to imaging. Neurons were imaged on a LSM 510 Confocal microscope (Zeiss) taking a picture every 0.5 sec for 25 minutes. A baseline was recorded for two minutes and then 2 µM Thapsigargin was added. The data analysis was performed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>). The corrected total cell fluorescence per cell was determined for 10 neurons per experiment and plotted over time to evaluate the calcium signal. To account for variability in Fluo4 signal between imaging sessions we always normalized the Fluo4 signal to the baseline (average Fluo4 signal before the addition of Thapsigargin). Only PINK1 KO clone 1 passed the D'Agostino & Pearson normality test. Therefore, ordinary two-way ANOVA was used for statistical analysis. The data was statically significant comparing genotype across time (****= $p < 0.0001$).

Flow cytometry experiments

Mature hDANs were carefully washed in PBS and then treated with Accumax (PAN Biotech) to remove them from the monolayer, quenched in PBS and centrifuged at

300 g for 5 minutes and then incubated in dye, buffer only or dye plus a control. For mitochondrial membrane potential, 200 nM Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE, from Thermo Scientific) in PBS or TMRE plus Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (CCCP, Sigma Aldrich) 10 μ M was used. Cells were measured using a MACSQuant® automated flow cytometer (Mitenyi Biotechnology) according to their mean average fluorescence signal. All mean average fluorescence values were divided by the background fluorescence in the same channel in the same unstained cells to account for auto-fluorescence. Statistics Figure 2D (left panel): Assuming normal distribution, the t test was performed. **= $p < 0.0026$. ns=not significant. nDiff=3.

Live Cell Kinetic Measurement of Mitochondrial Membrane Potential

Cells were seeded in Ibidi® dishes and the media exchanged for HBSS containing 200 nM TMRE stain (Thermo Scientific) for 15 minutes at 37°C with CO₂. The TMRE was removed and replaced with 360 μ L Hanks buffer. The cells were imaged using a Zeiss Inverted Confocal microscope at Excitation HeNe1, 543 nm and Emission LP 560 nm and brightfield for 20 \times 4s cycles. Followed by the addition of 360 μ L (0.25 mg/ml Oligomycin), measured for 20 \times 4s cycles, 180 μ L (10 μ M Rotenone), measured for 20 \times 4s cycles and 100 μ L (10 μ M FCCP) and measured for 20-40 \times 4s cycles. Using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>), each frame was analysed for TMRE fluorescence intensity, mean fluorescence and total area. The corrected total cell fluorescence (CTCF) over time was calculated using the formula: $CTCF = \text{fluorescence intensity} - (\text{cell area} \times \text{mean background fluorescence})$. Statistics:

Figure 2D (right panel): $p < 0.0001$, 2-way ANOVA (CTCF across genotype and time) for corrected total cell fluorescence from live imaging mean CTRL v PINK1 KO1 and KO2 hDANs. $n_{\text{Diff}}=3$.

ATP Assay

ATP was measured using the ViaLight™ Cell Proliferation BioAssay Kit according to the manufacturer's instructions (Lonza) and normalized to the total protein content in each well. Luminescence was detected using SpectraMax L plate reader (Molecular Devices). Technical replicates =3 in each assay and $n_{\text{Diff}}=4$. The mean data from each independent experiment comparing PINK1 KO1 and PINK1 KO2 to control was tested using the Mann Whitney U Test and the differences were not significant.

Mitochondrial turnover using MitoTimer™ plasmid

Mitotimer is a fluorescent reporter suitable for the investigation of mitochondrial turnover. Based on oxidation state, the fluorescent protein (dsRed mutant) targeted to the mitochondria shifts its emission spectra from green to red as the protein matures (Hernandez et al., 2013). Approximately 100,000 hDANs were seeded per 24-well well on coated coverslips in maturation medium. Mature hDANs were transfected with pMitoTimer from Zhen Yan (Addgene plasmid # 52659; <http://n2t.net/addgene:52659> ; RRID:Addgene_52659) using Fugene HD transfection reagent (Promega) according to manufacturer's instructions. Transfection efficiency ranged from 5-10% of total hDANs in the field of view. The hDANs were fixed 48 h post-transfection with 4% (v/v) PFA in PBS for 10 min RT.

Transfected hDANs were imaged for both green (Ex/Em, 488nm/518nm) and red channels (Ex/Em, 543nm/572nm) using a LSM 510 confocal (Zeiss). Z-Stack pictures were analyzed using IMARIS 8.3.1 (Bitplane). Statistics Figure S2B: Data were normalized to the isogenic control in each case and one outlier was removed. The student's t test was performed. $**=p0.0043$ and $*=p0.0222$. nDiff=3.

Tom22 flow cytometry

The Tom22 content in hDANs was measured using the Inside Stain Kit (Miltenyi Biotec). hDANs cultured on Matrigel (Corning) were pre-treated with N2 buffer 24 h prior to the experiment. Cells were harvested, suspended in PBS containing 0.5% BSA and 2mM EDTA and fixed using the same amount of Inside Fix solution (Miltenyi Biotec). After 20 min of incubation at RT neurons were centrifuged for 5 min at 300 g. The supernatant was removed and after a washing step the cell pellets were suspended in Inside Perm with Tom22-GFP antibody (diluted 1:10) or a mouse IgG control. The samples were incubated for 10 min at RT in darkness. To stop the staining samples were diluted with Inside Perm and centrifuged for 5 min at 300g. The supernatant was discarded, and the pellets were suspended in PBS containing 0.5% BSA and 2mM EDTA. Tom22-GFP fluorescence was measured using a MACSQuant Analyzer flow cytometer. IgG background control fluorescence was subtracted from the obtained Tom22-GFP fluorescence for analysis. Statistics Figure S2C: Data was normalised and no outliers removed. Assuming normal distribution, the t test was assigned and ns=not significant, $*=p<0.05$. nDiff=3.

Respiratory analyses

For the basic mitochondrial stress test, oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured in mature hDANs using a Seahorse™ XF96 Extracellular Flux Analyzer. Cells were seeded in Matrigel coated Seahorse cell plates 24-48h prior to the experiment. During the experiment, OCR/ECAR is measured before any injection of mitochondrial toxins. This is referred to as the basal state (The base media contains glucose, pyruvate and glutamine). The wells were then injected sequentially with 1µM Oligomycin (Santa Cruz Biotechnology) 'minimal respiration', 5µM FCCP (Santa Cruz Biotechnology) 'maximal respiration', and 1µM Antimycin A (Santa Cruz Biotechnology)/ 1µM Rotenone (Sigma Aldrich) 'mitochondrial inhibition'. Following the respiratory analysis, the media is removed and the cells are fixed with 4% (w/v) PFA containing Hoechst stain (1:10,000) for 5 minutes before washing and imaging for automated cell counting (BD Pathway 855, BD Bioscience). Normalisation was performed by counting the number of cells per well of the plate using a high content imager from BD Biosciences (BD Pathway 855). The cell counting was performed by the Pathway 855 using an in house written macro and the dimensions of the wells (0.358 cm²). Statistics Figure 3A: For each condition per experiment at least 6 wells are used for technical reproducibility. The mean average OCR/ECAR values for each independent experiment are plotted and the error bars show standard deviation (nDiff=4). The t test was used. ns=not significant. ***= $p < 0.0004$, *= $p < 0.0158$.

