Supplemental information

A mitochondrial inside-out iron-calcium signal

reveals drug targets for Parkinson's disease

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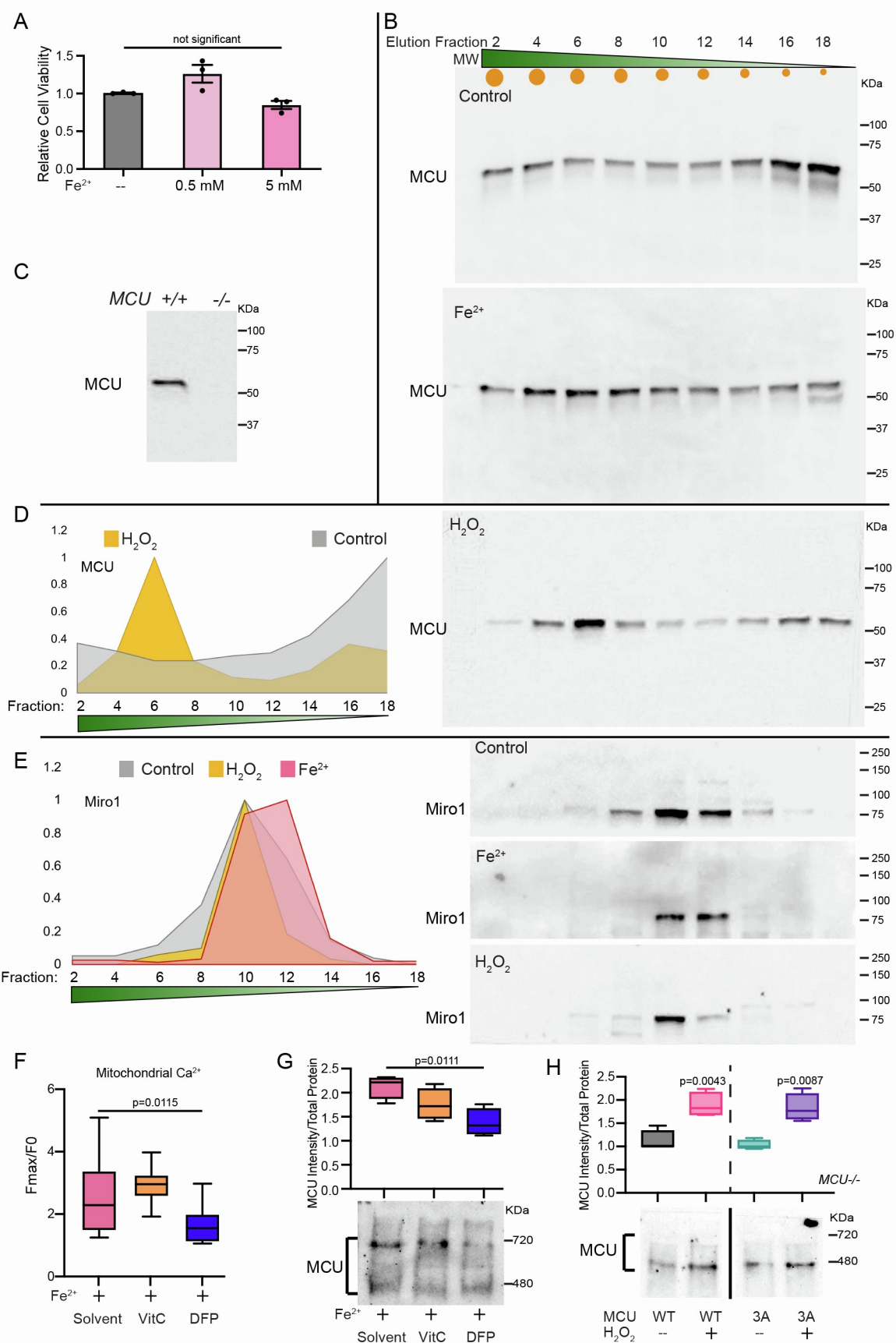
Figure S1. Cell Death Assay, SEC Blots, and ROS Assays, Related to Figure 1. (A) HEK cells were treated as indicated for 24 hours and cell viability was measured by the MTT assay. n=3. Not significant compared to "untreated". Two-tailed Welch's T Test. (B) HEK cells were treated as indicated and run through SEC. Each fraction containing differential MW of protein complexes was run in SDS-PAGE and blotted with anti-MCU. (C) Validation of anti-MCU using the SEC sample from wild-type HEK cells (control; *MCU+/+*) and lysate of *MCU-/-* HEK cells in SDS-PAGE. (D-E) Similar to (B) and Figure 1G. (D) MCU SEC profile of HEK cells treated with H_2O_2. (E) Miro1 SEC profiles. Note that Fe^{2+} or H_2O_2 treatment shifted the elution peak fractions of MCU from fractions "14-18" to "4-8", whereas Miro1's peak fractions remained in fractions "10-12" under all conditions. (F) Similar to Figure 1C-D, mitochondrial Ca^{2+} dynamics was measured. n=15 cells from 3 coverslips. (G-H) Similar to Figure 1E, MCU oligomerization was measured. n=4. (F-G) 500 μM Fe^{2+} was added for 24 hours. 100 μM VitC or DFP was added for 16 hours. One-Way Anova Post Hoc Tukey Test. (H) 100 μM H_2O_2 was added for 30 minutes. Two-tailed Welch's T Test. Compared within each genotype. The same volume of the solvent was added to controls in all panels.

