MicroRNA-26a/Death-Associated Protein Kinase 1 Signaling Induces Synucleinopathy and Dopaminergic Neuron Degeneration in Parkinson's Disease

Supplementary Information

Supplementary Methods and Materials

Cell culture

N2a and HEK293 cell lines, bought from ATCC (MD, USA), were cultured in DMEM (GlutaMAX™ supplement, Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Cultured cells were used for plasmid or mimics transfection by Lipofectamine 3000(Invitrogen, MA，USA) (1).

Western blot

Cell lysates were obtained using RIPA buffer (Thermo Fisher Scientific，MA，USA) supplemented with phosphatase and protease inhibitor (Thermo Fisher Scientific，MA，USA). 30 μg protein was heated at 95°C for 5 minutes then cooled on ice for 5 minutes. The protein was fractionated on a 4-12% SDS-PAGE Bis-Tris gel using the SDS-PAGE gel electrophoresis system (Biorad, CA, USA). Proteins were transferred onto Nitrocellulose membranes (Whatman, Kent, UK). Following blocking in 5% nonfat milk in 0.01% tween PBS (PBST), membranes were incubated with optimally diluted primary antibodies (Supplementary Table S1) overnight at 4°C. Following washing with PBST, membranes were

incubated with the appropriate anti-mouse or anti-rabbit secondary antibody 1:10,000 for 1 h at room temperature. Signals were detected using an Odyssey CLX instrument (2). The relative protein levels of specific proteins were quantified using Image Studio software (LI-COR Odyssey, NE, USA) compared with DM1A.

Immunofluorescence

The immunofluorescence assay was performed according to the manufacturer's instructions. The frozen cutting slices (30 ㎜) were rinsed by PBS (pH 7.4) 5 min for three times, and then incubated with 0.5% Triton in PBS for half an hour. After blocking with 0.5% BSA for 1 h, the cells were incubated with primary antibodies (Supplementary Table S1) overnight at 4°C. After rinsing with PBS, the slices were incubated with anti-rabbit IgG (H+L) (Alexa Fluor 488 and Alexa Fluor 546 dye were purchased from Life Technologies, USA) at room temperature for 1 h in the dark. After staining with DAPI for 5 min at room temperature, the slices were photographed using a confocal laser scanning microscope (LSM780, Carl Zeiss, Oberkochen, Germany) (3).

TH staining

Briefly, sections were rinsed in PBS and incubated in 3% hydrogen peroxide/10% methanol solution for 10 min to quench endogenous peroxidase activity. Sections were permeabilized/blocked in 5%BSA/0.1% Triton X-100/PBS for 1 h at room temperature. Sections were incubated overnight at 4°C with the following primary antibodies in PBS containing 5% BSA/0.01% Triton X-100. Biotinylated secondary antibodies (Vector

Laboratories, CA，USA) were used appropriately after incubation with streptavidin ABC solution (Vector Laboratories, CA，USA) as described before (4, 5). Immunostaining was visualized by diaminobenzidine (Sigma，CA，USA) chromogen. Sections were mounted on glass, dehydrated, and cover-slipped with cytoseal (Thermo Scientific). Digital images were captured with a Coolpix 5000 Nikon Camera. TH-immunostained sections were counterstained with thionin before dehydration and cover-slipped with cytoseal. Nissl (thionin)-stained and TH-positive neuronal counts were estimated within the substantia nigra by Stereoinvestigator software (Microbrightfield).

MPTP treatment

In the acute MPTP model, mice were injected intraperitoneally (i.p.) with MPTP (20 mg/kg, Sigma, M0896) or sterile saline solution (four times at 2 h intervals) (6). Nine-week-old male C57/Bl6 mice were used (n = 8-10 per group). In the chronic study, mice received a total of 10 doses of MPTP hydrochloride (25 mg/kg in saline, s.c.) in combination with an adjuvant, probenecid (250 mg/kg in dimethyl sulfoxide, DMSO, i.p.). The 10 doses were administered on a 5-week schedule, such that injections were given with an interval of 3.5 days between consecutive doses (7). One week after the last MPTP injection, mice were subjected to behavioral test. Only those showing behavioral dysfunction were used in the subsequent experiments.

