Activation of AMPK/mTORC1-Mediated Autophagy by Metformin Reverses Clk1 Deficiency-Sensitized Dopaminergic Neuronal Death

Qiuting Yan, Chaojun Han, Guanghui Wang, John L. Waddington, Longtai Zheng, and Xuechu Zhen

Jiangsu Key Laboratory of Translational Research and Therapy for Neuropsychiatric Diseases and College of Pharmaceutical Sciences (Q.Y., C.H., G.W., J.L.W., L.Z., X.Z.), and College of Pharmaceutical Sciences and the Collaborative Innovation Center for Brain Science (Q.Y., C.H., G.W., L.Z., X.Z.), Soochow University, Suzhou, Jiangsu, China; and Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin, Ireland (J.L.W.)

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ABSTRACT

The autophagy-lysosome pathway (ALP) plays a critical role in the pathology of Parkinson's disease (PD). Clk1 (coq7) is a mitochondrial hydroxylase that is essential for coenzyme Q (ubiquinone) biosynthesis. We have reported previously that Clk1 regulates microglia activation via modulating microglia metabolic reprogramming, which contributes to dopaminergic neuronal survival. This study explores the direct effect of Clk1 on dopaminergic neuronal survival. We demonstrate that Clk1 deficiency inhibited dopaminergic neuronal autophagy in cultured MN9D dopaminergic neurons and in the substantia nigra pars compacta of Clk1^{+/-} mutant mice and consequently sensitized dopaminergic neuron damage and behavioral defects. These mechanistic studies indicate that Clk1 regulates the AMP-activated protein kinase (AMPK)/rapamycin

complex 1 pathway, which in turn impairs the ALP and TFEB nuclear translocation. As a result, Clk1 deficiency promotes dopaminergic neuronal damage in vivo and in vitro, which ultimately contributes to sensitizing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neuronal death and behavioral impairments in Clk1-deficient mice. Moreover, we found that activation of autophagy by the AMPK activator metformin increases dopaminergic neuronal survival in vitro and in the MPTP-induced PD model in Clk1 mutant mice. These results reveal that Clk1 plays a direct role in dopaminergic neuronal survival via regulating ALPs that may contribute to the pathologic development of PD. Modulation of Clk1 activity may represent a potential therapeutic target for PD.

Introduction

Parkinson's disease (PD) is a common aging-related neurodegenerative disease with progressive loss of dopaminergic neuron in the substantia nigra pars compacta (SNc) (Savitt et al., 2006; Ye et al., 2013; Kalia and Lang, 2015). Although neuroinflammation, oxidative stress, and mitochondrial dysfunction are believed to contribute to the damage of dopaminergic neurons, the precise pathologic mechanism remains unknown (Dias et al., 2013; Camilleri and Vassallo, 2014; Menzies et al., 2015; Moon and Paek, 2015; Morris and Berk, 2015; Segura-Aguilar and Kostrzewa, 2015). As a mitochondrial hydroxylase responsible for coenzyme Q (ubiquinone)

biosynthesis, Clk1 (coq7) plays an important role in electron transference in the mitochondrial respiratory chain (Nakai et al., 2001; Lapointe and Hekimi, 2008). Clk1^{+/-} mutant mice exhibit a series of changes in mitochondrial metabolism such as reduced mitochondrial oxygen consumption, reduced electron transport, and mitochondrial ATP synthesis (Hekimi, 2013). Moreover, Clk1 deficiency induces apoptosis associated with mitochondrial dysfunction, which may lead to embryonic lethality in mice around E10.5 (Takahashi et al., 2008). We reported recently that Clk1 deficiency sensitizes microglia-mediated neuroinflammation by altering metabolic reprogramming in microglial cells and subsequently increasing dopaminergic neuronal death induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment, suggesting that Clk1 plays an important role in the survival of dopaminergic neurons via modulating microglia activation (Gu et al., 2017). However, the direct functional role of Clk1 in dopaminergic neurons remains unknown.