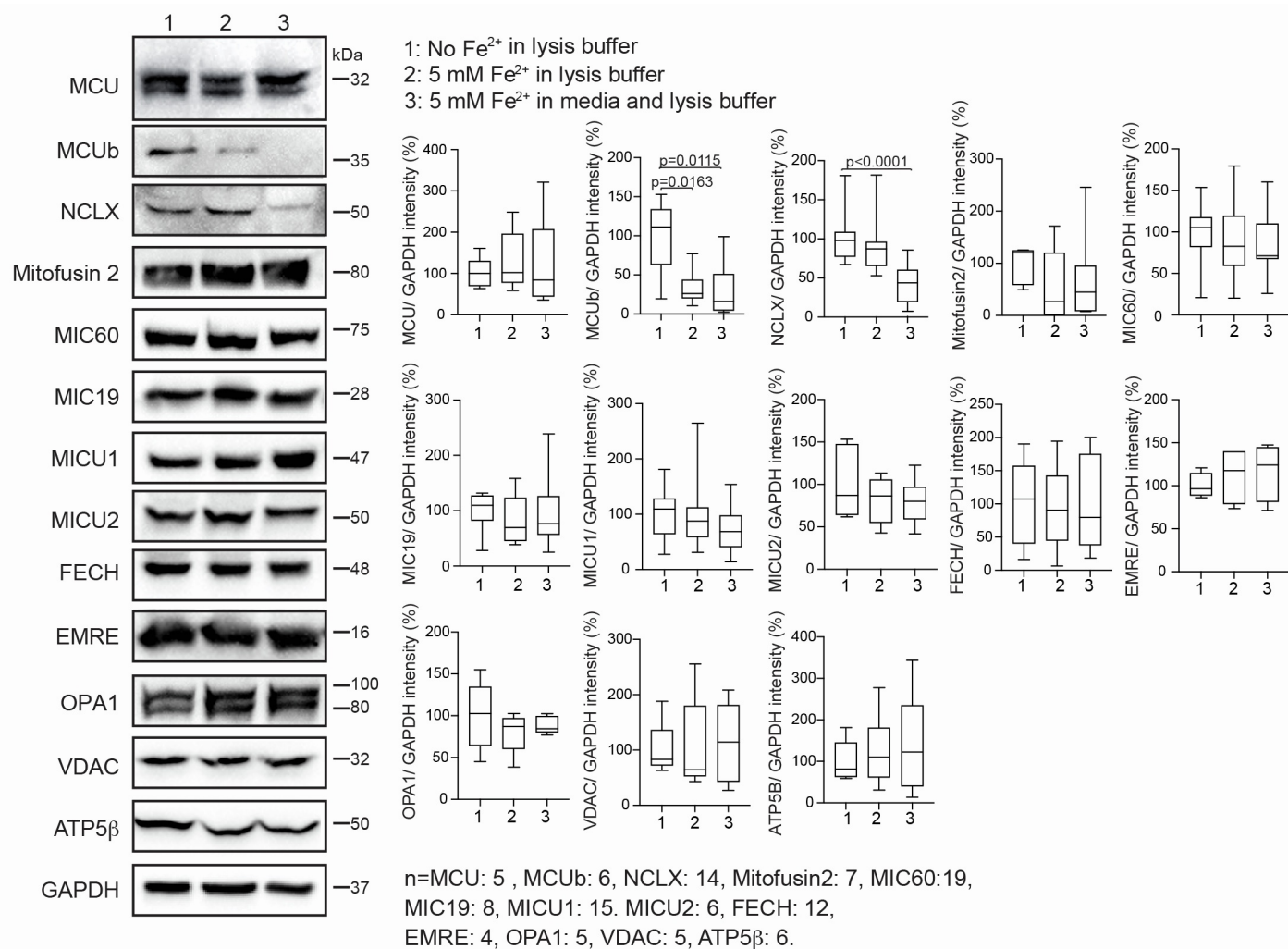**Figure S2. Mitochondrial Protein Responses to Iron.** HEK cells were treated as indicated, lysates were run in SDS-PAGE, and blotted. The band intensity was normalized to that of GAPDH from the same blot. n=5-19 