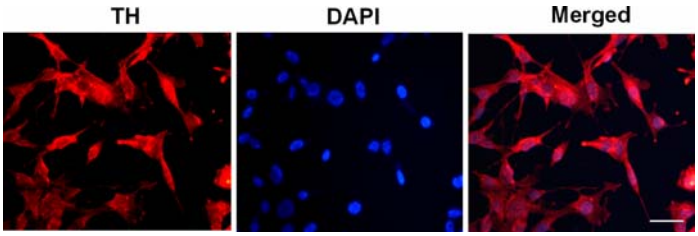
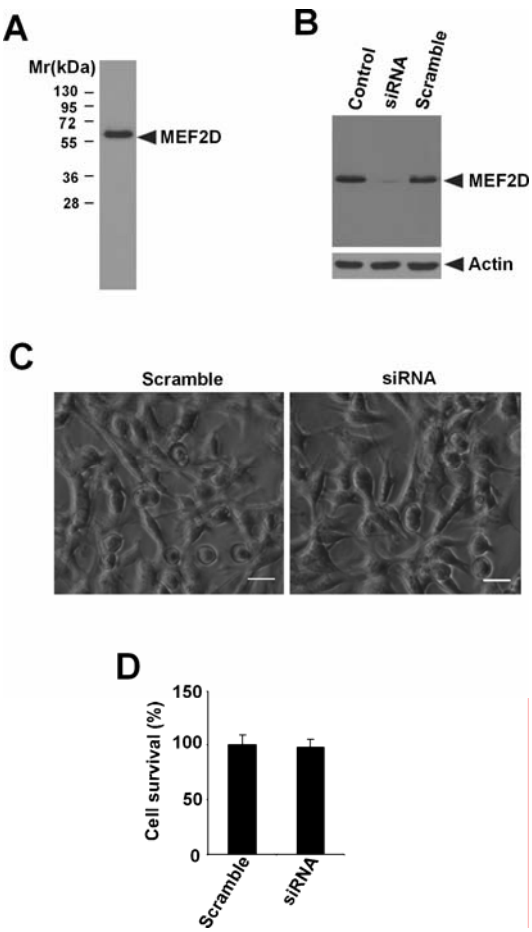
Supplemental Information

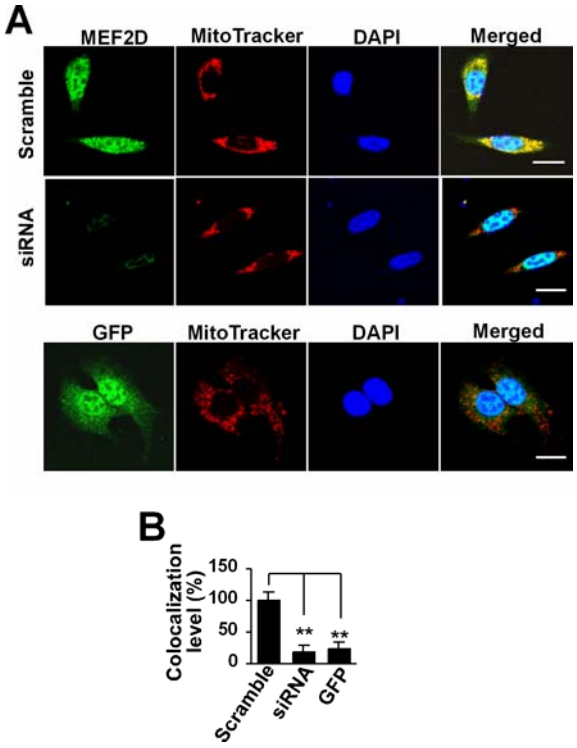
Supplementary Figures



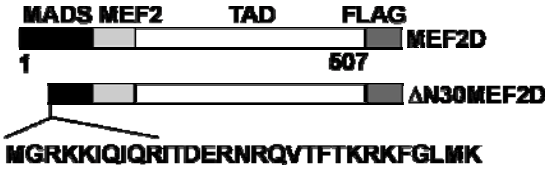
Supplemental Figure 1. Expression of DA Neuronal Marker Tyrosine Hydroxylase (TH) in SN4741 Cells. Immunocytochemistry was carried out as described in Methods. Anti-TH antibody was from Aves Labs (#: TH). N>4; scale bar, 20μm.