Crude mitochondrial enrichment

Crude mitochondrial enrichment: Fresh cell pellets on ice were suspended in mitochondrial isolation buffer (10 mM HEPES pH 7.4, 50 mM sucrose, 0.4 M

mannitol, 10 mM KCl, 1 mM EGTA, phosphatase and protease inhibitors (Roche)) and passed through 20G, 27G and 30G needles 8 times to disrupt cells. Note: because of the size of hDANs and large amounts of axonal material, we found needle homogenization in the absence of detergents to yield the best mitochondrial enrichments. Fractionation was then achieved by several centrifugation steps. First, samples were centrifuged for 5 min at 1000 xg at 4°C and the supernatant was saved. The pellet was suspended in 500 µL mitochondrial isolation buffer and passed through a 30G needle 8 times before centrifuging 5 min at 900 g at 4°C. The second supernatant was pooled with the first and centrifuged for 15 min at 9000 g at 4°C. The obtained pellet comprises the mitochondrial fraction.

Complex I and citrate synthase activity

Complex I activity was measured in crude mitochondrial enrichments from hDANs previously described in (Fitzgerald et al., 2017) and based on the method of (Hargreaves et al., 2007). Following isolation of crude mitochondria, a total protein content >0.7mg/mL is required to have enough active mitochondria for each sample replicate in each independent assay which needs to be duplicated for the addition of rotenone as a negative control. Citrate synthase activity was measured on the same sample sets and independent differentiations according to (Hargreaves et al., 2007). The citrate synthase activity is used to normalise complex I activity (to account for mitochondrial mass). To later measure the whether citrate synthase activity alone was significantly altered between the groups citrate synthase activity was measured again, this time measuring the exact input protein per well. Citrate synthase is then expressed as a change in absorbance per min per µg protein. The SpectraMax® M

microplate reader (Molecular Devices) was used. Statistics Figure 3B/C. Outlier tests were performed in Graphpad Prism and no outliers were removed. Distribution analysis (D'Agostino & Pearson test) was not possible. The student's t test was used. ns=not significant. nDiff=6 (CI) and nDiff=3 (CS).

NAD⁺ and NADH measurement

Mature hDANs were cultivated in N2 medium 24 h before the experiment. Whole cell NAD⁺ and NADH-levels were determined using the NAD/NADH Assay Kit (Fluorometric) from Abcam according to manufacturer's instructions. NADH reaction mixture was incubated for 1 hour and fluorescence (Ex/ Em = 540/ 590 nm) was measured using a SpectraMax® M microplate reader (Molecular Devices). Statistics Figure 3D. Outlier tests were performed in Graphpad Prism and no outliers were removed. Distribution analysis (D'Agostino & Pearson test) was not possible. The student's t test was used. *=p0.0222, ***=p0.0004. nDiff=5. NADH levels were slightly increased in PINK1 KO hDANs but not significantly (data not shown).

α Ketoglutarate dehydrogenase activity assay

Mature hDANs were pretreated with N2 medium for 24 hours prior to the experiment. Mitochondria were extracted using the protocol for crude mitochondrial enrichment. Measurement of alpha-ketoglutarate dehydrogenase activity in mitochondria was performed using the alpha-Ketoglutarate Dehydrogenase Activity Colormetric Assay Kit (Sigma-Aldrich) according to manufacturer's instructions. Absorbance (A₄₅₀) of the samples was measured in kinetic mode every minute for 1h at 37°C on a

SpectraMax®M microplate reader (Molecular Devices). Statistics Figure 3E. Outlier tests were performed in Graphpad Prism and no outliers were removed. The student's t test was used. ns=not significant. nDiff=3.

Mitochondrial Aconitase activity

*m*Aconitase activity was measured in mature hDANs using a protocol from (Pierik et al., 2009). Briefly, hDANs were washed with PBS and the cell pellet suspended in 200µl TNEG buffer + protease inhibitors. Then dounced on ice using a loose then a tight fitting glass pestle. The homogenates were centrifuged at high and low speeds with rigorous vortexing inbetween (Pierik et al., 2009). The resulting supernatant is then centrifuged for 10 minutes at 13,000g at 4°C. The resulting supernatant is then protein estimated before being used in the assay. Set up of the assay plate: Per well: 95µl triethanolamine buffer, 10µl cis aconitic acid (20mM), 20µl Isocitrate dehydrogenase (4U/ml), 10µl NADP (0.1M) and 40µg hDAN supernatant containing the enzyme. Blank = no cis aconitic acid, no isocitrate dehydrogenase. Read absorbance at 340nm, every 30s for 10 mins. We used a SpectraMax® M microplate reader (Molecular Devices). Statistics Figure 3F: Activity rates are normalised to the healthy control. No outliers were removed. The t test was used. $\ast=p<0.05$. ns= not significant. nDiff=3, Hela n=3.

Nuclear magnetic resonance (NMR) based metabolomics

hDAN metabolite extraction: hDAN pellets were collected, washed with PBS buffer and quenched in 400 µL ice-cooled ultrapure methanol. The cell suspensions were

then transferred to 2 mL glass tubes (Covaris Adaptive Focused Acoustics AFA™) and added to 1000 µL of *tert*-butyl methyl ether (MTBE), well mixed and submitted to AFA ultrasound metabolite extraction protocol (Covaris E220 Evolution Woburn, USA). Ultrasonication programme setup: two treatment cycles, 1st: 30s, Peak Power 125.0, Duty Factor 32.0, Cycles/Burst 400, Avg. Power 40.0. 2nd: 30s, Peak Power 100.0, Duty Factor 30.0, Cycles/Burst 800, Avg. Power 30.0. The ultrasonication was carried out in cooled water bath, temperature range 5.0 to 15.0 °C. Each cycle was repeated 5 times per sample, total run time per sample was 5 min. Following extraction, 400 µL of ultrapure water were added to the extraction mixture, thoroughly vortexed and centrifuged at 12'000 g for 10 min for optimum phase separation. After centrifugation, the two phases were manually separated: the top lipid (MTBE) layer was transferred to 2 mL HPLC (High-Performance Liquid Chromatography) glass vials; the bottom aqueous phase was moved to 1.5 mL *Eppendorf* cups. Those were then submitted to another centrifugation step at 30'000 g for 10 min to separate any undissolved cell culture residue. Following centrifugation, the aqueous supernatant was transferred to fresh *Eppendorf* cups and evaporated to dryness by a vacuum concentrator (Thermo Fisher Speedvac XYA). **NMR sample preparation:** Dried metabolite pellets were re-suspended in 45 µL 1M K₂HPO₄ (phosphate) buffer (pH = 7.4, containing NaN₃ and 1mM internal NMR standard TSP), thoroughly mixed and then centrifuged for 5 min with 30'000 g. 40 µL of the supernatant was filled into 1.7mm NMR tubes that are compatible to Bruker auto-sampler. **NMR spectra acquisition:** Metabolomics data were acquired on a 14.10 Tesla (600 MHz) ultra-shielded NMR spectrometer (Avance III HD, Bruker BioSpin, Karlsruhe, Germany) equipped with a 1.7 mm room temperature triple resonance probe (¹H, ¹³C, ³¹P). Spectra were recorded at 298 K. A quick