independent experiments. Precise p and n are in the figure. One-Way Anova Post Hoc Tukey Test. Condition 1 contained EDTA. For condition 3: 5 mM Fe^{2+} was added to the media 20-22 hours before cells were lysed.
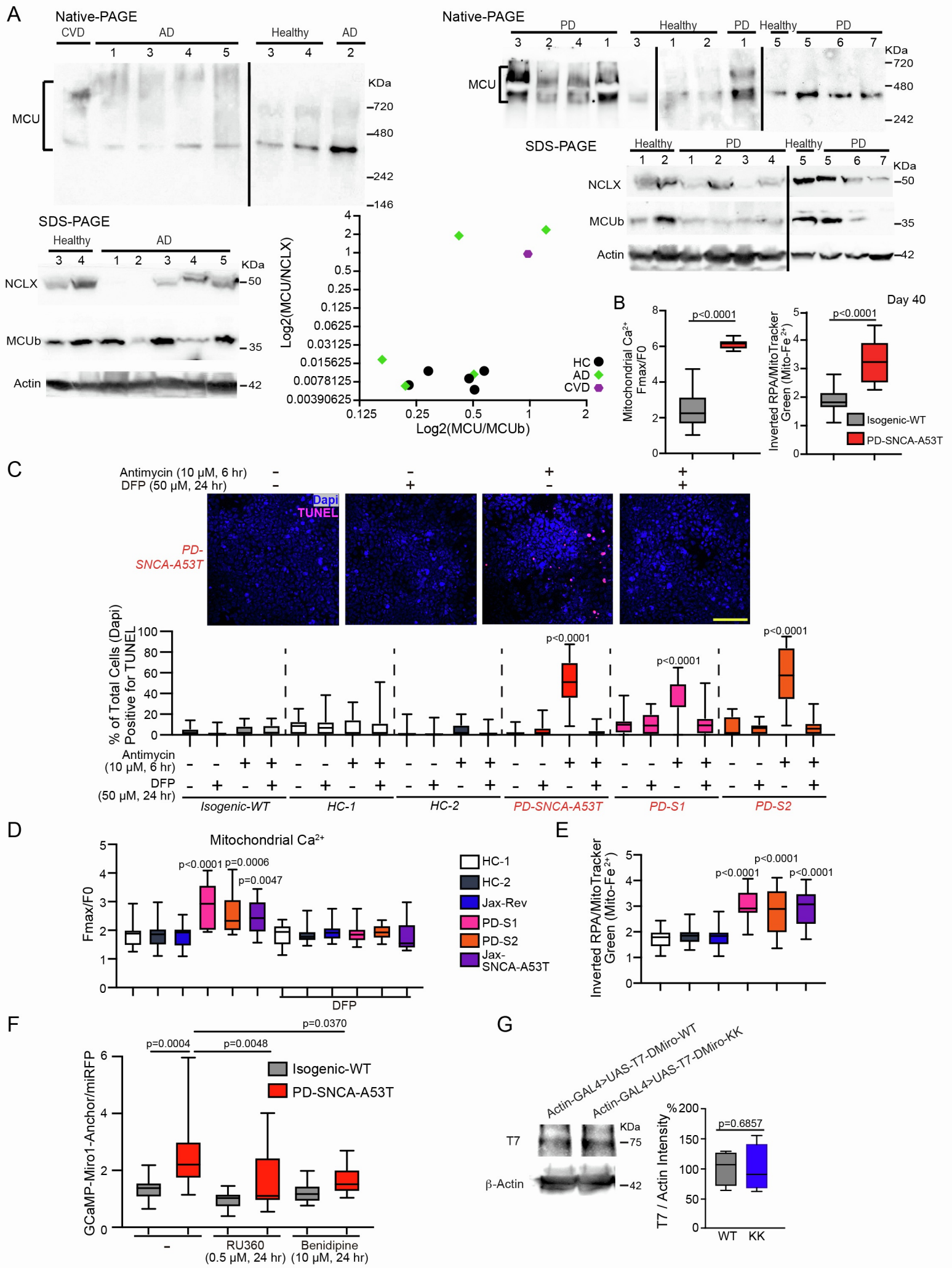
Figure S3