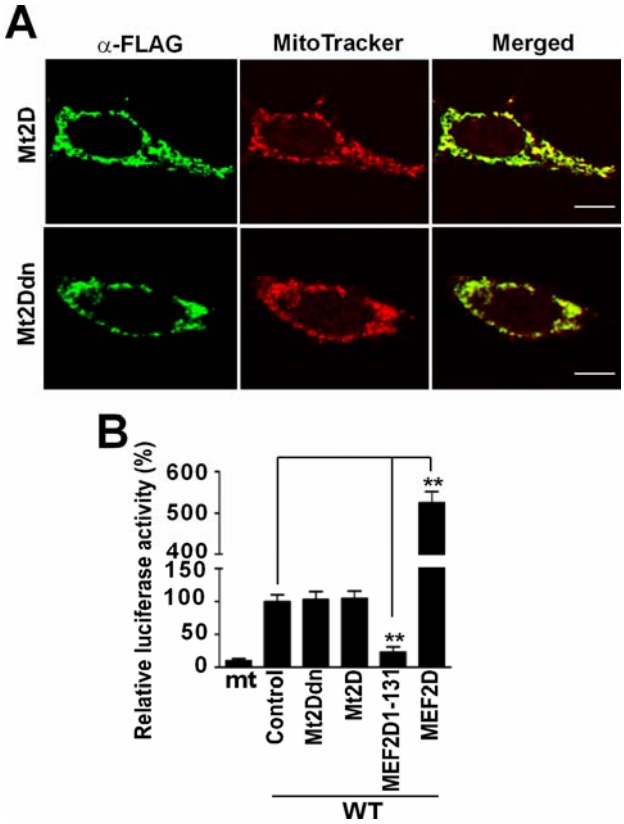
Supplemental Figure 2. Characterization of Anti-MEF2D Antibody. (**A**) The anti-MEF2D monoclonal antibody (BD Biosciences, cat.no.610775) recognized a single band by western blotting (n>10). (**B**) Knock-down of MEF2D expression in SN4741 cells by siRNA significantly reduced the signal recognized by the anti-MEF2D antibody (n=4). Control indicates untreated. (**C**) Knockdown of MEF2D by siRNA in SN4741 cells had no overt effect on the cellular morphology of SN4741 (n=4; scale bar, 20 μm). (**D**) Cellular viability wasn't affected by MEF2D knockdown under basal condition (n=4).