Reverse transcription and quantitative PCR (qPCR)

Total RNA was extracted from cells using TRIzol reagent (Life Technologies, Shanghai,

China), and the miscript cDNA synthesis kit (GeneCopoeia，Rockville, MD) was used for the reverse transcription reaction according to the manufacturer's instructions. MiRNA was extracted using miRNA isolation kit (Tiangen, Beijing, China). For qPCR all specific primers were purchased from Ribo Bio (Supplementary Table S2). Quantitative real-time PCR was performed using CFX Connect Real-Time System (BioRad, CA，USA). The amplification and analysis were performed using an iCycler iQ Multicolor Real-Time PCR Detection System (BioRad, CA，USA). Samples were compared using the relative CT method. The fold increase or decrease was determined relative to a vehicle-treated control after normalizing to a housekeeping gene using $2^{-\Delta\Delta CT}$, where ACT is (gene of interest CT) - (U6 CT), and ΔΔCT is (ΔCT treated) - (ΔCT control). All experiments were conducted in triplicate (8).

Luciferase reporters

A wild-type and a mutant DAPK1 3′ UTR were cloned into the SV40-driven *renilla* luciferase reporter plasmid (*p*siCHECK™-2, Promega). HEK293 cells (3×10^5 cells per well) were co-transfected with the pre-miRNA constructs or the empty control vector (*p*cDNA3.2/V5, 500 ng), or pre-miR-26a-5p and wild-type or the mutant DAPK1 3′ UTR plasmid (200 ng). Cells were harvested and cell lysates were assayed for firefly and renilla luciferase activities using the dual-luciferase reporter assay system (Promega，WI USA) according to the manufacturer's protocol. The normalized values (Renilla/firefly activity) were used for analysis (9).

Open field tests

Adult male mice, 90 ±2 days old, were used in this study. Mice were placed in the center of

an open field arena (40×40×30 cm^3) for 60 min and then habituated daily in the same chamber for 10 min per day for three consecutive days. After the 3-day habituation, general locomotion was assessed for a 10 minutes period as previously described (10). Specifically, we measured the distance moved in the arena, mean velocity, rearing time and mobility time.

Rotarod test

The rotarod training was performed at the same time for 10 min during 5 consecutive days. On the first day, mice were placed on the rotating rod at 4 rpm for 5 min. Every 30 s, the rod speed was increased by 1 rpm up to 15 rpm, and this speed was maintained for 1 min. On the second day, a 4 rpm speed was used for 1.5 min. The rod speed was increased by 1 rpm every 30 s until it reached 20 rpm, and this speed was maintained for 10 min. On the third, fourth, and fifth days, the latencies of falling off the rod were measured three times with a linear increase in rod speed from 4 rpm up to 32 rpm for 5 min, and the latency times were averaged. The actual test was performed after the treatment. The test protocol was the same as the last three days of the training protocol. The ORP was calculated according to a previous report (11).

Administration of peptides

The mice were administered 10 mg/kg i.v. Tat-siP-Syn (Tat-YEMPSEEGYQD) or the scrambled control peptide once every three days for 6 weeks. The peptides with 95.79% purity were synthesized by ChinaPeptides (Shanghai, China). The Tat-siP-Syn or scrambled peptides were numbered, and the animal handlers were unaware of which one was applied in

all experiments.

Phosphorylation assay by using recombinant proteins

The recombinant human DAPK1 (aa 1-363) (11966-H20B) and human SNCA/a-Synuclein proteins (12093-HNAE) were purchased from Sino Biological. Phosphorylation of a-Synuclein by recombinant DAPK1 was performed in buffer containing 20 mM 4-morpholinepropane sulfonic acid, pH 7.4, 30 mM $MgCl_2$, 100 mM ATP salt (MACKLIN, 987-65-5), and 20 nmol/min/mg using synthetic R11-S6-Peptide (R11-IAKRRRLSSLRASTSKSESSQK). Human SNCA/a-Synuclein protein was incubated with or without recombinant human DAPK1(20-40mU/ul) at 30°C for 30min. The DAPK1 inhibitor TC-DAPK 6 (MCE,HY-15513) was added at the concentration of 69nM as suggested by the manufacture instruction. The reaction was terminated by boiling in 4xSDS sample buffer. Western blotting was performed as described previously (12).

