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Supplemental Information

STAT3 Inhibitor OPB-51602 Is Cytotoxic to Tumor Cells Through Inhibition of Complex I and ROS Induction

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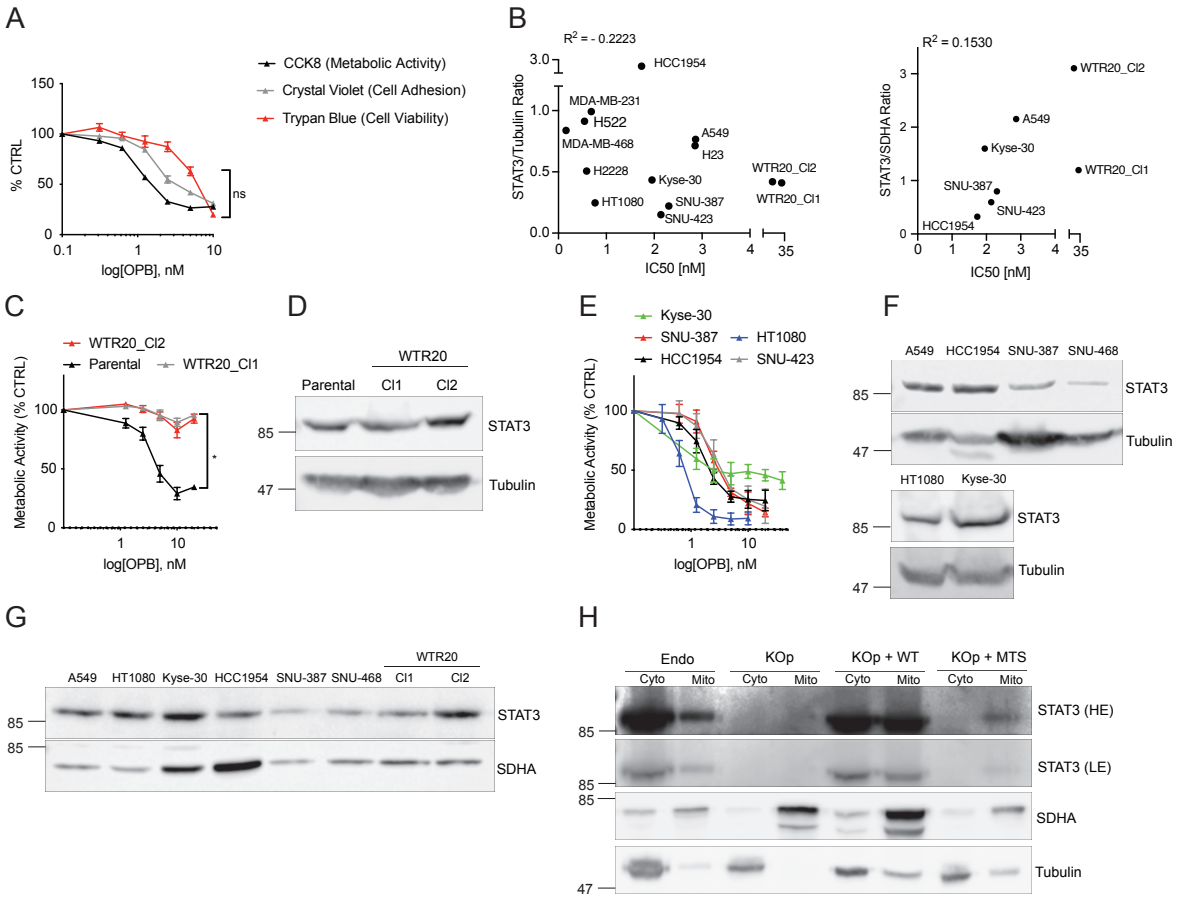
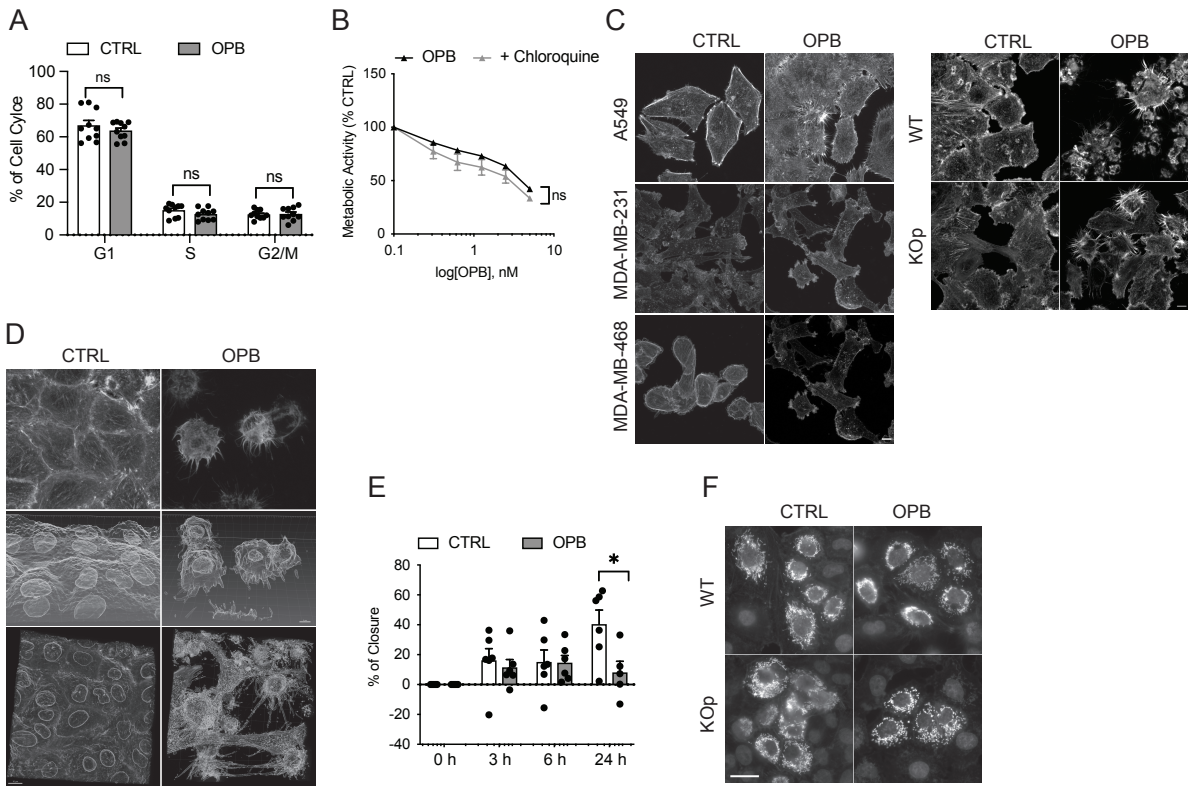
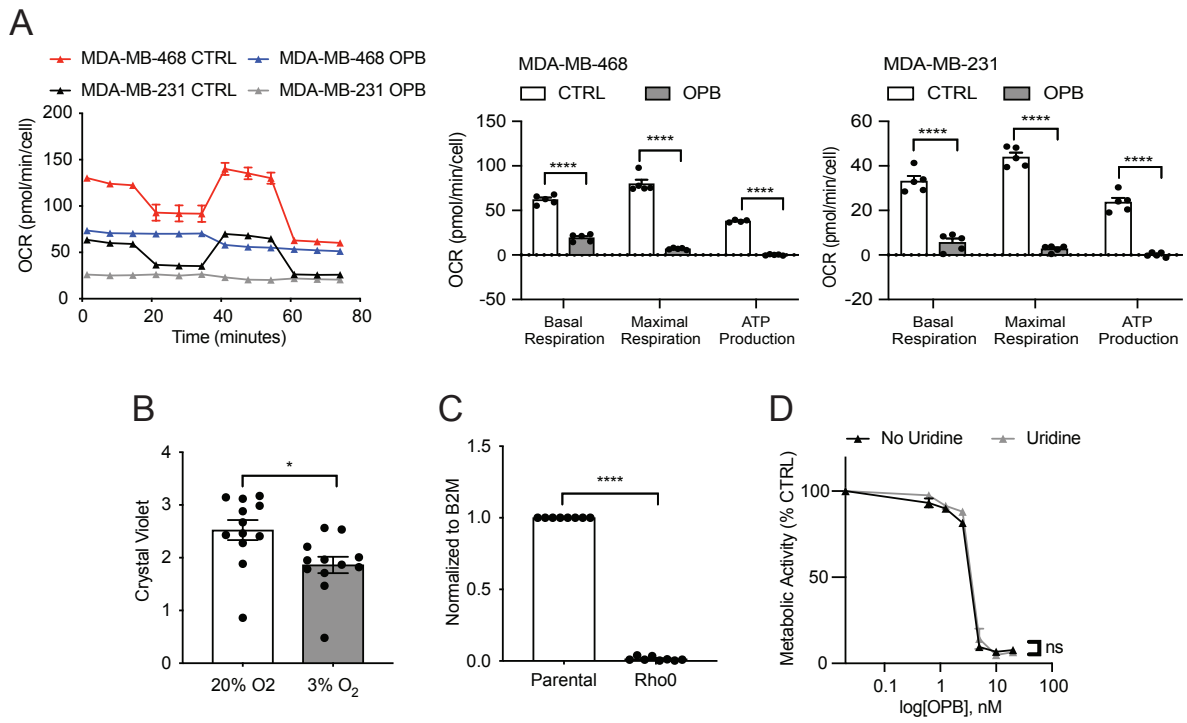
Supplemental Information