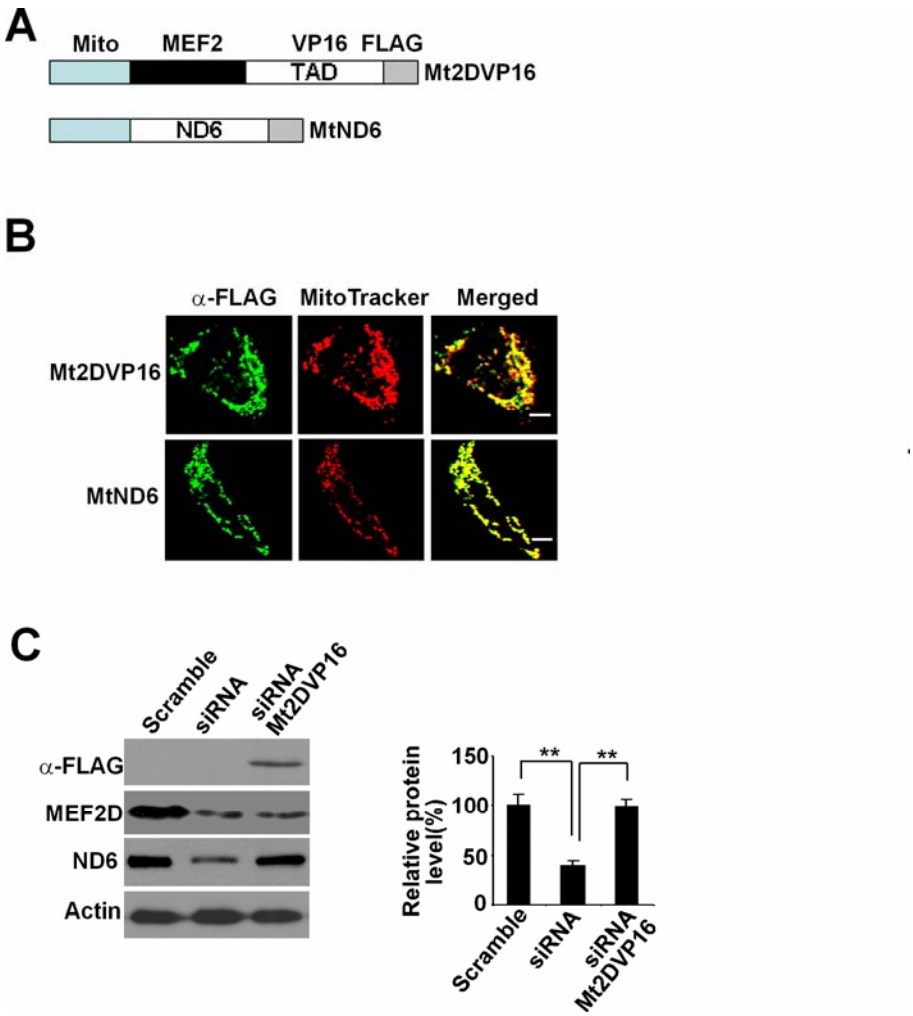
Supplemental Figure 3. Specific Localization of MEF2D in Mitochondria. **(A**) Endogenous MEF2D co-localized with mitochondrial marker MitoTracker in SN4741 cells; siRNA knockdown of MEF2D expression in SN4741 cells greatly reduced the co-localization signal. Green fluorescent protein (GFP) showed poor co-localization with MitoTracker (n=4; scale bar, 10 μm). (**B**) Statistical analysis of co-localization signals in (**A**) (n=50 cells, $^{**}P<0.01$). Experiments were repeated four times.



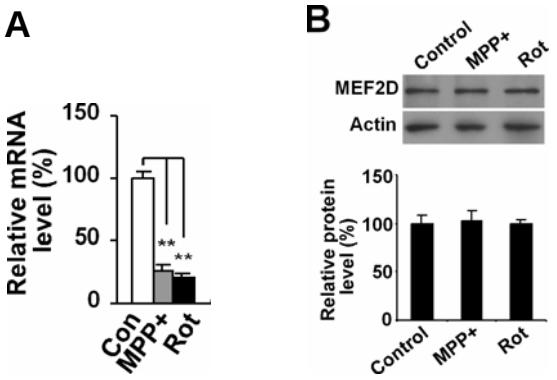
Supplemental Figure 4. FLAG-tagged wild type MEF2D and ΔN30MEF2D. Constructs of FLAG-tagged wild type mouse MEF2D and mutant MEF2D with its N-terminal 30 amino acid (aa) residues deleted (ΔN30MEF2D).