simple ZG experiment was performed followed by a 1D NOESY (Nuclear Overhauser Effect Spectroscopy) aiming to optimise offset and shim parameters. A CPMG (Carr-Purcell-Meiboom-Gill) experiment was used to suppress residual background signals from remaining macromolecules in the solution and water (time domain = 64k points, sweep width = 20 ppm, 2024 scans, 4 hours per sample). **NMR data analysis and statistics:** The recorded free induction decays (FIDs) were Fourier-transformed (FT) and NMR spectra were processed by Bruker TopSpin 3.6.1 software (automated baseline correction, phase correction and spectra offset adjustment). Metabolite annotation and quantification was done with ChenomX NMR Suite 8.5 software containing the additional HMDB (Human Metabolome Data Base) library. The MetaboAnalyst 4.0 web server (R-based online analysis tool, www.metaboanalyst.ca) was used for statistics. To make samples and features comparable, all data was normalised by a reference sample (probabilistic quotient normalisation (PQN) to account for dilution effects and scaled by the Pareto scaling method (mean-centred and divided by the square root of the standard deviation of each variable). Statistics Figure 3H, initially one-way ANOVA (analysis of variance) with $p < 0.05$. Then the student's t test was applied to compare metabolite concentrations between the control and each PINK1 KO clone. We used Principal component analysis (PCA), sparse partial least squares discriminant analysis (sPLS-DA), pattern hunter and heatmap tools for data visualisation. Independent biological experiments $n=4$ and $n_{\text{Diff}}=2$.

Mitochondrial import Assay

Radiolabelled proteins were synthesized in rabbit reticulocyte lysate in the presence of ^{35}S -methionine after in vitro transcription by SP6 polymerase from pGEM4 vector (Promega). Radiolabelled precursor proteins were incubated at either 30°C (pSu9-DHFR) or 4°C (Fis1) in import buffer (250 mM sucrose, 0.25 mg/ml BSA, 80 mM KC1, 5 mM MgCl_2 , 10 mM MOPS-KOH, 2 mM NADH, 4 mM ATP, pH 7.2) with crude mitochondria isolated from mature hDANs. Non-imported pSu9-DHFR molecules were removed by treatment with proteinase K (PK, 50 $\mu\text{g/ml}$) for 30 min on ice and then PK was inhibited with 5 mM Phenylmethanesulfonyl fluoride. Membrane integration of Fis1 molecules was confirmed by resistance to alkaline extraction (incubation on ice for 30 min with 0.1 M Na_2CO_3 followed by centrifugation to obtain membrane-embedded proteins in the pellet). Finally, samples were heated at 95°C for 5 min before their analysis by SDS-PAGE and autoradiography. Representative images of four independent experiments are shown. Quantification of band densitometry was performed with the AIDA software (Raytest). No outliers were removed. Error bars show standard deviation between independent experiments. Test for normality were not possible. The student's t test was performed comparing control to PINK1 KO hDANs at multiple time points. ns=not significant. nDiff=4.

Cytosolic ROS

Medium was changed to N2 medium 24 h prior to the experiment. On the following day mature hDANs were incubated with N2 medium or N2 medium containing 10 μM Rotenone, 1mM Buthionine sulfoximine (BSO) or 50 μM Mn-Tbap (a mitochondrial superoxide dismutase 2 mimic) (for 4h before measurement. 100 μM dihydroethidium (DiHET) (Santa Cruz Biotechnology) was added to all wells and emission was

measured at 610nm emission (535nm excitation) (chromatin-bound, oxidized DiHET) was measured every 30 secs for 30 min on a SpectraMax® M microplate reader (Molecular Devices). No outliers were removed. Error bars show standard deviation between independent experiments. Tests for normality were not possible. The student's t test was performed and the data showed no statistically significant differences between control and PINK1 KO hDANs. nDiff=3.

Glutathione assay

Neurons on a 96-well plate were treated with different treatment conditions (untreated N2 medium only, 10µM Rotenone, 1mM BSO, and 10µM Rotenone plus 1mM BSO) in N2 medium 24 h prior to the experiment. Cells were briefly washed with HBSS (Gibco) and incubated with 100µL HBSS containing 50µM Monochlorobimane (Sigma) for 40 minutes @37°C, 5% CO₂. Blanks were treated the same way but did not contain cells. After washing with HBSS, cells were imaged with a SpectraMax® M microplate reader (Molecular Devices) (Ex 390nm/Em 478nm) to detect the fluorescent adduct of Monochlorobimane and reduced Glutathione (GSH). Background fluorescence obtained from blanks was subtracted for analysis. No outliers were removed. Error bars show standard deviation between independent experiments. Tests for normality were not possible. The t test was performed and the data showed no statistically significant differences between control and PINK1 KO hDANs. nDiff=3.

RNA sequencing

Approximately 5 million hDANs were lysed in 350ml RTL buffer and homogenized using QIAshredder® homogenizer (Qiagen). RNA isolation was performed using RNeasy Mini Kit (Qiagen). RNA was eluted in 30µl RNase-free water. RNA quality was assessed with an Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano kit (Agilent). Samples with very high RNA integrity number (RIN > 9) were selected for library construction. For polyA enrichment, a total of 200ng of total RNA was subjected to polyA enrichment and cDNA libraries were constructed using the resulting mRNA and the Illumina TruSeq Stranded mRNA kit (Illumina). Libraries were sequenced as single reads (65 bp read length) on a HighSeq 2500 (Illumina) with a depth of >22 million reads each. Library preparation and sequencing procedures were performed by the same individual and a design aimed to minimize technical batch effects was chosen. Read quality of raw RNA-seq data in FASTQ files was assessed using QoRTs (v1.2.37) to identify sequencing cycles with low average quality, adaptor contamination, or repetitive sequences from PCR amplification. Reads were aligned using STAR (v2.5.3a) allowing gapped alignments to account for splicing to the Ensembl Homo sapiens GRCh37 reference genome. Alignment quality was analyzed using ngs-bits (v0.1) and visually inspected with the Integrative Genome Viewer (v2.4.19). Normalized read counts for all genes were obtained using subread (v1.5.1) and edgeR (v3.24.3). Transcripts covered with less than 1 count-per-million in at least 5 out of 6 samples were excluded from the analysis leaving >13,000 genes for determining differential expression in each of the pair-wise comparisons between experimental groups. For statistics, we used the edgeR statistical framework for the calculations (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>). In short, the methods models read counts per gene using the negative binomial distribution

(capturing the abundance and variability between replicates). Variability is estimated for each gene and on a global scale. After establishing these models, using a generalized linear model (GLM) coefficients are fitted and each gene is tested individually using quasi-likelihood F-test to yield a significance value (p value).

Statistics: For sample size n , we used minimum 3 replicates per group. This experiment is following a 2x2 design with 2 samples ($n_{\text{Diff}}=2$) in Valinomycin treatment groups and $n_{\text{Diff}}=1$ sample in control groups for each genotype. **Pathway Analysis:** The heatmap shows the top significant genes (<0.03 p -value) in contrast to untreated PINK1 KO vs. untreated isogenic control. Colored squares show the gene expression as per-row normalized (scaled and centered, i.e. mean=0, standard deviation=1) cpm (counts per million as a measure for gene expression strength) values - not log FC values. CPM data were refined by log2 fold change values (logFC) of PINK1 KO hDANs compared to control hDANs for Ingenuity Pathways Analysis (IPA, QIAGEN). First, twenty genes with the highest and lowest log2 fold change were listed. The gene ontology pathway analysis GOrilla was used to generate p values and maps of affected processes according to GO terms.