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ABBREVIATIONS: ALP, autophagy-lysosome pathway; AMPK, AMP-activated protein kinase; LV, lentivirus; MPP⁺, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mTOR, mammalian target of rapamycin; mTORC1, rapamycin complex 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PD, Parkinson's disease; RLU, relative light unit; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

The autophagy-lysosome pathway (ALP) is a critical cellular quality control system that involves degradation of dysfunctional cellular components and organelles. Altered ALP is known to be associated with the pathologic development of various neurodegenerative diseases including PD (Maiuri et al., 2007). Enhancement of autophagy with overexpression of beclin1 effectively reduced the accumulation of α -synuclein and protected neurons in an animal model of PD (Spencer et al., 2009). Furthermore, deletion of genes essential for autophagy, such as ATG7, resulted in PD-like neurodegeneration in mice (Komatsu et al., 2006). Moreover, MPTP treatment leads to defective autophagosomal clearance due to impairment of lysosomal function in a model of PD (Dehay et al., 2010). Therefore, ALP is essential for the survival of neuronal cells in response to an increased burden of misfolded protein or neurotoxicity (Decressac et al., 2013).

An *in vivo* study with CCI-779, a derivative of rapamycin, reduced the accumulation of α -synuclein by activation of autophagy, indicating that the mammalian target of rapamycin (mTOR) pathway plays an important role in the pathologic development of neurodegenerative diseases (Ravikumar et al., 2004; Malagelada et al., 2006; Santini et al., 2009; Tain et al., 2009; Bar-Peled and Sabatini, 2014). AMP-activated protein kinase (AMPK) is an upstream target of rapamycin complex 1 (mTORC1), and activation of AMPK inhibits mTORC1 activity, thereby promoting autophagy (Choi et al., 2010). Furthermore, the AMPK activator metformin protects dopaminergic neurons in SNc in a mouse PD model via enhancement of AMPK-mediated autophagy, suggesting that the AMPK signaling pathway may constitute a potential target for PD therapy (Sardiello et al., 2009; Wu et al., 2011). Recently, it has been shown that mTORC1 is a key regulator of the cellular localization and activity of TFEB (Wong and Cuervo, 2010; Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Martina and Puertollano, 2013). TFEB is a major transcriptional regulator of the ALP pathway by regulating the expression of autophagic gene products such as ATG5, beclin-1, and ATG9B, and lysosomal gene products such as LAMP1 and cathepsins (Peña-Llopis et al., 2011; Settembre et al., 2011). Altered TFEB function is involved in neurodegenerative diseases through regulating cargo recognition, autophagosome-lysosome fusion, and TFEB nuclear localization in ALP (Decressac et al., 2013; Chua et al., 2014; Cortes et al., 2014).

The present study is designed to investigate the direct functional role of Clk1 in the regulation of dopaminergic neurons. We found that loss of Clk1 strongly increased MPTP neurotoxicity and inhibited autophagy through the AMPK/mTORC1 pathway. Our data reveal a novel mechanism of Clk1-regulated dopaminergic neuronal survival.

Materials and Methods

Materials. Cell culture reagents were purchased from Hyclone (Thermo Fisher Scientific, Waltham, MA). 1-Methyl-4-phenylpyridinium (MPP⁺), MPTP, bafilomycin A1, puromycin, rapamycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay reagents were purchased from Sigma-Aldrich (St. Louis, MO). Metformin was purchased from Beyotime (Shanghai, China). Quantitative reverse-transcription polymerase chain reaction (PCR) primers were synthesized by Sangon Biotech (Shanghai, China). Antibodies to P62, phosphor-AMPK (threonine 172), AMPK, phosphor-mTOR

(serine 2448), mTOR, phosphor-p70s6k (threonine 389), and p70s6k were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for LC3 and LAMP1 were purchased from Abcam (Cambridge, MA). Antibodies for actin were purchased from Sigma-Aldrich. The antibody for tyrosine hydroxylase (TH) was obtained from Millipore (Billerica, MA) and the antibody for Clk1 was obtained from Proteintech Technology (Rosemont, IL). All drugs were freshly prepared before each experiment. MPTP was dissolved in saline. MPP⁺ was dissolved in phosphate-buffered saline (PBS) and added to cells for a final concentration of 250 μ M. Metformin was dissolved in saline for injection into mice at 50 mg/kg. For cell cultures, metformin was prepared in PBS at 1 mol/l stock solution and kept at -20°C ; the stock solution was diluted with cell culture medium to a designated concentration of 2 mmol/l. Rapamycin was dissolved using dimethylsulfoxide to a 100 mmol/l stock solution and kept at -20°C ; the stock solution was diluted with cell culture medium to a designated concentration of 200 nmol/l. The final concentration of dimethylsulfoxide was no more than 0.1%.

Animals. Wild-type and Clk1^{+/-} mutant mice were obtained from Rugen Therapeutics (Suzhou, China). Mice were maintained in plastic cages with free access to food and water in specific-pathogen-free conditions (temperature: $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$; air exchange per 20 minutes; 12 hour/12 hour light-dark cycle). Animals were allowed free access to a standard laboratory diet and water. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Soochow University and were in compliance with the Guidelines for the Care and Use of Laboratory Animals.