CHX chase assay

Cycloheximide (CHX), a protein synthesis inhibitor, was used to evaluate the stability of α-synuclein. HEK293 cells were transfected with plasmids of wt a-synuclein and wtDAPK1 or vector control (pcDNA). Twenty-four hours later, cells were treated with 10 μM CHX (Sigma, CA, USA) (13). Proteins were extracted at the indicated time points, and western blot with quantitative analysis was performed as described above.

Filter trap assay

The amount of α-synuclein aggregates following the assembly or disassembly reactions was determined via a filter trap assay as previously described (14). Reactions were diluted in TBS such that they contained 20 ng of protein in 300 µL. For heparin-induced α-synuclein aggregation reactions, the reactions were diluted in TBS to contain 60 ng of protein in 300 µL. Solutions were passed through a nitrocellulose membrane using a vacuum in a dot blot apparatus. The membranes were washed three times with TBS-0.05% Tween-20 (TBST) and then blocked in 5% nonfat dry milk in TBST for 1 h. The aggregates trapped on the membrane were detected by general antibodies and analyzed by a western blotting analysis system. Statistical analyses were performed using unpaired t-tests to compare the triplicate values to control values.

Supplementary Tables

Supplementary Table S1. Antibody information

	Company	Catalog No.	Dilution
Anti-a-synuclein	CST	#2628	1:1000 WB
Anti-a-synuclein p-Ser129	Abcam	ab184674	1:1000 WB/1:200 IF
Anti-DAPK1	CST	#3008S	1:1000 WB
Anti-DAPK1	Acris		1:200 IF
Anti-DAPK1	ORIGENE	TA324907	1:100 IF
Anti-tyrosine hydroxylase	Abcam	ab112	1:100 IHC
Anti-tyrosine hydroxylase	Abcam	ab113	1:100 IHC
Anti-C/EBPα	Proteintech	18311-1-AP	1:1000 WB
Anti-DM1A	CST	#3873	1:1000 WB
Anti-pMLC	Abcam	ab130770	1:1000 WB

WB: Western blot; IF: Immunofluorescence; IHC: Immunohistochemistry

Supplementary Table S2. The information of qPCR primers, mimics, agomirs, antagomirs and probes.

Name	Catalog
qPCR primers	
mmu-miR-141-3p	miRQ0000153-1-1
mmu-miR-98-5p	miRQ0000545-1-1
mmu-miR-26a-5p	miRQ0000533-1-1
mmu-miR-26b-5p	miRQ0000534-1-1
mmu-miR-124-5p	miRQ0004527-1-1
mmu-let-7a-5p	miRQ0000521-1-1
mmu-let-7b-5p	miRQ0000522-1-1
mmu-let-7c-5p	miRQ0000523-1-1
mmu-let-7d-5p	miRQ0000383-1-1
mmu-let-7e-5p	miRQ0000524-1-1
mmu-let-7f-5p	miRQ0000525-1-1
mmu-let-7i-5p	miRQ0000122-1-1
hsa-miR-26a-5p	miRQ0000082-1-1
hsa-miR-26b-5p	miRQ0000083-1-1
has-miR-141-5p	miRQ0004598-1-1
mimic, agomir and antagomir	
mmu-miR-26a-5p mimics	miR10000533-1-5
negative control mimics	miR01201-1-5
mmu-miR-141-3p mimics	miR10000153
negative control mimics	miR01101
mmu-miR-26a-5p agomir	miR40000533-1-10
miRNA agomir control	miR04201-1-10
mmu-miR-26a-5p antagomir	miR30000533-1-10
miRNA antagomir control	miR03201-1-10
probes	
mmu-mir-26a-5p	WHP2018060022082

Supplementary Table S3. The potential regulation of 3'UTR of dapk1 by miRNAs predicted by Targetscan and miRNA.org