RNA extraction from PINK1 KO mice brain and PINK1 Q456X PD patient hDANs for *PNPO* expression analysis

RNA was prepared from the striatum of three healthy control mice and three PINK1 knockout mice (homogenized using QIAshredder® homogenizer (Qiagen). RNA isolation was performed using RNeasy Mini Kit (Qiagen)) and kindly provided through collaboration with Daniela Vogt-Weisenhorn and Wolfgang Wurst (Helmholtz

Zentrum München, German Research Center for Environmental Health, Institute of Developmental Genetics, Munich-Neuherberg, Germany).

Human iPSC-derived hDANs from three distinct PD patients carrying the PINK1 Q456X mutation and their corresponding gene-corrected isogenic controls were collected at day 30 of differentiation and subjected to RNA extraction by using the RNeasy mini kit (QIAGEN). Briefly, cells were washed twice in PBS and immediately lysed in RTL buffer supplemented with beta-mercaptoethanol. Lysate homogenization was obtained by using the QIAshredder system (QIAGEN), followed by on-column DNase digestion to remove DNA contamination. RNA was finally eluted in nuclease-free water and purity assessed by Nanodrop (A260/A280 and A260/A230 ratios). The cell models were kindly provided by Christine Klein and Philipp Seibler (University of Lubeck, Germany). Their corresponding gene-corrected controls were generated by Jens Schwamborn and Javier Jarazo (University of Luxembourg, Luxembourg).

Proteomics: Quantitative Hyper Reaction Monitoring (HRM)-based mass spectrometry

For lysis, mitochondrial pellets were suspended in 100 µl 6M urea, 100 mM Ammonium bicarbonate pH 8.0 and incubated for 30 min at 4°C. After clearance, the concentration of each lysate was determined by Bradford and 50µg protein per biological replicate were precipitated by methanol chloroform precipitation and processed as described previously (Gloeckner et al., 2009). Briefly, dried protein precipitates were re-dissolved in 30mM Ammonium bicarbonate pH8.0 supplemented with 0.2% RapiGest™ (Waters) surfactant and reduced/ alkylated by

DTT/ Idoacetamide prior to over-night proteolysis with trypsin (Promega). Proteolysis was followed by hydrolysis of the surfactant by TFA according to the manufacturer's protocol. Prior to MS analysis, samples were pre-cleaned with StageTips. For the final dataset two biological replicates (individual differentiations) have been analyzed in three technical replicates. For the generation of the assay library, three DDA runs per condition and biological replicate were acquired. Vacuum-dried samples were re-dissolved in 0.5% TFA and mixed with 2 μ l iRT standard peptide mix (Biognosis). Extracted peptides were subsequently subjected to LC-MS/MS-analysis by a 180 min standard method: Tryptic peptide mixtures were injected automatically and loaded at a flow rate of 30 μ l/min in 0.1% trifluoroacetic acid in high performance liquid chromatography (HPLC)-grade water onto a nano trap column (300 μ m inner diameter \times 5 mm precolumn, packed with Acclaim PepMap100 C18, 5 μ m, 100 Å; Thermo Scientific). After 3 min, peptides were eluted and separated on the analytical column (75 μ m inner diameter \times 25 cm, Acclaim PepMap RSLC C18, 2 μ m, 100 Å; Thermo Scientific) by a linear gradient from 2% to 30% of buffer B (80% acetonitrile and 0.08% formic acid in HPLC-grade water) in buffer A (2% acetonitrile and 0.1% formic acid in HPLC-grade water) at a flow rate of 300 nl/min over 147 min. Remaining peptides were eluted by a short gradient from 33% or 30% to 95% buffer B in 5 or 10 min. Eluting peptides were analysed on an Q-Exactive Plus mass spectrometer (Thermo Fisher). The HRM acquisition method was adapted from (Bruderer et al., 2015). To improve the quantification performance, 15 instead of the 19 variable windows have been used covering an MS range from M/Z 457 to M/Z 914 (457-483, 481-506, 504-531, 529-554, 552-576, 574-600, 598-624, 622-650, 648-676, 674-704, 702-735, 733-771, 769-810, 808-856, 854-914). Each HRM sequence was preceded by a full-scan. MS1 and MS2 spectra were acquired in the profile

mode. MS2 spectra were acquired with a stepped collision energy (10% at 25%). DDA MS2 spectra for the assay library creation were acquired with a TOP10 method and in the centroided mode with a fixed collision energy of 25%.

HRM-workflow and statistical analysis

Assay library. The assay library was created with the trans-proteomic-pipeline (TPP v5.1) following published protocols (Schubert et al., 2015). The Thermo RAW files were converted into the mzXML format using MSConvert (Proteowizard v3.0.10765) with the TPP compatibility switch set. The files were filtered for the 150 most intense peaks to reduce the size of the datasets. Briefly, results from four search engines (Mascot [v.2.5.1], Comet [v.2017.01 rev. 1], Myrimatch [v.2.2.10165] and X!Tandem [v.2013.06.15.1]) with trypsin as enzyme, a peptide tolerance of 20 ppm and 3-Methoxythyramine as fixed modification. Individual searches were performed against the human subset of the Swissprot database (release 2016_05, 20201 entries). The masses of the iRT standard (Escher et al., 2012) and decoys (reversed sequences) were added. The assay library was generated by the published TPP workflow. Remaining decoy sequences were manually removed and protein names were replaced by the Uniprot identifiers and the final library was processed and converted into the PQP format by OpenMS (v. 2.4.0). The final assay library contained shuffled decoy sequences in the same amount as the targets. **OpenSWATH workflow.** The openSWATH analysis was performed as previously described (Rost et al., 2017). Briefly, prior to quantification the HRM RAW files were converted into the mzML format at 64bit precision. The files were processed by the OpenSWATHWorkflow (OpenMS v. 2.4.0; Revision: 103a38b) and subsequently analyzed by pyProphet

(v2.01) performing local, experiment-wide and global-statistics for filtering. Files exported to the legacy tsv format were re-aligned by TRIC (Rost et al., 2017).

MSStats. The TRIC output was processed with SWATH2stats R package (v.1.12.0) (Blattmann et al., 2016) to generate the MSstats format. The final analysis and group comparison was performed using MSStats R package (v.3.12.3) (Choi et al., 2014). Final tables were generated by Perseus (v.1.6.1.3) (Tyanova et al., 2016) to extend the annotation by gene and protein names. **Data analysis.** Statistical significance for each gene Log2FC for control versus PINK1 KO clone 1 and control versus PINK1 KO clone 2 was assigned by an asterisk * $p < 0.05$ ($n_{\text{Diff}}=3$). Filtering to minimize the top hits list for the figure was achieved by removing all significant proteins in the CTRL v PINK1 comparison that did not appear significant in both PINK1 KO lines. Then a cutoff point of $\pm \text{Log2FC } 1$ to limit only relevant fold changes and then an arbitrary $\pm 1.7 \text{ Log2FC}$ cut off (-1.7 to $+1.7 \text{ Log2FC}$ was removed) to limit the number of hits shown because of space. Qiagen Ingenuity and GOrilla was used to generate a pathway analysis using the LogFC as metrics. The pathway analysis files are available as supplementary Excel files. For the comparison of the mitoproteome with gene expression, RNASeq data were merged on the SWATH proteomic dataset using the 'matching rows by name' function of Perseus.