Figure S3. PD Postmortem Brains and Validation of Models, Related to Figure 3-5. (A) Postmortem brains were run in Native- or SDS-PAGE and blotted. The band intensity normalized to the total protein level measured by BCA is divided by that of the universal control (CVD, cardiovascular disease) on the same blot, which was included on every blot. The MCU oligomer bands in Native-PAGE and the NCLX and MCUb bands in SDS-PAGE (average of 3 replicates) are used in the plot here and in Figure 3A. HC: healthy control. (B) Similar to Figure 3C and E, Fmax/F0 of the Rhod-2 intensity (left) and the inverted RPA/MitoTracker intensity was measured in neurons day 40 after differentiation. n=20 cell bodies from 3 independent coverslips. Two-tailed Welch's T Test. (C) iPSC-derived neurons treated as indicated, were immunostained with TUNEL and Dapi, and imaged under a confocal microscope. Scale bar: 50 μm. Below: Quantification of the percentage of TUNEL-positive neurons. n=20 images from 3 independent coverslips. Comparison within each genotype (p values are significant compared to every other condition; non-significant p not shown, One-Way Anova Post Hoc Tukey Test). (D) Similar to Figure 4A, iPSC-derived neurons with or without treatment of 100 μM DFP for 24 hours were stimulated with thrombin, and mitochondrial Ca^{2+} (Rhod-2) was measured. Quantification of the peak fluorescent intensity normalized to the baseline within each cell body. (E) Similar to Figure 3E, the inverted RPA/MitoTracker intensity was measured. (D-E) n=15 cell bodies from 3 independent coverslips. Compared to "HC-1". One-Way Anova Post Hoc Dunnett's Test. (F) In iPSC-derived neurons transfected with miRFP670_P2A_GCaMP6f-Miro1-Anchor and treated as indicated, the intensity of GCaMP-Miro1-Anchor is normalized to miRFP670 within the same cell body. n=15 cell bodies from 3 coverslips. One-Way Anova Post Hoc Tukey Test. Data without treatment is the same as in Figure 4G. (G) Flies with indicated genotypes were lysed and run in SDS-PAGE. n=4 independent experiments. Two-tailed Mann-Whitney Test.
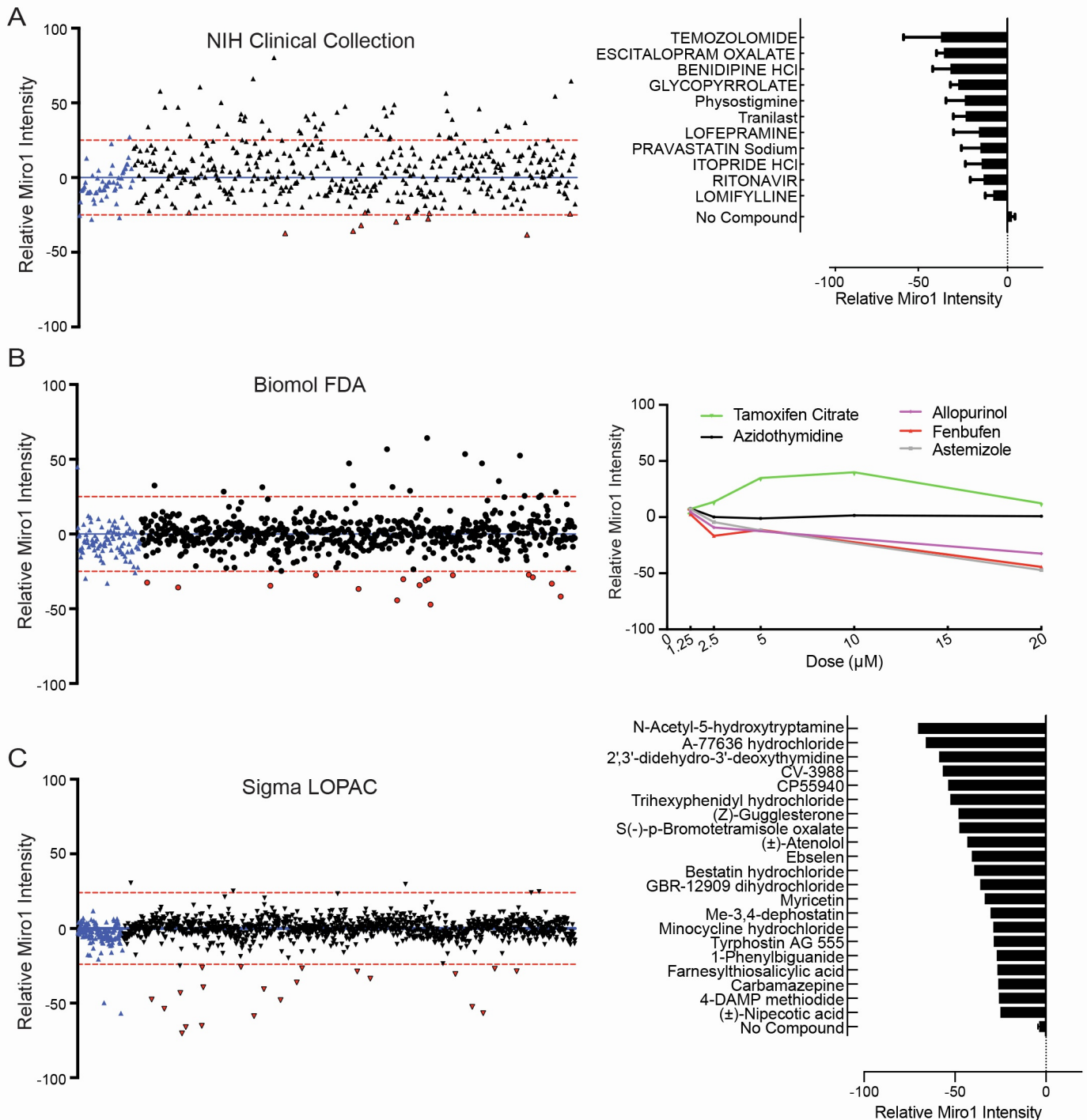
Figure S4. HTP Screens Identify Hit Compounds That Reduce Miro1 upon Depolarization, Related to Figure 6. Miro1 values are modulated by compounds from 3 libraries. The calculation of Relative Miro1 Intensity is described in Method. Negative values indicate Miro1 reduction. Each black or red dot represents the Miro1 intensity value from ICC on the sporadic PD cell line treated with each unique compound combined with FCCP. Each compound was applied 10 hours before application of 20 μM FCCP for another 14 hours. Blue dots represent the Miro1 values of the same sporadic PD cell line treated only with FCCP for 14 hours from multiple independent experiments. (A) NIH Clinical Collection (screening concentration=10 μM, n=4 biological repeats). Right: Eleven primary hits; mean±SEM are shown. (B) Biomol FDA (5-fold screening concentrations, n=2 biological repeats; data of 20 μM are shown). Right: Three primary hits (purple, red, gray line) in a dose-dependent manner. The green line shows data from a compound that increases Miro1 levels, and the black line shows data from a non-responsive compound. IC$_{50}$: Allopurinol: 1.338 μM; Fenbufen: 6.260 μM; Astemizole: 12.63 μM. (C) Sigma LOPAC (screening concentration=20 μM, n=1 biological repeat). Right: Twenty-one primary hits.