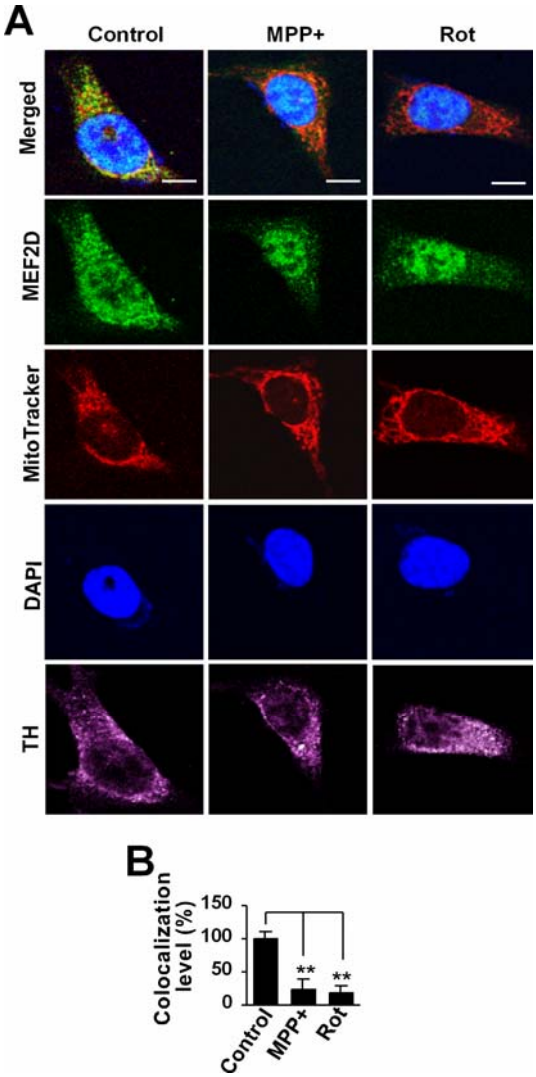
Supplemental Figure 5. Targeting Mt2D and Mt2Ddn to Mitochondria. **(A)** Immunocytochemical staining showed that Mt2D and Mt2Ddn were targeted exclusively to mitochondria in SN4741 cells (n=3; scale bar, 2.5 μm). **(B)** MEF2 reporter assay showed that Mt2D and Mt2Ddn did not affect nuclear MEF2 activity. MEF2D1-131 is a dominant-negative form of MEF2D which interferes with nuclear MEF2 function (n=3, $^{**}P<0.01$). Control indicates the group co-transfected with control vector.



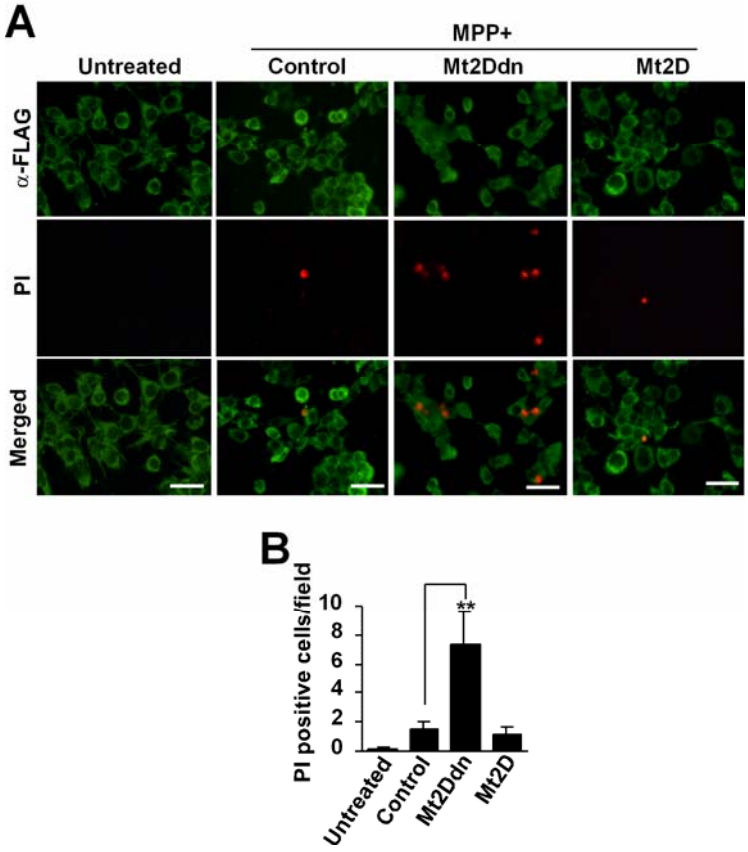
Supplemental Figure 6. Targeting Mt2DVP16 and MtND6 to Mitochondria. (**A**) Diagrams of Mt2DVP16 and MtND6**.** Mt2DVP16 was constructed by fusion of transactivation domain (TAD) of VP16 with DNA-binding domain of MEF2D and a mitochondria-targeting sequence (Mito). (**B**) Immunocytochemical staining shows that Mt2DVP16 and MtND6 are targeted exclusively to mitochondria in SN4741 cells (n=3; scale bar, 2.5 μm). (**C**) Over-expression of Mt2DVP16 attenuated MEF2D siRNA-induced reduction of ND6 expression (n=3, $^{**}P$<0.01).