Measurement of biogenic amines by HPLC-ED

Mature hDANs were expanded during differentiation into 175cm^2 Matrigel coated flasks. For each HPLC experiment, approximately 2-3 175cm^2 flasks were required. Although endogenous dopamine levels could be detected in hDANs derived from much larger cell volumes, we employed $50\mu\text{M}$ L-DOPA treatment overnight (16h) to

enhance dopamine metabolism without risk of dopamine toxicity (Allen et al., 2013, Burbulla et al., 2017). Following detachment of hDANs with Accumax, the cell suspensions were washed in PBS and cells counted. PBS was used to normalize the number of cells in the suspension. An aliquot of the normalized suspension was taken for preparation of protein lysate and RNA to determine the relative amount of TH positive cells in each experiment and differentiation for each cell line. Fresh (unfrozen) cell pellets kept on ice were suspended in 350µl of a standard HPLC elution buffer that does not contain detergents (Thermo Scientific). The suspensions were homogenized using 5mm stainless steel beads and the tissue lyser LT (Qiagen) for 4 minutes with 50Hz. The cell homogenate was centrifuged at 14000 g for 10 min and the supernatant was filtered through a 0.2µm nylon membrane. Samples were analyzed for catecholamine and indolamine content by ion-pair reverse phase HPLC with colorimetric detection (Ultimate 3000 LC with electrochemical detection ECD3000RS, Thermo Fischer Scientific, California, USA). A hypersil C18 column was used (150x3 mm, 3 µm) and the system was run with a Test mobile phase containing 10% acetonitrile and 1% phosphate buffer (Thermo Fischer Scientific, California, USA) at a flow rate of 0.4 ml/min at 30 °C. The potential of the first channel was set to +350 mV, the second channel to -250 mV. Epinephrine, Norepinephrine, Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindol-acetic acid (HIAA), 3-Methoxytyramine (3-MT) and 5-hydroxytryptamine/serotonin (5-HT), concentration was determined by comparing peak areas of the samples with those of standards using Chromeleon 7 chromatography data system software. The neurochemicals in standards were determined with a high correlation linearity ($r^2 = 0.98$) and good reproducibility in retention time (0.03%). The limit of detection was <1 pg on column for all the

metabolites analyzed. Statistics Figure 6A: The data was not normalized. One outlier for dopamine was removed from the control hDAN dataset. The data was normally distributed. The Student's t test was performed, $**=p0.0045$ (DOPAC), $p0.0023$ (DA), ns=not significant. nDiff=8. Figure 6B: The data was not normalized. One outlier for dopamine was removed from the control hDAN dataset. The data was normally distributed. The Student's t test was performed, $*=p0.0130$ (DOPAC/DA), $p0.0144$ (DOPAC+HVA/DA), ns=not significant. nDiff=8. Figure 6C: The Student's t test was performed, ns=not significant. nDiff=3. Figure 6D: The data was not normalized. No outliers were removed. Data distribution analysis was not possible. The Student's t test was performed, $**=p0.0036$ (L-DOPA, CTRL v KO), $p0.046$ (DOPAC+MAOi, CTRL v KO), ns=not significant. nDiff=4.

Neurotransmitter Uptake Assay

Neurotransmitter transporter activity in hDANs was measured using the Neurotransmitter Transporter Uptake Assay Kit (Molecular Devices) according to the manufacturer's instructions. Mature hDANs were seeded in Matrigel coated black, clear bottom 96-well plates prior to the assay at a density of 60,000/well in triplicates. Wells containing no cells were used as an internal control. hDANs were treated with a specific VMAT2 inhibitor (Tetrabenazine, 10 μ M), a TH inhibitor (3-londol-L-Tyrosine, 300 μ M), or a combination of MAO and COMT inhibitors (Tranylcypromine 10 μ M and Tolcapone 100nM respectively). These compounds were added in media containing L-DOPA (50 μ M) or without L-DOPA. A subset of hDANs triplicates were treated with media alone or media containing L-DOPA (50 μ M). All treatments were incubated on the cells at 37°C for 20 minutes prior to the addition of the substrates.

For the NT uptake in Figure 6G; hDANs were plated in the same way and either untreated or treated with 1mM BH4 for 2h prior to addition of substrates. Uptake fluorescence was measured using the SpectraMax M2^e microplate reader in kinetic mode (Molecular Devices) measuring every 30s or as an endpoint after 30 minutes incubation with the substrates). After the assay, the hDANs were washed and fixed in 4% (v/v) PFA containing Hoechst to account for cell number in each well and then counterstained for TH to account for any large differences in TH positive hDANs in the culture wells. No outliers were removed. Statistics Figure 6E: Linear regression plotted, 2-way ANOVA was performed. Genotype across times was statistically significant ***= $p < 0.0001$ nDiff=3. Figure 6F: In the presence of L-DOPA, THi and MAO/COMTi statistically significantly different to PINK1 KO hDANs using 2-way ANOVA (multiple comparisons). **= $p 0.0071$ (*L-DOPA+THi, KO1), **= $p 0.0048$ (*L-DOPA+THi, KO2), *= $p 0.049$ (*L-DOPA+COMTi/MAOi, v KO1), **= $p 0.010$ (*L-DOPA+COMTi/MAOi, v KO2) nDiff=3. Figure 6G: No outliers were removed. Neurotransmitter uptake was measured as a FL endpoint and normalized to the untreated control hDANs in each experiment, Welch's t test was performed. **= $p 0.0024$ (untreated CTRL v PINK1 KO), *= $p 0.023$ (untreated GC CTRL v PINK1 Q126P), ns=not significant. nDiff=3.

Monoamine oxidase (MAO) Activity Assay

MAO activity was monitored using a radiometric assay with ¹⁴C-tyramine hydrochloride as substrate as previously described (Ugun-Klusek et al., 2019). Data were normalized for protein content and rates expressed as disintegrations of

14C/min/μg protein. The student's t test identified no significant differences. ns=not significant, nDiff=3.

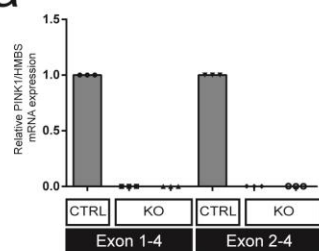
Dopamine (Catecholamine) oxidation assay

The catecholamine oxidation assay was performed according to (Burbulla et al., 2017) using Biodyne® B 0.45μm membranes (Pall corporation) and detection using a LiCOR fluorescent scanning device. The quantification performed using Image Studio from LiCOR. Representative blots and standard curve are shown. Mean FL units were plotted for each genotype, fraction and treatment. No outliers were removed. The student's t tests were not significant (CTRL v PINK1 KO in each condition). 2-way ANOVA found significance across the genotypes (*=p0.144) including all the conditions. nDiff=3.