A

DMSO | FCCP

Healthy

Sporadic PD

Sporadic PD with Benidipine HCl

Miro1
Dapi

B

	DMSO	FCCP
Healthy	100	63
Sporadic PD	100	91
N-Acetyl-5-hydroxytryptamine	99	116
2'3'-didehydro-3'-deoxythymidine*	72	84
CP55940	147	143
Trihexyphenidyl hydrochloride	115	102
(Z)-Gugglesterone	88	91
S(-)-p-Bromotetramisole oxalate	103	117
(±)-Atenolol	97	119
Bestatin hydrochloride	89	89
Temozolomide*	72	74
Escitalopram Oxalate*	61	70
Myricetin*	70	74
Benidipine HCl*	97	77
Me-3,4-dephostatin	96	117
Minocycline hydrochloride*	66	71
Tyrphostin AG 555*	84	80
Glycopyrrolate	70	97
1-Phenylbiguanide*	65	66
Farnesylthiosalicylic acid	85	93
Carbamazepine*	85	74
4-DAMP methiodide*	82	82
(±)-Nipecotic acid	104	123
Physostigmine*	82	74
Tranilast*	101	78
Lofepramine*	69	79
Pravastatin Sodium*	73	76
Itopride HCl	102	124
Astemizole	86	132
Ritonavir*	53	82
Allopurinol	97	127
Lomifylline	73	90

150
125
100
75
50

C

Library	Unique compounds	Screening concentration (µM)	Biological replicates	Primary Hits	Primary Hit rate (%)	Confirmed Hits	Confirmed Hit rate (%)
NIH CC	377	10	4	11	2.92	8	72.73
Biomol FDA	175	1.25-2.5-5-10-20	2	3	1.71	0	0
Sigma LOPAC	1269	20	1	21	1.65	7	33.33
Total	1821			35	1.92	15	42.86