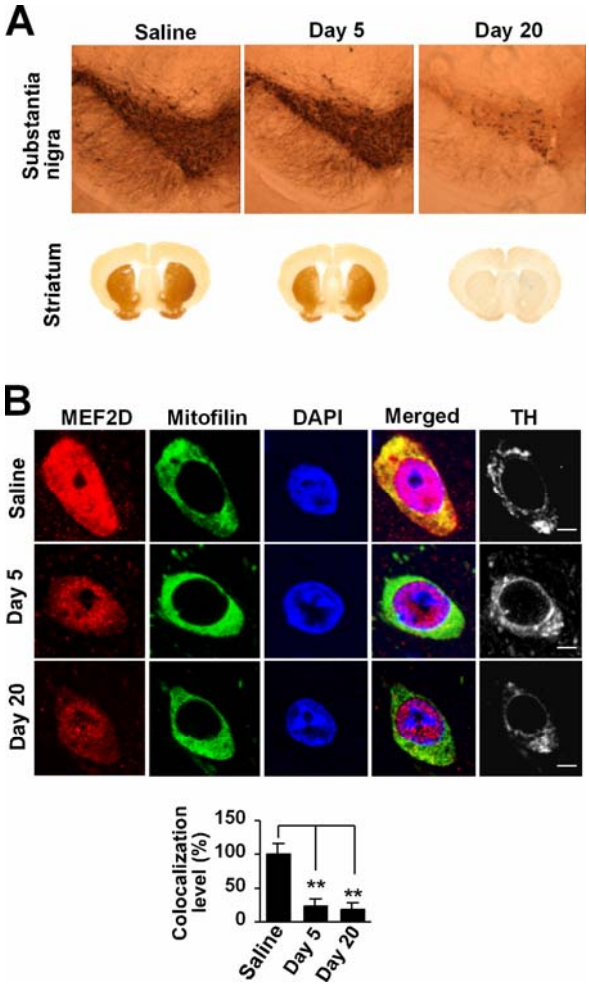
Supplemental Figure 7. Characterization of MEF2D Protein Level and ND6 mRNA Level in SN4741 Cells Following MPP+ or Rot Treatment. (**A)** Reduction of ND6 mRNA in SN4741 cells after MPP+ or rotenone treatment. SN4741 cells were treated with MPP+ (25μM) or Rot (100 nM) for 12 hours. Effects of MPP+ and rotenone (Rot) on the mRNA level of ND6 was analyzed by real-time quantitative PCR (n=4, $^{**}P$<0.01). (**B)** The total MEF2D level in whole cell didn't change in 12 hours following MPP+ (25μM) or Rot (100 nM) treatment (n=4). Control indicates untreated.



Supplemental Figure 8. Immunocytochemical Analysis of Mitochondrial MEF2D in Primary Midbrain DA Neurons after MPP+ or Rotenone Treatment. **(A**) Primary midbrain DA neurons were treated with MPP+ (25 μM) or Rot (100 nM) for 12 hours and immunocytochemical staining was performed (*n*=4; scale bar, 5 μm). TH, tyrosine hydroxylase, is a marker for dopaminergic neurons. (**B**) Effects of MPP+ and Rot on colocalization of MEF2D and MitoTracker (*n*=50 cells, ***P*<0.01). Experiments were repeated four times. Control indicates untreated.



Supplemental Figure 9. A, Sensitization of SN4741 cells to MPP+ toxicity by over-expression of Mt2Ddn. (**A**) SN4741 cells were infected with lentiviruses for 24 hours and followed by exposure to MPP+ (5 μM) for 24 hours. Cell death was analyzed by PI (propidium iodide) staining (n=4; scale bar, 30 μm). The control indicated the control vector. (**B**) Quantification of the number of PI positive cells in **(A)** (n=50 cells, $^{**}P$<0.01). Experiments were repeated four times. Control indicates the group transfected with the control vector.