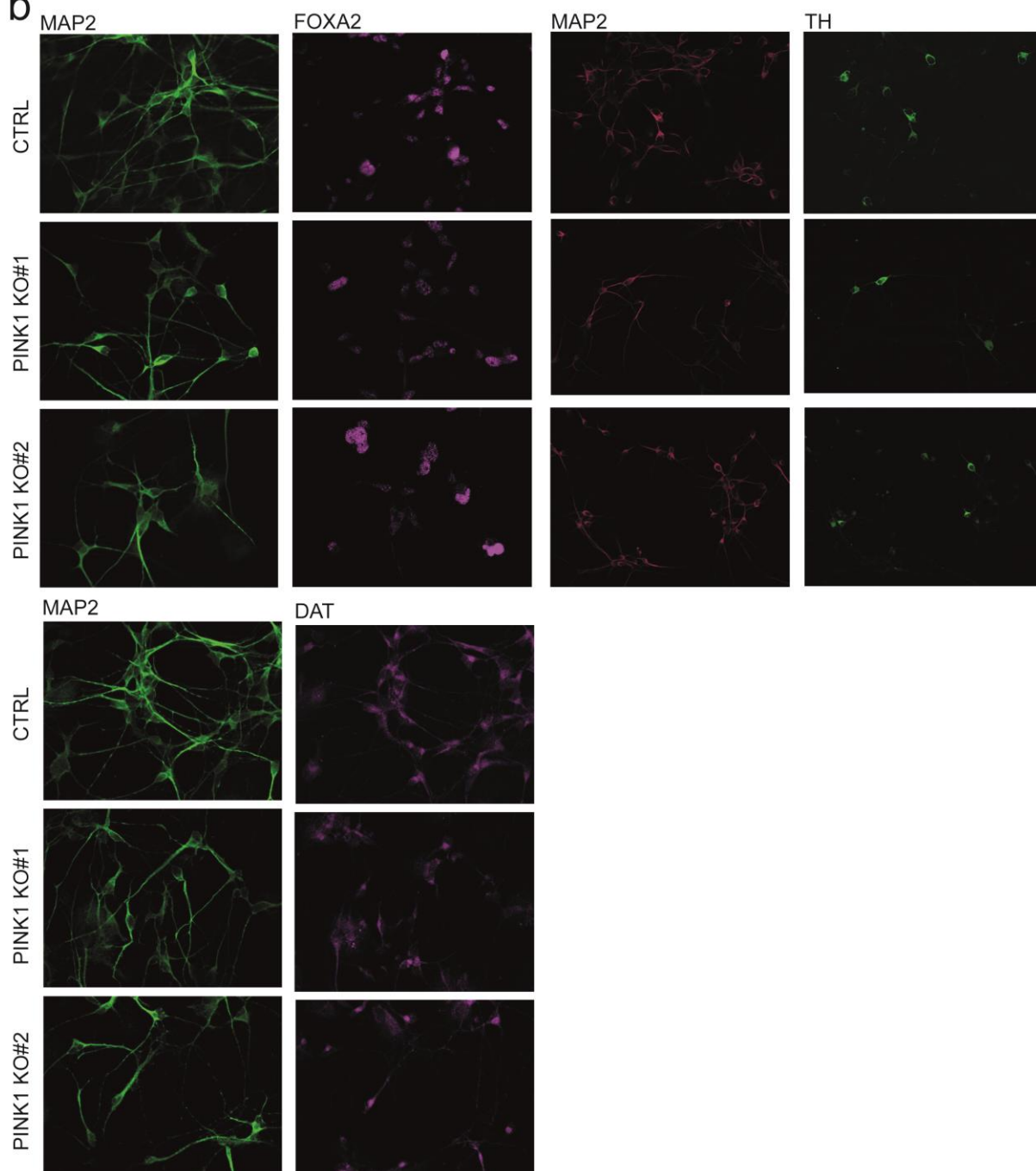
Supplementary Figures and Legends

Bus et al. Supplementary Figure 1

a

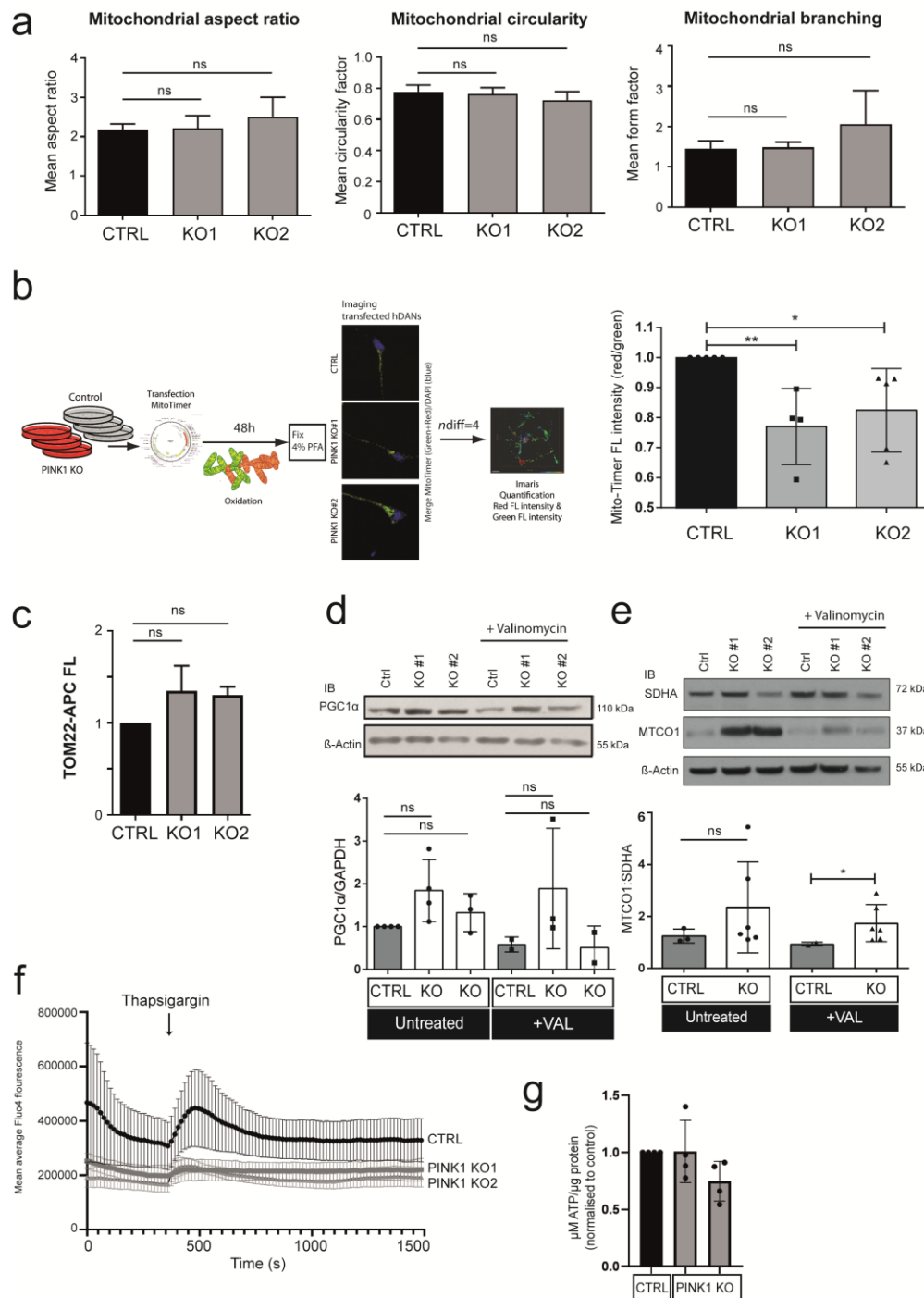


b



Supplementary Figure S1, related to Figure 1: S1A) Example immunocytochemistry staining of MAP2, TH and FOXA2 in fixed hDANs. Representative images are shown (nDiff=4). **S1B)** Relative PINK1 gene expression in control and PINK1 KO hDAN clones using primers directed to exon1-4 and exon 2-4 (nDiff=3).