*fresh compounds of only 34 out of 35 hits were available and tested

Figure S5. Independent Validation of Primary Hits, Related to Figure 6. (A) Representative images of a healthy (upper row) or a sporadic PD fibroblast cell line (middle and lower rows), treated as indicated, and stained with anti-Miro1 and Dapi. Scale bar: 100 µm. (B) Heat map shows the mean Miro1 protein intensities calculated from images similar to (A). Each mean Miro1 value from the sporadic PD line treated with a unique compound is expressed as a percentage of the mean value from the same PD line with no treatment (DMSO; 100). Green shows Miro1 reduction and magenta shows Miro1 elevation. n=4 (drug treatment), 16 (PD, FCCP), and 18 (the rest) independent experiments. Red asterisks indicate hits. More information is in Table S2. Please note that 5 compounds were not shown since 4 compounds were toxic and 1 was not available to retest. (C) Summary of the hit discovery rate of this study.
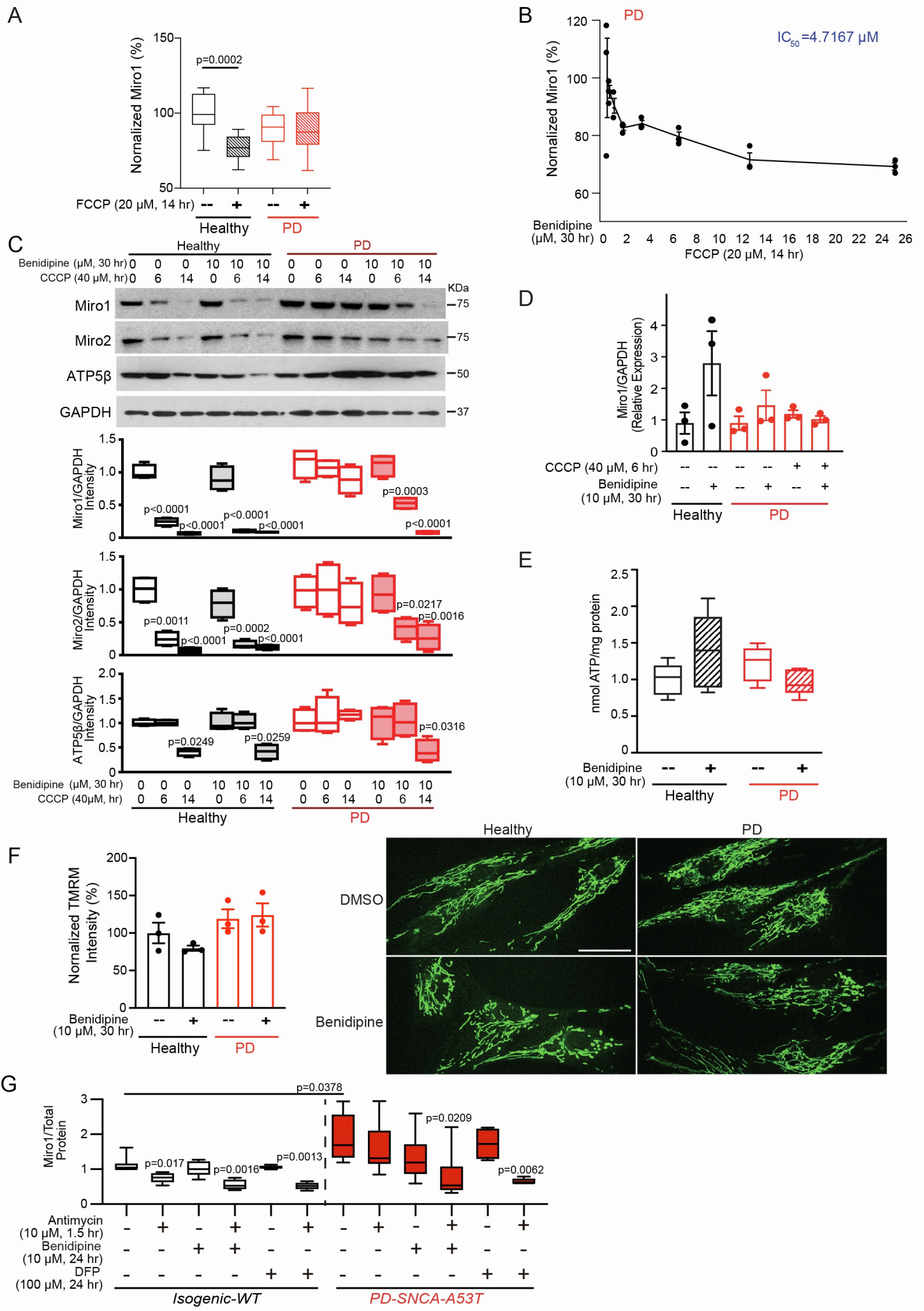
Figure S6. Benidipine Rescues the Miro1 Phenotype, Related to Figure 7. (A-B) Similar to Figure S5, Miro1 intensity is measured in conditions indicated. (A) n=4 independent experiments. (B) n=3 independent experiments. (C) Fibroblasts were treated as indicated and run in SDS-PAGE. Quantification of the band intensity to that of GAPDH from the same blot. Compared to the far-left bar. n=4 independent experiments. Further explanation: Here we depolarized mitochondria with a different uncoupler, CCCP [1], instead of FCCP, because it had been used successfully in Western blotting on