Supplemental Figure 10. Reduction of Mitochondrial MEF2D Protein in DA Neurons of MPTP Treated Mouse. **(A)** Loss of TH signals in MPTP treated mouse brain. Images show immunohistochemical staining of TH in substantia nigra and striatum in mice treated with saline (day 20) or MPTP (day 5 and 20) (n=3). (**B**) Effects of MPTP treatment on mitochondrial MEF2D. Images are representative immunohistochemical studies of MEF2D and MitoTracker signals in TH positive neurons in the substantial nigra from saline (day 20) or MTPT (day 5 and 20) treated mice (n=50 cells; $^{**}P<0.01$). TH tyrosine hydroxylase. Mitofilin is a known mitochondrial protein. Experiments were repeated three times.

Supplementary Methods

Cell Culture

SN4741 cells were cultured at 33°C with 5% CO2 in RF medium (DMEM supplemented with 10% FBS, 1% D-glucose, 1% penicillin-streptomycin, and 140mM L-glutamine) (1). When cells reached 70% confluence (usually 3 days), split it into 3 plates. Experiments were usually done in 12-well plate when cells reached 50-60 confluence. Primary mouse midbrain dopaminergic (DA) neurons were cultured as described by Son *et al* (2). Briefly, mice were sacrificed and mesencephalic regions from E13.5 embryos were surgically removed under sterile condition in L-15 medium (Invitrogen). Mesencephalic tissues were cut into small pieces around 1 mm^3, mechanically triturated in 5ml L-15 medium containing Trypsin-EDTA (final concentration, 0.1%), and incubated at 37°C for 20 min. The reaction was stopped by adding 5 ml RF medium. The cells were peletted by centrifuged 5 min at 1,000×g and cultured in poly-l-lysine coated 12-well plate (5×10^5 cells/well) at 37°C with 5% CO2 in RF medium. Medium was half changed every 3 days. Experiments were done after DIV 7.

Plasmids and Lentiviruses

Flag-tagged mouse MEF2D and ΔN30MEF2D were constructed by cloning PCR fragments into Nhe I/Not I sites of pcDNA3.1(+) (Invitrogen). Mitochondria-targeting MEF2D (Mt2D) and dominant-negative MEF2D (Mt2Ddn) were constructed by cloning mouse MEF2D1-493aa or MEF2D1-131aa into Age I/Not I sites of pDsRed2-Mito (Clontech), respectively. For lentivirus production, Mt2D and Mt2Ddn were subcloned into Xba I site of pFUGW using primers 5'-GATCCGCTAGCATGTCCGTCC-3' (forward) and 5'-CGTCTAGACTAT

TTATCGTCATCGTCTTTGTAG-3' (reverse). Mt2DVP16 was constructed by fusion of TAD domain of VP16 with DNA-binding domain of MEF2D and mitochondria-targeting sequence (Mito) from pDsRed2-Mito (Clontech). MtND6 was constructed by fusion of Mito with ND6 sequence. All clones were confirmed by sequence. Lentiviruses were prepared by standard methodology.

Mitochondrial Complex Activity Assays and Functional Assays

Mitochondrial complex I activity was evaluated initially by blue native-polyacrylamide gel electrophoresis (BN-PAGE) and in-gel activity staining (3). This assay measures total complex I activity. Rotenone sensitive complex I activity and activities of other mitochondrial complexes were further measured by methods described by Antoni *et al* (4). Briefly, parallel assays were performed to measure complex I activity at 340 nm using 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone (DB) (50 μM) as acceptor and NADH (0.8 mM) as donor, in 50 mM Tris (pH 8.0) buffer supplemented with 5 mg/ml BSA either without or with the addition of 4 μM rotenone, which gave total and rotenone insensitive complex I activities, respectively. The difference in value between the two provided a quantified measure of the rotenone-sensitive activity. Mitochondrial membrane potential was detected using a kit (cat.no.280002) from Stratagene following the procedures provided by the manufacturer. The fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) stains mitochondria red in a membrane potential dependent manner. Briefly, cells were seeded in 96-well plates at 4 x 10^4 cells/well with 100 μl culture medium and incubated at 33°C with 5% CO_2. After treatment, 10 μl/well of premixed JC-1 staining solution was added, and the cells were incubated for an additional