Cell Culture. Murine dopaminergic MN9D cells (Choi et al., 1992) were cultured in Dulbecco's modified Eagle's medium (Gibco, New York City, NY) containing 5% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C under 5% CO₂ air environment. MN9D cells were grown until 70%–80% confluent before further treatment in all experiments.

Plasmid and Lentivirus (LV). EGFP-N3-tagged TFEB plasmid was provided by one of the authors (Guanghui Wang). LV gene transfer vectors encoding shClk1 (LV2-shClk1, 5'-TGCCTTGTTGAA-GAGGATTAT-3') and scrambled shRNA used as a negative control (LV2-NC, 5'-TTCTCCGAACGTGTCACGTTTC-3') were synthesized by GenePharma (Shanghai, China). The titer of LV used was $\geq 3 \times 10^8$ U. Polybrene was employed to promote the transduction of LV and puromycin was applied to select successfully infected cells. After LV2-shClk1 was stably expressed, total RNA and proteins were extracted from MN9D cells to confirm the knockdown efficiency. For Clk1 overexpression and EGFP-TFEB assays, cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions; total RNA and proteins were isolated from MN9D cells.

Cell Viability Assays. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, MN9D cells were seeded in 96-well plates to a final density of 2×10^4 cells/well. After treatment with the indicated drugs for 24 hours, 30 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (0.5 mg/ml) was added to each well. The plates were incubated at 37°C for 2 hours in the dark. Then, after addition of 100 μ l dimethylsulfoxide, the absorbance was read at 570 nm using a spectrophotometer.

Immunofluorescence. MN9D cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Then, the cells were incubated with blocking solution containing 3% bovine serum albumin and 0.3% Triton-X 100 in PBS for 1 hour, followed by incubation overnight at 4°C with primary antibodies anti-LC3 (1:500). Subsequently, cells were washed three times with PBS and subjected to incubation with secondary fluorescent antibodies (1:400) for 2 hours at room temperature. After three washes with PBS, samples were dyed with 4',6-diamidino-2-phenylindole for 30 minutes at 37°C , followed by three more washes. Confocal microscopy (Carl Zeiss, Oberkochen, Germany) was applied to the resultant images.

Western Blotting. Cells or tissues were lysed in radioimmuno-precipitation assay buffer (Cell Signaling Technology) on ice and incubated at 95°C for 10 minutes. Nuclear protein extraction was performed according to the manufacturer's instructions (Thermo Fisher Scientific). Protein was quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Denatured protein was loaded into SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% milk for 2 hours, the membranes were incubated overnight at 4°C with primary antibodies against Clk1 (1:1000), TH (1:1000), LC3 (1:3000), P62 (1:1000), LAMP1 (1:1000), phosphor-AMPK (1:1000), AMPK (1:1000), phosphor-mTOR (1:1000), mTOR (1:1000), phosphor-p70s6k (1:1000), p70s6k (1:1000), or internal reference antibodies actin (1:10000). Then, the membranes were washed three times in Tris-buffered saline containing 0.1% Tween 20 and subjected to incubation with anti-rabbit or anti-mouse IgG polyclonal secondary antibodies (Sigma-Aldrich). Following incubation with enhanced chemiluminescence (Millipore), the band was determined with a ChemiScope 3300 Mini (CLINX, Shanghai, China).

RNA Isolation and Real-Time Quantitative PCR. Total RNA was isolated by TRIZOL reagent (Takara, Shiga, Japan) according to the manufacturer's instructions. After assessment with NanoDrop (ND-2000C; Thermo Fisher Scientific), cDNA was reverse synthesized from 1000 ng RNA using oligo (deoxythymine), RNase-free water, 10 Mm deoxynucleotide triphosphate, Moloney murine leukemia virus buffer, Moloney murine leukemia virus, and recombinant RNase inhibitor (Takara). Quantitative assay of mRNA was performed using SYBR Green PCR Master Mix (Takara) in a 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA). The cycling parameters were as follows: 50°C, 2 minutes; 95°C, 10 minutes; 95°C, 10 minutes; 95°C, 15 seconds; 40 cycles; and 60°C, 1 minute. The primer sequences for each gene are listed as follows:

GAPDH, forward primer: 5'-GACAAGCTTCCCGTTCTCAG-3',
reverse primer: 5'-GACTCAACGGATTGGTTCGT-3';
Clk1, forward primer: 5'-GATTGCATTCAGGGTCCGAC-3',
reverse primer: 5'-CTTCCATCAGCATGCGGATC-3';
ATP6v1h, forward primer: 5'-CATTCGAGGTGCTGTGGATG-3',
reverse primer: 5'-TGCTTGCTCCTCGGAATTCT-3';
CTSA, forward primer: 5'-TCCCAGCATGAACCTTCAGG-3',
reverse primer: 5'-AGTAGGCAAAGTAGACCAGGG-3';
CTSD, forward primer: 5'-TGCTCAAGAACTACATGGACGC-3',
reverse primer: 5'-CGAAGACGACTGTGAAGCACT-3';
CTSF, forward primer: 5'-AGAGAGGCCCAATCTCCGT-3',
reverse primer: 5'-GCATGGTCAATGAGCCAAGG-3';
HEXA, forward primer: 5'-ACGTCCTTTACCCGAACAAC-3',
reverse primer: 5'-CGAAAAGCAGGTACGATAGC-3';
LAMP1, forward primer: 5'-CAGATGTGTTAGTGGCACCCA-3',
reverse primer: 5'-TTGGAAAGGTACGCTGGATG-3';
ATG5, forward primer: 5'-TGGATGGGACTGCAGAATGA-3',
reverse primer: 5'-GATCTCCAAGTGTGTGCAGC-3'; and
ATG9B, forward primer: 5'-TGGCATCACATCCAGAACCT-3',
reverse primer: 5'-CATTGTAATCCACGCAGCGA-3.

Gene expression was normalized to GAPDH in each sample.

ADP/ATP Ratio Assay. The ADP/ATP ratio was measured using an ADP/ATP Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Cells were seeded in a 96-well plate to a final density of 10^3 – 10^4 cells/well. The ATP reagent was prepared with assay buffer 95 μ l, substrate 1 μ l, cosubstrate 1 μ l, and ATP enzyme 1 μ l; 90 μ l of the ATP reagent was added to each well and the plate was tapped briefly to mix. The plate was incubated for 1 minute at room temperature. Luminescence activities were read and determined using a luminescence Reporter Assay System (Promega, Madison, WI) for the ATP assay [relative light unit (RLU)_A]. The ADP reagent was prepared with 5 ml water and 1 μ l ADP enzyme. After incubation for 10 minutes, luminescence was read as for ATP (RLU_B). Immediately following the reading of RLU_B, 5 ml ADP reagent was added to each well and mixed by tapping the plate or pipetting. After 1 minute,

luminescence was read as above (RLU_C). The ADP/ATP ratio was calculated using the formula $\text{ADP/ATP ratio} = \text{RLU}_C - \text{RLU}_B/\text{RLU}_A$.

MPTP Administration. The MPTP-induced mouse model of PD was prepared according to our previously described procedures (Ren et al., 2016; Gu et al., 2017), in which mice received intraperitoneal injections of 25 mg/kg free base MPTP or saline for seven consecutive days. Briefly, wild-type and Clk1^{+/-} mutant mice were divided into three treatment groups: 1) saline, 2) MPTP (25 mg/kg), and 3) metformin (50 mg/kg) + MPTP (25 mg/kg). For the metformin-treated group, metformin (50 mg/kg i.p.) was given 3 days prior to and during each of the subsequent seven daily MPTP injections. On day 11, behavioral tests were conducted. After the behavioral tests, mice were sacrificed and brain tissue was prepared for immunohistochemistry or western blotting.

Rotarod Test. One day before testing, mice were trained until they could remain on the rotarod (UGO Basile, Comerio, Italy) for 120 seconds without falling. During testing, mice were placed on the rotarod and the rotation speed was increased from 5 to 40 rpm for 5 minutes. Latency (time) to fall from the rotarod was automatically recorded. Each mouse underwent three independent trials with a 20-minute intertrial interval, with latency to fall calculated as the average of the three trials (Ren et al., 2016).

Pole Test. Mice were placed head down on the top of a vertical wooden pole (60 cm in length, 2 cm in diameter) with a rough surface. Latency for the mice to climb down from the top of the pole to the base was measured. Trials were considered a failure if the mouse jumped or slid down the pole. Each mouse underwent three trials, with latency to climb down calculated as the average of the three trials (Ren et al., 2016).