	Targetscan(Pct)	miRNA.org (mirSVR score)
miR-124-3p.1	0.8	-1.1153
let-7-5p	0.56	-0.3608
miR-98-5p	0.56	
miR-26-5p	N/A	-0.6596
miR-141	0.36	-0.4813
miR-451	<0.1	-0.8164
miR-361		-1.2585
miR-301a		-1.2004
miR-448		-1.0897
miR-216a	<0.1	-1.0404
miR-342-3p		-0.8372
miR-544		-0.7733
miR-26a		-0.6596
miR-362-3p		-0.5951
miR-329		-0.5915
miR-324-5p		-0.5884

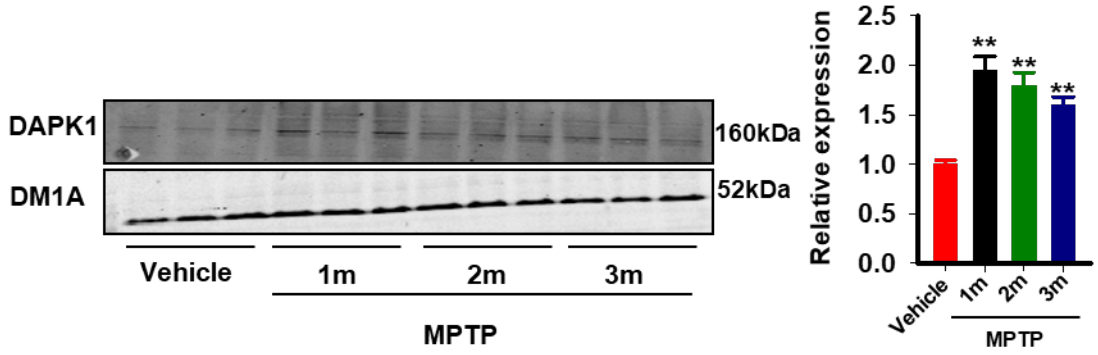
Notes: The miRNAs labeled by red color were chosen for Q-PCR validation.

Supplementary Table S4. Human IHC case list

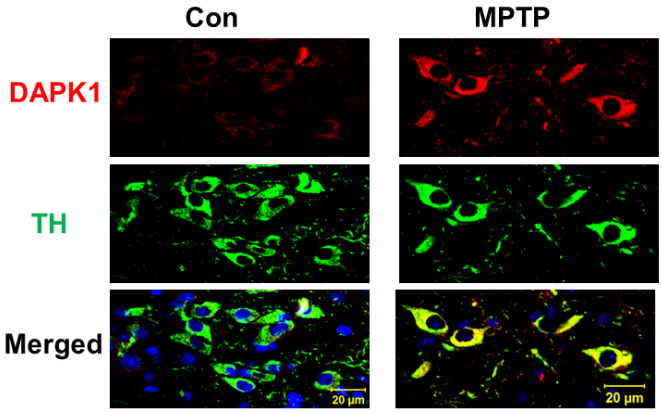
Case	Diagnosis	Age	PMI	LBs	Nucleoli	Neurons	Processes
A95-108	Control	51	16	-	+/-	+/-	++
098-52	Control	67	7	-	+/-	+	+/-
A95-83	Control	72	14	-	-	++	+
P01-11	Control	78	unk	-	-	+/-	+/-
A84-458	PD	66	19	+	++	+	++
P14-19	PD	75	19	+	++	+/-	++
P14-11	PD	76	16	-	++	+	+/-
A85-148	PD	79	11	-	++	+/-	++
A78-126	PD	85	unk	-	+	+	++
085-34	PD	unk	unk	+	++	+	+