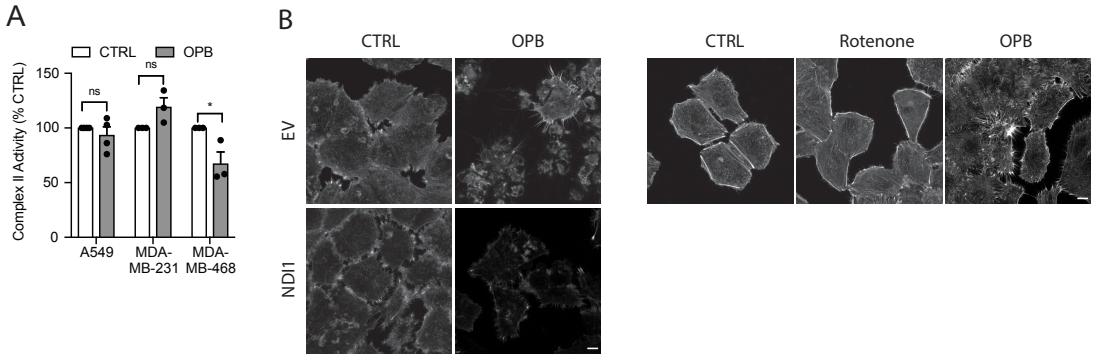
Supplementary Figure 1: Comparison of cell viability assays, STAT3 protein levels in several cell lines and STAT3 constructs localization. Related to Figure 1. A: Comparison of OPB-51602 effects on cell viability with different assays. **B**: Correlation of IC50 and STAT3 expression in total cell lysate (left, normalized to tubulin) and mitochondrial fraction (right, normalized to SDHA) in different cell lines. Cell viability of A549 parental versus drug-resistant isolated clones (WTR20, **C**) and different tumor cell lines (**E**) incubated with OPB-51602 for 16 h and analyzed with CCK8. Basal STAT3 protein expression in WTR20 (**D** and **G**) and different tumor cell lines (**F** and **G**) analyzed by WB. **H**: Subcellular localization of different constructs of STAT3 analyzed by WB. In **A, B** and **C** data are represented as mean ± SEM; n ≥ 3. Unpaired two-tailed Student's t-test; *P<0.05. Molecular weight is expressed in KDa.



Supplementary Figure 2: OPB-51602 effects on cell cycle, migration and actin rearrangement. Related to Figure 2. **A**: Cell cycle analysis of A549 cells treated with 10 nM OPB-51602 for 16 h measured by PI incorporation (n=10). **B**: Cell viability of A549 cells incubated with OPB-51602 for 16 h in the presence or absence of 50 µM Chloroquine, analyzed with CCK8. **C**: Additional figures of actin filaments in different cell lines, related to Fig 2. **D**: Interference reflection microscopy of actin protrusions. **E**: Cell migration of A549 STAT3 KO treated with 10 nM OPB-51602 (n=6). **F**: Mitochondrial morphology in A549 after OPB-51602 treatment analyzed with DsRed2-Mito-7. In **A, B** and **E** data are represented as mean ± SEM; n ≥ 3. Unpaired two-tailed Student's t-test; *P<0.05. In **C, D** and **F** scale bar is 10 µm.