30 min under the same conditions. After thorough washes, 100 µl assay buffer was added to each well. Fluorescence signal was detected with excitation and emission at 520 nm and 590 nm, respectively, on a multi-well plate reader (Bio-Tek). Cellular ATP and H_2O_2 were measured by Bioluminescent Somatic Cell ATP Assay Kit (Sigma) and Hydrogen Peroxide Assay Kit (Cayman), respectively.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (5). Briefly, 10 ng of purified proteins was used to incubate with ^{32}P-ATP labeled specific probe or mutant probe on ice for 30 min. The reaction complexes were separated by a 5% non-denature polyacrylamide gel electrophoresis and visualized by autoradiography. The probe for the MEF2 site in *ND6* was 5′-CTAAACCCCCAT**AAA**TAGGAGAAGGCTT-3′; for the mutant probe, the 3 nucleotides in bold were mutated to GGC.

MEF2 Luciferase Reporter Assay

MEF2 luciferase reporter assay was done as described previously (6). Briefly, cells were transiently transfected with various constructs with MEF2 luciferase reporter plasmid (WT, reporter with wild type MEF2 DNA binding sites; mt, reporter with the MEF2 DNA binding sites mutated) using Lipofectimane 2000 (Invitrogen) following procedures provided by the manufacturer. A β-galactosidase expression plasmid was used to determine the efficiency in each transfection. The total amount of DNA for each transfection was kept constant by using control vectors. Cell lysates

were analyzed for luciferase and β-galactosidase activity according to the manufacturer's instructions (Roche, cat.no.11669893001).

WST-1 Assay

WST-1 assay was done using a kit (cat.no.11644807001) from Roche and following procedures provided by the manufacturer. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is a water-soluble tetrazolium salt whose cleavage by cellular enzymes correlates with cell viability. Cells were seeded in 96-well plates and treated as indicated. Than 10 μl/well of premixed WST-1 Cell Proliferation Reagent was added, and the cells were incubated for an additional 4 hr under the same conditions. Absorbance at 450 nm was measured on a multi-well plate reader (Bio-Tek).

1. Yang, Q., She, H., Gearing, M., Colla, E., Lee, M., Shacka, J.J., and Mao, Z. 2009. Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. *Science* 323:124-127.
2. Son, J.H., Chun, H.S., Joh, T.H., Cho, S., Conti, B., and Lee, J.W. 1999. Neuroprotection and neuronal differentiation studies using substantia nigra dopaminergic cells derived from transgenic mouse embryos. *J Neurosci* 19:10-20.
3. Dabbeni-Sala, F., Di Santo, S., Franceschini, D., Skaper, S.D., and Giusti, P. 2001. Melatonin protects against 6-OHDA-induced neurotoxicity in rats: a role for mitochondrial complex I activity. *FASEB J* 15:164-170.
4. Barrientos, A., Kenyon, L., and Moraes, C.T. 1998. Human xenomitochondrial cybrids. Cellular models of mitochondrial complex I deficiency. *J Biol Chem* 273:14210-14217.
5. Wang, X., Tang, X., Gong, X., Albanis, E., Friedman, S.L., and Mao, Z. 2004. Regulation of hepatic stellate cell activation and growth by transcription factor myocyte enhancer factor 2. *Gastroenterology* 127:1174-1188.
6. Wang, X., Tang, X., Li, M., Marshall, J., and Mao, Z. 2005. Regulation of neuroprotective activity of myocyte-enhancer factor 2 by cAMP-protein kinase A signaling pathway in neuronal survival. *J Biol Chem* 280:16705-16713.