Miro1. We detected Miro1 and mitochondrial markers at 6 and 14 hours after CCCP treatment. We had previously demonstrated that in healthy control fibroblasts following CCCP treatment, Miro1 was degraded earlier (6 hours) than multiple other mitochondrial markers (14 hours) [1,2], consistent with the observation of proteasomal degradation of Miro1 prior to mitophagy [1-4]. This figure confirmed that both Miro1 degradation and subsequent damaged mitochondrial clearance were impaired in the PD cell line we used for screens. Importantly, Benidipine promoted Miro1 degradation after 6 hours following CCCP treatment and facilitated mitochondrial clearance as was evidenced by the degradation of ATP5β at 14 hours post-treatment. (D) RT-qPCR results of *Miro1* normalized to *GAPDH* from fibroblasts treated as indicated. n=3. (E) ATP levels were measured in fibroblasts as indicated. n=5. (F) Right: Representative confocal images of TMRM staining in fibroblasts. Left: Quantification of the TMRM intensity normalized to background. Scale bar: 25 μm. n=3 independent coverslips. (A, C, D, E, F) One-Way Anova Post Hoc Tukey Test. (G) Neurons were treated as indicated and Miro1 protein was detected by an ELISA normalized to the total protein amount [5,6] (Figure S7C-D). n=4 for DFP treated, 6 for the rest. Two-tailed Welch's T Test. Comparison within each cell line with "untreated", except otherwise labeled. Note that at baseline, Miro1 protein amount is increased in PD neurons, as reported in [7]. The same volume of solvent was added to controls.
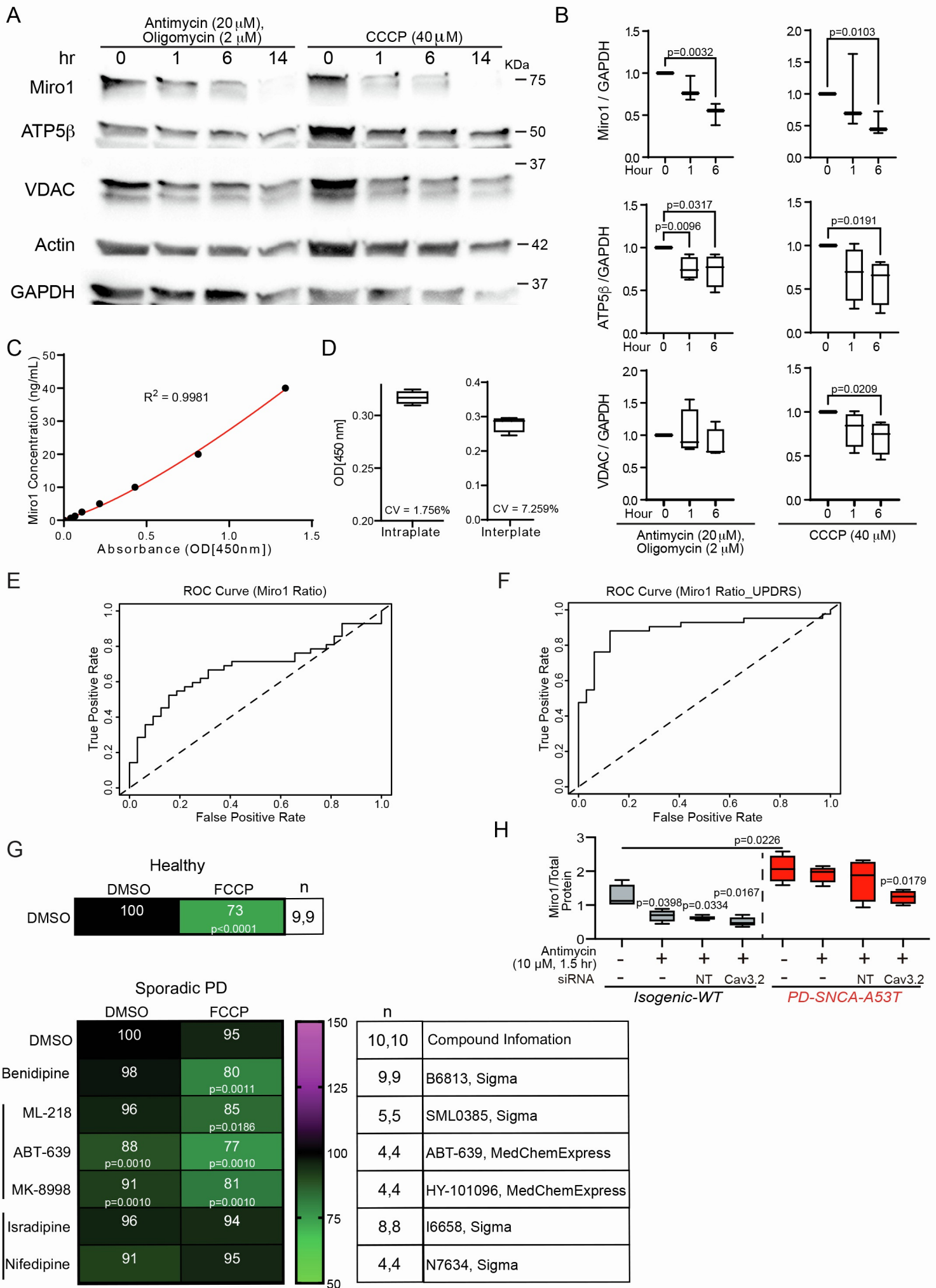
Figure S7