Supplementary Figure 3: Mitochondrial respiration in MDA-MB-231 and MDA-MB-468 and characterization of Rho0 cells. Related to Figure 3. A: Oxygen Consumption Rate (OCR) in MDA-MB-231 and MDA-MB-468 cells treated with 50 nM OPB-51602 for 2 h (n=12). **B**: Cell growth of A549 in high (20%) and low (3%) oxygen tension analyzed with Crystal Violet (n=12). **C**: mitochondrial DNA content of A549 parental and Rho0 cells analyzed by Real Time PCR. **D**: Cell viability of A549 parental cells incubated with OPB-51602 for 16 h in the presence or absence of uridine, analyzed with CCK8. In **A-D** data are represented as mean ± SEM; n ≥ 3. Unpaired two-tailed Student's t-test; *P<0.05, ****P<0.0001.



Supplementary Figure 4: Effect of OPB-51602 on complex II activity and on actin rearrangement. Related to Figure 4. A: Activity of Complex II in A549, MDA-MB-231 and MDA-MB-468 STAT3 WT cells treated with OPB-51602 at 50 nM for 4 h (n=4). **B**: Additional microscope images of larger fields, related to Fig. 4. In **A** data are represented as mean ± SEM; n ≥ 3. Unpaired two-tailed Student's t-test; *P<0.05. In **B** scale bar is 10 μm.

Transparent Methods

Cell lines and plasmids

HEK293T, A549, H522, H2228, H23, HT1080 STAT1 KO, MDA-MB-231 and MDA-MB-468 were purchased from American Type Culture Collection (ATCC) and maintained in DMEM (Fisher Scientific, cat# SH30243.01) supplemented with 10% Bovine Calf Serum (BCS) (Sigma-Aldrich, cat# C8056) and Gentamicin (Cellgro, cat# 30-005-CR). HT1080, Kyse-30, SNU-387, SNU-423 and HCC1954 were kindly obtained from Dr. R. Possemato and maintained in DMEM supplemented with 10% Bovine Calf Serum and Gentamicin. MCF 10A cells were purchased from ATCC and maintained in DMEM F-12 (Gibco, cat# 11320033) supplemented with 5% horse serum (Gibco, cat# 16050114), 20 ng/ml hEGF (PeproTech, cat# AF-100-15), 0.5 mg/ml hydrocortisone (Sigma-Aldrich, cat# H0888), 100 ng/ml cholera toxin (Sigma-Aldrich, cat# C8052) and 10 μg/ml insulin (Sigma-Aldrich, cat# I0516). HBEC3-KT cells were purchased from ATCC and maintained in Defined Keratinocyte SFM supplemented with Defined Keratinocyte-SFM Growth Supplement (Gibco, cat#). Cell lines stably expressing short-hairpin RNA against either STAT3 (shSTAT3, Open Biosystem) or a nonspecific sequence (shEV) were maintained in the above media containing 5 ug/mL of puromycin. A549 stably expressing Cas9 and guide RNA against either STAT3 (gRNA sequence: AGATTGCCCGGATTGTGGCC) or a nonspecific sequence (siEV) were maintained in the above media containing 5 ug/mL of puromycin. A549 stably expressing STAT3 WT or MTS were maintained in the above media containing 200 ug/ml of hygromycin. A549 stably expressing pMXS-IRES-NDI1 (Dr R. Possemato) were maintained in the above media containing 10 ug/ml of Blasticidin. A549 stably expressing pCHAC-mt-mKeima (Dr M. Philips) or DsRed2-Mito-7 (Dr D. Bar-Sagi) were maintained in the above media without selection. Recombinant retroviruses were generated in HEK293T cells. A549 Rho0 cells were generated by growing the cells for 10 days in the presence of ddC (5 μM) and Uridine (10 mg/ml). A549 OPB-51602 resistant clones (WTR20_Cl1 and WTR20_Cl2) were made by growing the cells in complete media supplemented with increasing concentrations of OPB-51602 and then maintaining them in 20 nM OPB-51602.