unk:unknown

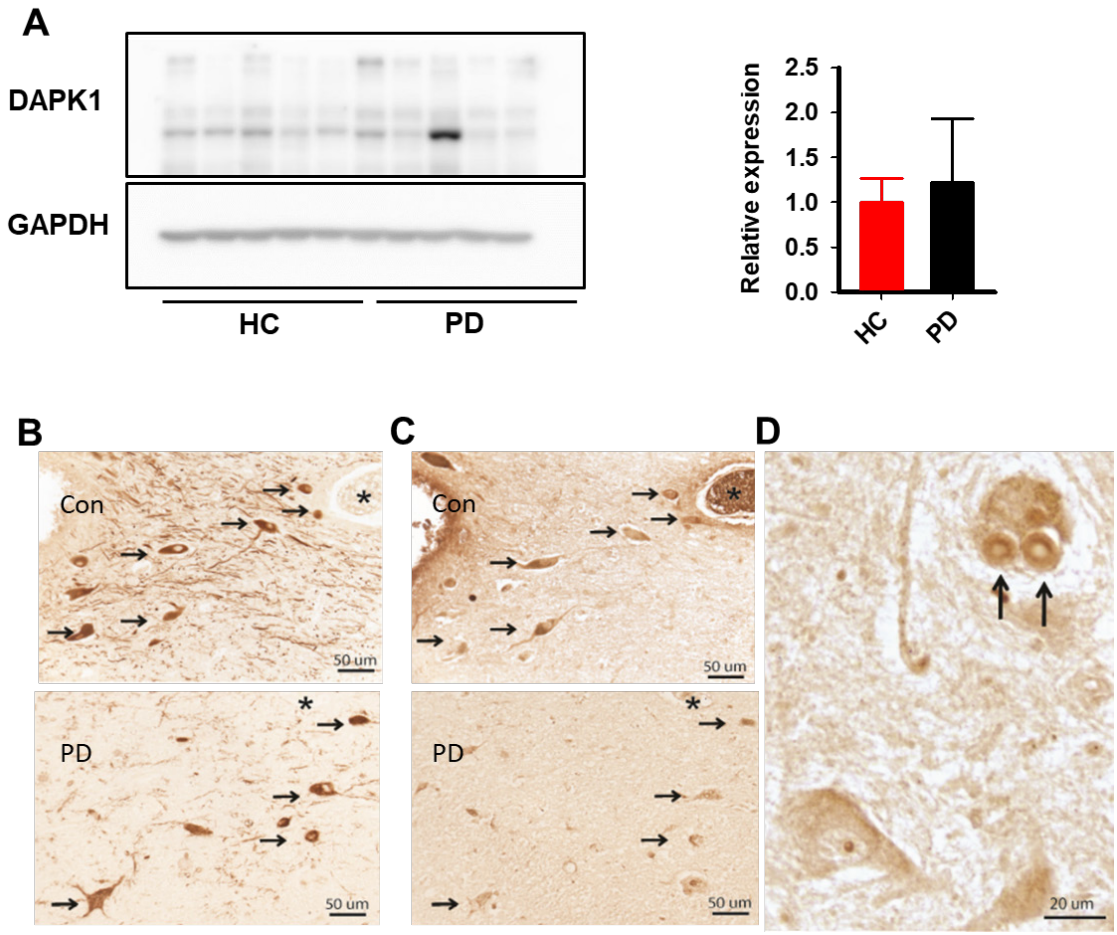
Supplementary Figures



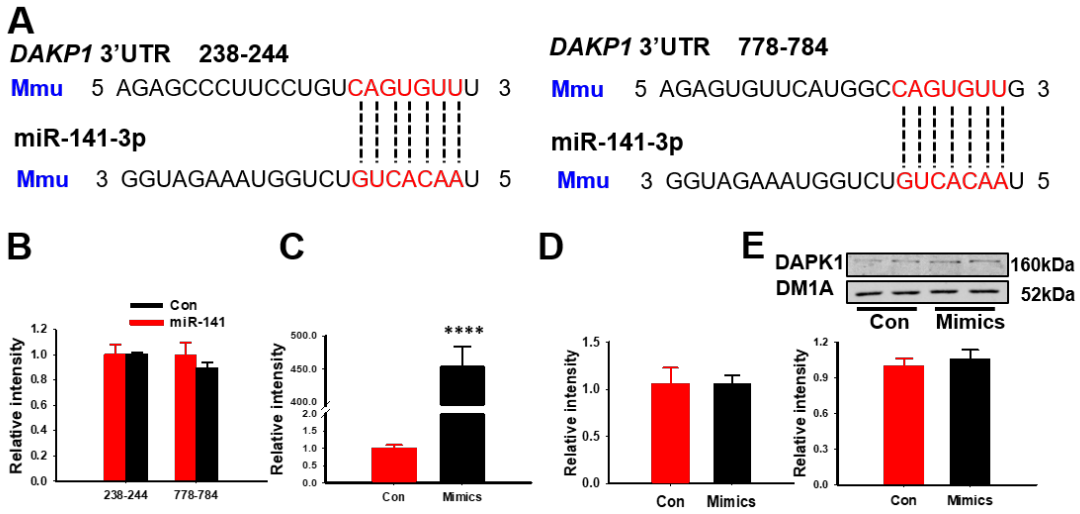
Supplementary Figure S1. DAPK1 level is increased in chronic MPTP treated mice
Protein from the SN of the chronic MPTP-injected mice at indicated timepoint were extracted and subjected to western blot with anti-DAPK1 and anti-DM1A antibodies. Representative images are shown, and the quantitative analysis was performed with Student's t-test. **P<0.01 vs vehicle-injected mice; N=3 independent experiments by using 6 mice per group.



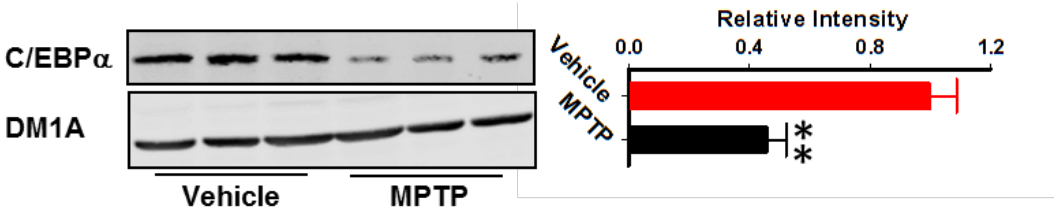
Supplementary Figure S2. The double-immunofluorescence staining of DAPK1 (Red) and TH (G) in the SNc of control or MPTP treated mice.