A

Antimycin (20 μM), Oligomycin (2 μM) | CCCP (40 μM)

hr: 0 1 6 14 | 0 1 6 14 KDa

Miro1 — 75
ATP5β — 50
— 37
VDAC
Actin — 42
GAPDH — 37

B

Miro1 / GAPDH: p=0.0032 (Hour 0 1 6); p=0.0103 (0 1 6)
ATP5β /GAPDH: p=0.0096, p=0.0317 (Hour 0 1 6); p=0.0191 (0 1 6)
VDAC / GAPDH: (Hour 0 1 6); p=0.0209 (0 1 6)

Antimycin (20 μM), Oligomycin (2 μM) | CCCP (40 μM)

C Miro1 Concentration (ng/mL) vs Absorbance (OD[450nm]), $R^2 = 0.9981$

D OD[450 nm]: CV = 1.756% Intraplate; CV = 7.259% Interplate

E ROC Curve (Miro1 Ratio) — True Positive Rate vs False Positive Rate

F ROC Curve (Miro1 Ratio_UPDRS) — True Positive Rate vs False Positive Rate

G

Healthy

	DMSO	FCCP	n
DMSO	100	73 p<0.0001	9,9

Sporadic PD

	DMSO	FCCP	n	Compound Infomation
DMSO	100	95	10,10	Compound Infomation
Benidipine	98	80 p=0.0011	9,9	B6813, Sigma
ML-218	96	85 p=0.0186	5,5	SML0385, Sigma
T ABT-639	88 p=0.0010	77 p=0.0010	4,4	ABT-639, MedChemExpress
MK-8998	91 p=0.0010	81 p=0.0010	4,4	HY-101096, MedChemExpress
L Isradipine	96	94	8,8	I6658, Sigma
Nifedipine	91	95	4,4	N7634, Sigma

H

Miro1/Total Protein

p=0.0226
p=0.0398 p=0.0334 p=0.0167
p=0.0179

Antimycin (10 μM, 1.5 hr): − + + + | − + + +
siRNA: − − NT Cav3.2 | − − NT Cav3.2

Isogenic-WT | *PD-SNCA-A53T*

Figure S7. Miro1 in PBMCs and Miro1's Relation with Selective T-Type Ca^{2+}-Channel Blockers, Related to Figure 7. (A) PBMCs from a healthy donor (SBC, Table S4) were cultured and treated as indicated, and lysates were run in SDS-PAGE. (B) From blots as in (A), the band intensity is quantified by normalizing it to GAPDH from the same blot. n=3 independent experiments for Miro1 and 4 for ATP5β and VDAC. Two-tailed T Test. Left: Antimycin A and Oligomycin. Right: CCCP. (C) A representative standard curve using the Miro1 ELISA kit. Sigmoidal 4PL is used. (D) Intraplate and interplate variability of the ELISA kit was demonstrated to be low. Intraplate: the same fibroblast cell lysate was run 4 times in the same plate. Interplate: the same protein standard (1.25 ng/ml) was run in 4 different plates. (E-F) ROC plots for Miro1 Ratio alone (E) and Miro1 Ratio combined with UPDRS (F). (G) Similar to Figure S5, fibroblasts were treated as indicated and Miro1 and Dapi were imaged. Heat map shows the mean Miro1 protein intensities calculated from the images. Each mean Miro1 value is expressed as a percentage of the mean Miro1 value from the same cell line with DMSO treatment alone. n=3-10 independent experiments. Precise p and n are in the figure. Two-tailed Mann-Whitney Test, compared to "DMSO alone". (H) Neurons were transfected with siRNA and treated as indicated. Miro1 was detected by ELISA as above. Comparison with "untreated" within the same line unless labeled. n=4. Two-tailed Welch's T Test. Compared to non-targeting (NT) siRNA, remaining *Cav3.2* after *Cav3.2* siRNA was 37.5-39.0% by RT-qPCR.

Supplementary References

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