Antibodies and chemicals

The antibodies against the following proteins were obtained from commercial sources: STAT3 (Cell Signalling, cat# 8768S, dilution 1:2000), SQSTM1/p62 (ABClonal, cat# A11483, dilution 1:2000), SDHA (ABClonal, cat# A2594, dilution 1:2000), NDUFV2 (ABClonal, cat# A7442, dilution 1:2000), NDUFS2 (ABClonal, cat# A12858, dilution 1:2000), Actin (Millipore, cat# MAB1501, dilution 1:2000), Tubulin (Sigma-Aldrich, cat# T9026, dilution 1:2000) and STAT1 antibody was a gift from Chien-Kuo Lee, National Taiwan University (dilution 1:2000). The following chemicals were used: OPB-51602 (Otsuka Pharmaceuticals, Japan), APC-Annexin V (BioLegend, cat# 640920), PI (Biolegend, cat# 421301), FITC-Phalloidin (AAAT Bioquest, cat# 23115), rotenone, (Sigma-Aldrich, cat# 83-79-4), DCFDA (Sigma-Aldrich, cat# 287810), MitoSOX (Thermo Fisher Scientific, cat# M36008), Antimycin A (Sigma-Aldrich, cat# A8674),

Metformin, (Thermo Fisher Scientific, cat# ICN15169101), FCCP (Cayman Chemical Company, cat# 15218), Chloroquine (Sigma-Aldrich, cat# C-6628), GSH-EE (Sigma-Aldrich, cat# G1404), DCIP (Sigma-Aldrich, cat# D1878), DCU (Sigma-Aldrich, cat# D7911), β-NADH (Sigma-Aldrich, cat# N8129), rATP (Sigma-Aldrich, cat# FLASS), ddC (Sigma-Aldrich, cat# D5782), Uridine (Sigma-Aldrich, cat# U3750), Hygromycin (Corning, cat# MT30240CR), Puromycin (Thermo Fisher Scientific, cat# A1113803), Blasticidin (Sigma-Aldrich, cat# 15205).

Immunoblotting (WB)

Cells were lysed in 25 mM Tris-HCl (pH 7.4), 150 mM KCl, 5 mM EDTA, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS, protease inhibitor cocktail (Bimake, cat# B14002), 2 mM Na_3VO_4, and PMSF (Sigma-Aldricht, cat# 41947), resolved by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, cat# 10600023), and probed with antibodies diluted in Tris-buffered saline (TBS)–0.1% Tween 20 overnight at 4°C. Blots were developed with a 1:10,000 dilution of fluor-conjugated secondary antibody (LI-COR).

RT-PCR

RNA isolated with TriZol (Invitrogen) was used to generate cDNA with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Transcripts were then quantified by qRT-PCR with SYBR green (Molecular Probes) using the following primers: human B2M: F 5'_TGCTGTCTCCATGTTTGATGTATCT_3', R 5'_TCTCTGCTCCCCACCTCTAAGT_3' and human mitochondrial tRNA-Leu gene: F 5'_GATGGCAGAGCCCGGTAATCGC_3', R 5'_TAAGCATTAGGAATGCCATTGCG_3'
Relative expression was determined by comparison to a standard curve generated from serial dilutions of cDNA containing abundant target sequences and normalized to the expression of B2M. Data were represented as the mtDNA/B2M ratio and the mean ± standard deviation between triplicate samples of a representative experiment were shown.

Cell viability

Cells were plated in 96-well plates in 25 mM or 5 mM glucose DMEM supplemented with 10% serum and treated with indicated compounds after 24 h. Cell viability was assessed after 16 h using the Cell Counting Kit-8 (CCK8) assay (Bimake, cat# B34304) or Crystal Violet (Fisher Scientific, cat# C581) or Trypan Blue (Kodak, cat# C123850).

Cell cycle analysis

Cells were plated in 10-cm dishes in 25 mM glucose DMEM supplemented with 10% serum and treated with 10 nM OPB-51602 for 16 h. Next, cells were trypsinized and fixed in cold 70% ethanol for at least 16 h at -20C. Cells were spun at 2 400 g for 5 minutes, washed in cold PBS and the pellet was resuspended in PI and Ribonuclease at 4 C for at least 30 minutes. Samples were analyzed by flow cytometry.