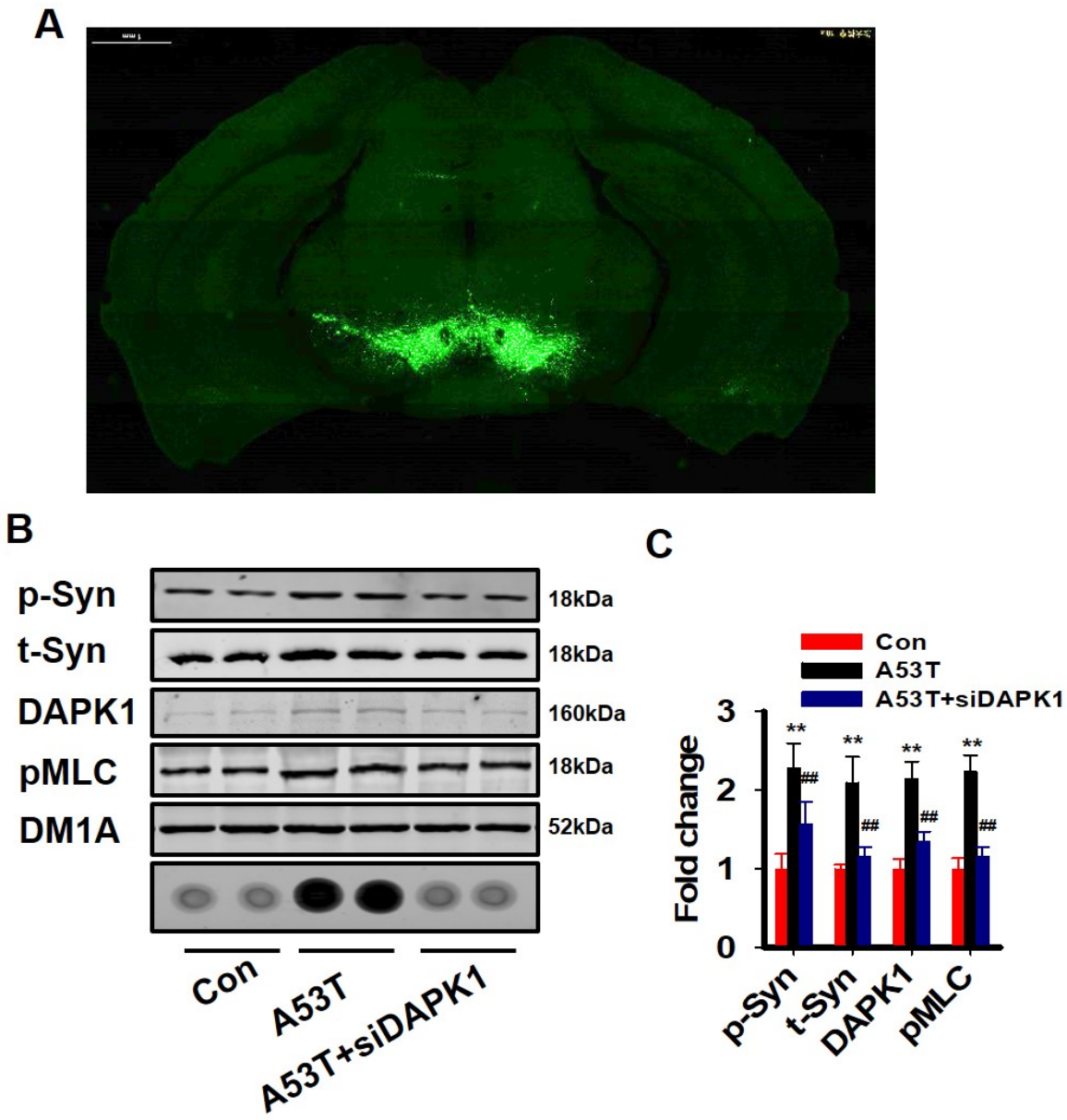
Supplementary Figure S3. The expression of DAPK1 in the PD postmortem brain.
(A) The representative blot from PD patients (PD) and health control (HC). (B-D) DAPK1 immunoreactivity in the brain of Parkinson disease patients and age-matched control patients. In brain stem sections of PD and control cases, adjacent serial sections were stained for tyrosine hydroxylase (B) and DAPK1 (C). Asterisks (*) denote landmark vessels. Arrows point to the same neurons in adjacent section. In PD cases, Lewy bodies were occasionally stained for DAPK1 (D).



Supplementary Figure S4. miR-141 doesn't regulate DAPK1.
(A) The predicted binding sites of mouse miR-141 with 3'UTR of DAPK1. (B) Luciferase experiment by using reporter constructs with different 3'UTR region of DAPK1. (C) The expression of miR-141 upon the treatment of miR-141 mimics. **** p<0.0001, vs scrambled control (con). (D) The relative mRNA level of DAPK1 upon the treatment of miR-141 mimics in N2a cells. (E) The representative blot (upper) of DAPK1 and the quantification (lower) in the cell lysis that treated with miR-141 mimics or scrambled control.



Supplementary Figure S5. The alteration of C/EBPa in MPTP mouse.
The homogenates from the SN and were used to detect C/EBPa expression. **P<0.01 vs vehicle; N=3 independent experiments; Student's t-test.



Supplementary Figure S6. Effect of DAPK1 silencing on the synucleinopathy in aged A53T mutant mice.

The lentivirus that contains the effective shRNA (5'-GGTCAAGGATCCAAAGAAG -3') for DAPK1 or the scrambled control were injected into the SNc of A53T mice or wild type littermates at 18 Months. One month later, the homogenates were prepared for western blotting (upper three blots) and the filter trap analysis (lower dot blot). The representative virus infection image (A) and blots are shown (B), and the quantitative analysis for p-syn and t-syn are shown (C). **P<0.01 vs Con; ##P<0.01 vs A53T; N=6; one-way ANOVA with Bonferroni post hoc test was used.

Supplementary References

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