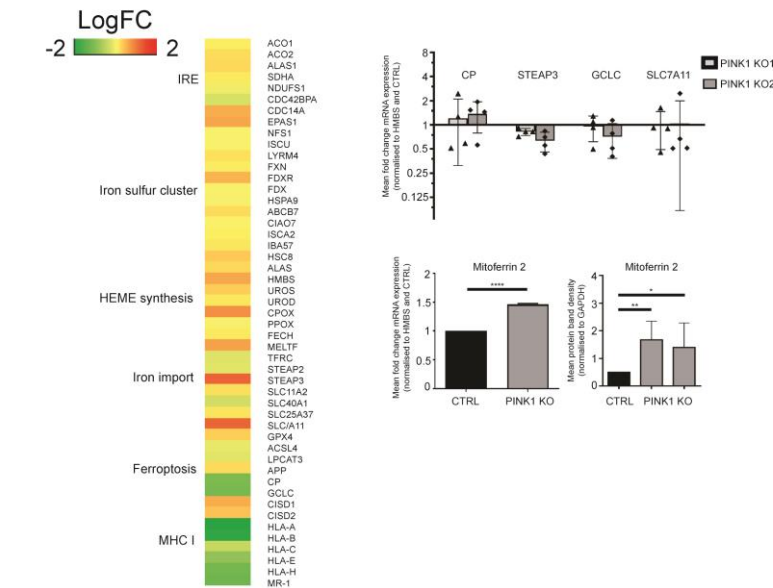
Bus et al. Supplementary Figure 2



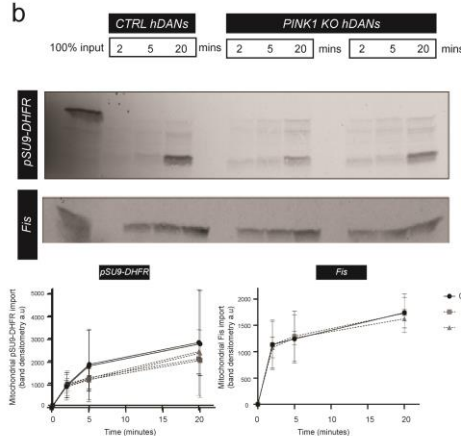
Supplementary Figure S2, related to Figure 2: S2A) Readouts of mitochondrial morphology in hDANs from live cell imaging (nDiff=3, ns=not significant). **S2B)** Diagram showing work flow for MitoTimer experiments in hDANs. Right panel: MitoTimer live cell imaging red/green fluorescence intensity ratio of PINK1 KO hDANs normalised to the isogenic control **=p0.0043 and *=p0.0222 (nDiff=3, error bars=SD). **S2C)** Tom22 fluorescence (APC-FL) detected by inside staining and flow cytometry in hDANs. ns=not significant (nDiff=3, error bars=SD). **S2D)** Upper panel respective Western blot of PGC1alpha in PINK1 KO hDANs and isogenic control under untreated conditions (UT) or valinomycin (VAL) 1µM, 24h (UT n=diff3-4; VAL n=2-3diff, error bars=SD). Lower panel densitometric quantification of PGC1alpha signal normalised to GAPD and isogenic control. ns=not significant. **S2E)** Upper panel respective Western blot of SDHA and MTCO1 in PINK1 KO hDANs and healthy control under untreated conditions or valinomycin (VAL) 1µM, 24h (nDiff=3-5). Lower panel densitometric signal ratio of MTCO1/SDHA of PINK1 KO hDANs and healthy control (ns= not significant, *=p<0.05, error bars=SD). **S2F)** Total corrected cell Fluo4 fluorescence (cytosolic calcium) calculated for every live image frame across 1500 seconds (nDiff=4, error bars=SD) for CTRL and PINK1 KO hDANs. **S2G)** Normalised ATP levels in CTRL and PINK1 KO hDANs (nDiff=4, error bars=SD, T-test).

Bus et al. Supplementary Figure 3

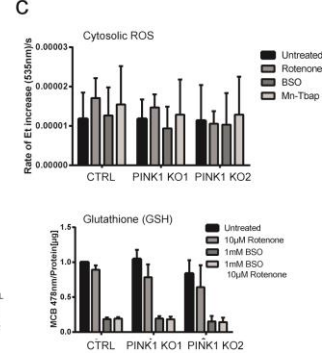
a



b



c



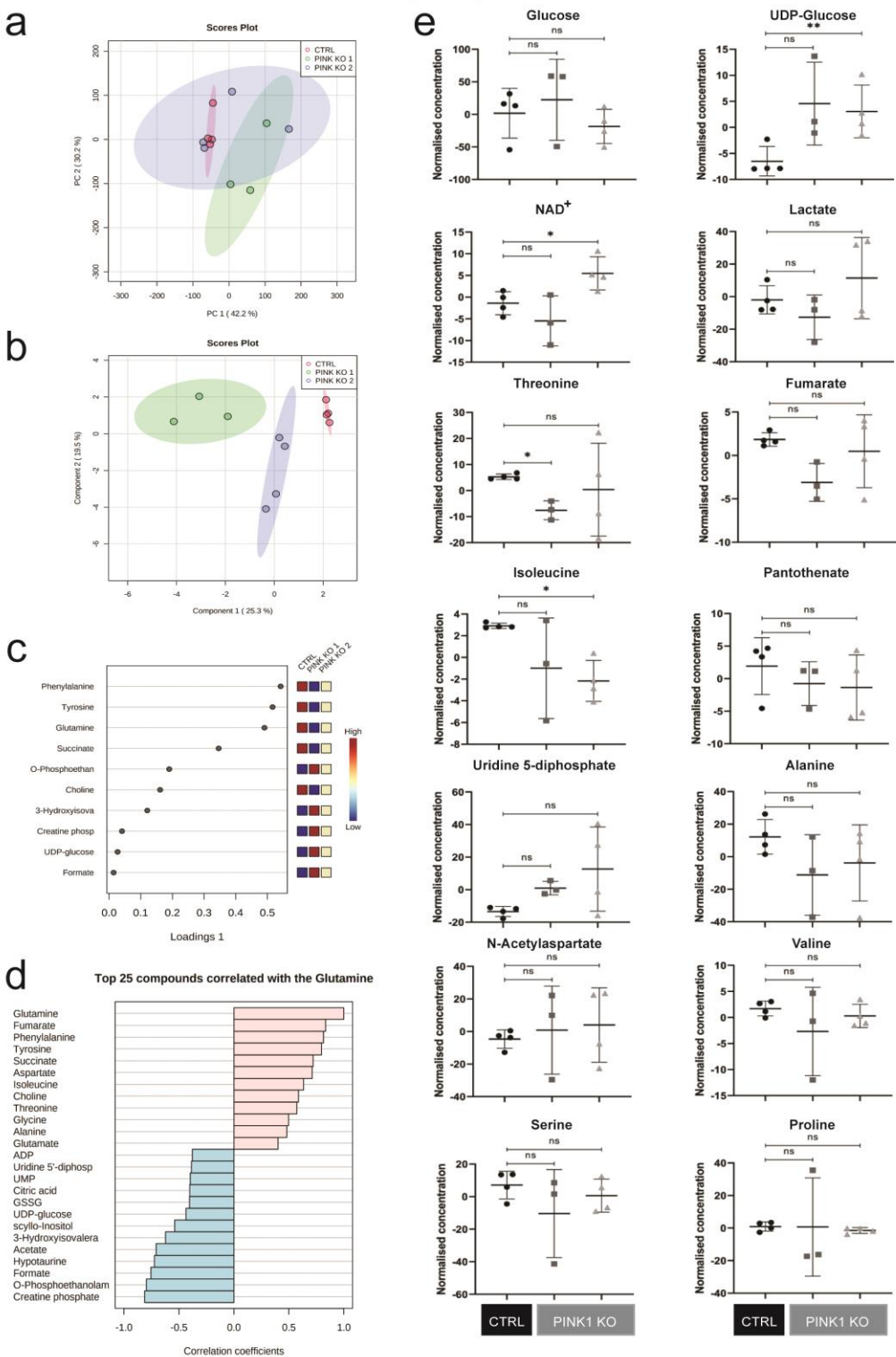
Supplementary Figure S3, related to Figure 3 A-F: S3A) Left panel: Genes identified from RNA sequencing related to iron metabolism. Log2FC values are shown for PINK1 KO hDAN clonal lines versus isogenic control (n=3, nDiff=1). Right panel: Mean fold change of mRNA expression of iron related genes; *CP*, *STEAP3*, *GCLC*, *SLC7A11* in PINK1 KO hDANs normalised to healthy control and HMBS (nDiff=4, error bars=SD). Lower panel: Expression of Mitoferrin-2. mRNA in PINK1 KO hDANs and isogenic control determined by qRT-PCR, results in mean fold change normalised to HMBS and control (nDiff=3; ****=p<0.0001). Western blot of Mitoferrin-2 in PINK1 KO hDANs compared to healthy control, mean protein band density normalised to GAPDH (nDiff=3-5, *=p<0.0179 **=p<0.0079). **S3B)** SDS-PAGE/autoradiography analysis showing import of radiolabelled proteins 5S-pSU9-DHFR and ³⁵S-