Apoptosis assay
Cells were seeded in 12-well plates for 24 h in 5 mM glucose DMEM and then treated with OPB-51602 for the indicated periods of time. Next, cells were trypsinized and incubated with APC-Annexin V and PI accordingly to the manufactures' instructions. Apoptosis was measured by flow cytometry.

Wound healing assay
Cells were seeded in 12-well plates in 25 mM glucose DMEM and after 72 h the cell layer was scratched by a 10 μL pipette tip and treated with 10 nM OPB-51602. Live cell images were acquired at 0-3-6-24 h, using a ZOE Fluorescent Cell Imager and the average extent of wound closure was quantified by ImageJ software.

Immunofluorescence
A549 cells were seeded onto 8-well NuncTM Lab-TekTM II Chanbered Coverglas (Nunc) in 5 mM glucose DMEM and treated with OPB-51602 10 nM or rotenone 1 nM for 16 h. Cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT), followed by permeabilization with 0.5% Triton X-100 in PBS for 20 minutes at RT. This was followed by incubation with AF-488 Phalloidin for 15 minutes at RT. Cells were visualized using the Zeiss AxioObserver Microscope.

Oxygen consumption rate
The oxygen consumption rate (OCR) was measured using the Seahorse XFp Analyzer (Seahorse Bioscience) under standard conditions and after addition of 1 μM oligomycin, 2 μM FCCP, and 1 μM rotenone/antimycin A with or without pre-treatment with OPB-51602 (50 nM for 2 h) according to manufacturer's instructions. Cells were seeded in the XFp miniplates and grown overnight. Test compounds were diluted in XF Base Medium containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose at pH 7.4 and were sequentially injected. OCR (picomoles per minute) was measured in basal condition and after injection of each test compound.

ROS measurement
Cells were seeded in 12-well plates for 24 h in 5 mM glucose DMEM and then treated with 50 nM OPB-51602 for 2 h and 500 nM rotenone for 4 h. ROS were measured by flow cytometry following treatment of cells with 5 μM DCFDA (15 minutes) or 5 μM Mito-SOX (30 minutes).

Mitochondrial preparation
Cells were harvested and the pellet was resuspened in 2X of Mitochondria Purification Buffer (MPB: 10 mM Tris-MOPS, 1 mM EGTA-Tris, 200 mM Sucrose, pH= 7.4 plus freshly added: protease inhibitors, 2 mM Na3VO4, 1 mM DTT and 1 mM Na-b-glycerophosphate) and incubated on ice for 10 minutes. Then, cell suspension was transferred to a homogenizer chilled on ice and cells were disrupted by the application of 40 strokes. Cells were spun at 800 g for 5 minutes to

pellet nuclei and unbroken cells and the supernatant was collected in a fresh tube and spun at 8 800 g for 10 minutes to pellet mitochondria. Mitochondria were resuspended in MPB with 5% digitonin and incubated on ice for 5 minutes, then spun at 10 000 g for 10 minutes. Mitochondria were washed 2X with MPB and protein content was quantified.

Complex I assay

2 ul of isolated mitochondria were incubated with 100 ul of Complex I assay Buffer (CIB: 25 mM Potassium Phosphate, 2 mM KCN, 3.5 g/L BSA, 60 μM DCIP, 70 μM DCU, 1 μM Antimycin A) with or without 1 μM rotenone for 10 minutes at 37 C. 5 mM NADH was added and absorbance was measured at 600 nm at 30 seconds intervals for 10 minutes. Complex I activity: 1U=1 μM DCIP reduced per minute per μg of protein.

Complex II assay

2 ul of isolated mitochondria were incubated with 100 ul of Complex II assay Buffer (CIIB: 80 mM Potassium Phosphate, 1 g/L BSA, 2 mM EDTA, 0.2 mM rATP, 80 μM DCIP, 50 μM DCU, 1 μM Antimycin A, 3 μM rotenone) for 10 minutes at 37 C. 10 mM Succinate and 0.3 mM KCN were added and absorbance was measured at 600 nm at 30 seconds intervals for 10 minutes. Complex II activity: 1U=1 μM DCIP reduced per minute per μg of protein.

Statistical analysis

Differences between experimental groups were analyzed for statistical significance using unpaired two-tailed Student's t-test. The Pearson correlation was used to evaluate the linear relationship between two continuous variables. Data shown are representative of at least three independent experiments. A $P < 0.05$ was considered to be statistically significant.