Fis1 in PINK1 KO and healthy control hDANs after different time points (2, 5 and 20 min), arrows indicate precursor and mature pSU9-DHFR protein (nDiff=4, error bars=SD). **S3C)**

Top panel: Mean rates of oxidised DiHET (Et) fluorescence signal increase (Ex 535nm/Em 610) per sec in PINK1 KO and isogenic control hDANs untreated or treated with 10µM Rotenone/ 1mM BSO/ 50µM Mn-Tbap indicating cytosolic ROS (nDiff=3, error bars=SD).

Bottom panel: Fluorescence signal increase of MCB-GSH adduct in PINK1 KO hDANs and isogenic control untreated or treated with 10µM Rotenone/ 1mM BSO/ 1mM BOS + 10µM Rotenone (nDiff=3). Values normalised per µg protein of cell lysates and to untreated healthy control.

Bus et al. Supplementary Figure 4

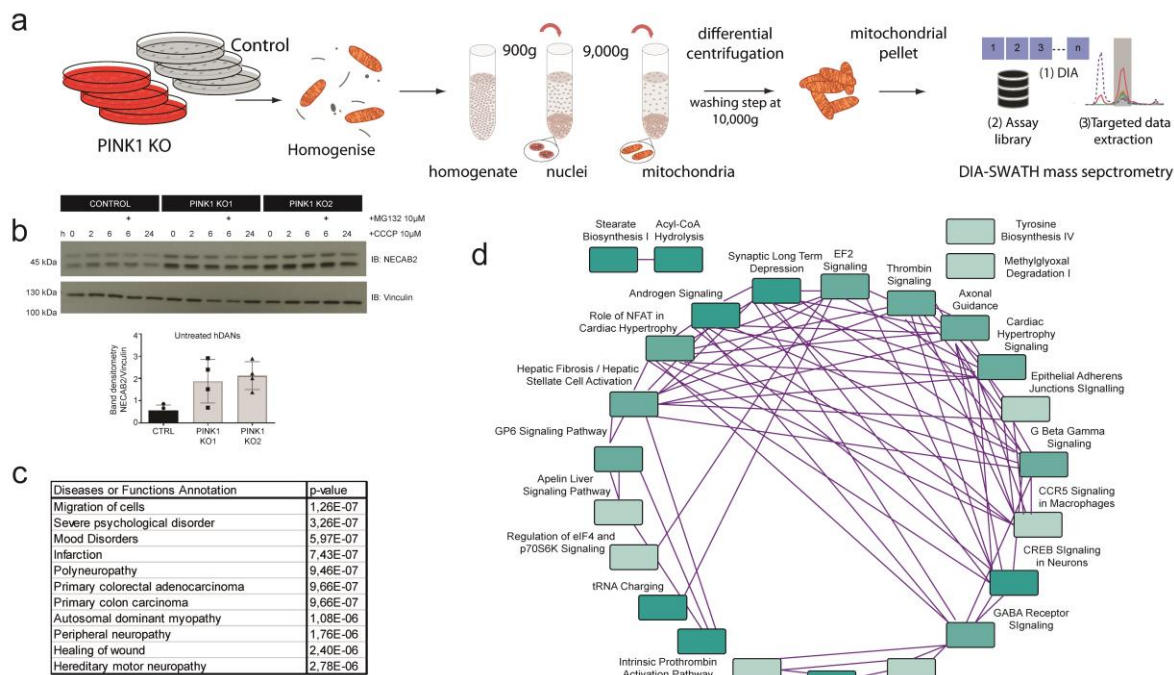


Supplementary Figure S4, related to Figure 3G-I: S4A) Principal Component Analysis

(PCA) was generated for the control, KO 1 and 2 metabolomics data comparison. Generally, all three groups have strong similarities between the first two principal components since the

overlap is almost complete. **S4B)** Sparse partial least squares discriminant analysis (sPLS-DA) algorithm allowed reducing the dimensional complexity and illustrated the changes driven by most significant features of the groups. Here, the group separation is significantly improved which correlates to multiple metabolites having significant p-values. **S4C)** sPLS-DA loadings plot illustrates the metabolites which drive the group differences. Here, as top scoring metabolites are phenylalanine, tyrosine, glutamine and succinate, which we identify as main statistical significant metabolite changes. **S4D)** Pattern hunter was generated for glutamine and illustrated the top 25 metabolites which correlate the most strongly with this compound in the dataset (Pearson r distance measure). Glutamine concentration changes in this metabolic setup have the strongest correlation with fumarate, phenylalanine, tyrosine, succinate, aspartate, isoleucine, choline, threonine, glycine, alanine and glutamate, most of which are the amino acids supplying TCA cycle. **S4E)** Scatter plots were generated for all the metabolites of interest based on their changes between the control and KO groups. ns=not significant, *=p<0.05, **=p<0.005 (n=4, nDiff=2, error bars=SD).

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Supplementary Figure S5, related to Figure 5: S5A) Diagram of workflow and mitochondrial preparations from hDANs for quantitative mass spectrometry. **S5B)** Representative Western blot of NECAB2 (MitoNEET) and vinculin (loading control) in PINK1 KO hDANs and healthy control untreated or treated with 10 μ M CCCP for 0, 2, 6 (+/- 10 μ M MG132) for 24 hours. Densitometric quantification of NECAB2 Protein in untreated PINK1 KO hDANs and isogenic control normalized to Vinculin. Not significant. (nDiff=4, error bars=SD). **S5C)** Top annotate hits according to disease or function using Qiagen Ingenuity software. listed with respective *p* values. **S5D)** Plot of interactions between annotated biological pathways determined by Qiagen Ingenuity.

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