Immunohistochemistry. Following behavioral tests, animals were anesthetized intraperitoneally with chloral hydrate (10%) and brain tissue was prepared as described previously. A freezing microtome was used to cut the midbrain into 20 μ m serial sections containing the SNc for subsequent immunofluorescence staining. For staining, the midbrain sections were rinsed three times with 0.01 mol/l PBS and incubated in PBS containing 3% bovine serum albumin and 0.3% Triton for 2 hours at room temperature. The sections were washed in PBS and then incubated with anti-TH antibody (1:400) at 4°C for 24 hours. After washing with PBS containing 0.3% Triton, secondary fluorescent antibodies (1:400) were added for 2 hours. The slides were then observed using a confocal microscope (Carl Zeiss).

Statistical Analysis. Data were expressed as means $\pm$ S.E.M. and analyzed using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA). One-way ANOVAs or two-way ANOVAs followed by Bonferroni post-hoc tests were utilized for multiple-group comparisons. Student's *t*-test was used for comparisons between two groups. $P < 0.05$ was considered statistically significant. If two or three measurements were conducted on the same sample, $P < 0.025$ or $P < 0.0167$, respectively, was considered significant. In some experiments, multiple measurements were conducted on different sets of cells or tissues (i.e. not from the same sample), in which case $P < 0.05$ was considered statistically significant.

Results

Clk1 Deficiency Promotes MPP⁺-Induced Dopaminergic Neuronal Death in MN9D Cells. Recently, we reported that Clk deficiency in microglia cells sensitized dopaminergic neuron toxicity in response to MPP⁺ treatment in a neuroglia coculture system (Gu et al., 2017). To explore the direct effect of Clk1 on dopaminergic neuronal survival, MN9D cells were cultured with the indicated concentrations of MPP⁺ for 24 hours. As expected, MPP⁺ produced neurotoxicity in MN9D cells (Fig. 1A). Interestingly, we detected a decrease in expression of Clk1 in MN9D cells treated with

Fig. 1. Clk1 deficiency promotes MPP⁺-induced dopaminergic neuronal death in MN9D cells. (A) MN9D cells were cultured with indicated concentrations of MPP⁺ for 24 hours before 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **P* < 0.05; ***P* < 0.001 vs. control group. (B) MN9D cells were treated with 125, 250, 500, and 1000 μM MPP⁺ for 24 hours. Cells were then harvested and protein levels of Clk1 and TH were determined by western blot analysis. **P* < 0.025; ***P* < 0.001 vs. control group. (C) MN9D cells were treated with 250 μM MPP⁺ for 12, 24, and 36 hours. Cells were then harvested and protein levels of Clk1 and TH were determined by western blot analysis. **P* < 0.025; ***P* < 0.001 vs. control group. (D) MN9D cells were transfected with LV2-shClk1 or LV2-NC for 48 hours. Cells were then collected for RNA extraction or lysis preparations, and used for quantitative PCR or western blot assay, respectively. **P* < 0.05 vs. LV2-NC. (E) MN9D cells were transfected as in (D); cells were treated with 250 μM MPP⁺ for a further 24 hours before MTT assay. **P* < 0.05 vs. LV2-NC+MPP⁺. Data are presented as mean ± S.E.M. from at least three independent experiments.

MPP⁺, suggesting that altered Clk1 expression may be involved in dopaminergic neuronal survival (Fig. 1, B and C). Knockdown of Clk1 with LV expressing Clk1 (LV2-shClk1) enhanced neuronal death induced by MPP⁺ relative to LV2-NC-transfected MN9D cells (Fig. 1, D and E). This indicated that deficient Clk1 in dopaminergic neurons enhanced MPP⁺-induced cell death.

Clk1 Regulates Autophagy in MN9D Cells. We next explored if Clk1 is involved in the regulation of autophagy. Clk1 deficiency resulted in decreased expression of LC3-II and lysosomal protein LAMP1 in Clk1-deficient MN9D cells. Decreased expression of LC3-II is known to result from either a decrease in the formation of autophagosomes or an increase in the flux of autophagosomes to autolysosomes. Effective autophagic flux will lead to a decrease in its substrates, such as P62. However, we found that expression of P62 was increased (Fig. 2A). This clearly indicated impairment in autophagy and lysosomal biogenesis in Clk1-deficient MN9D cells. To further confirm the effect of Clk1 on autophagy, we measured autophagic activity by immunofluorescence of LC3 and found a decrease in LC3 in Clk1-deficient MN9D cells (Fig. 2B). In contrast, overexpression of Clk1 increased expression of LC3-II and LAMP1, whereas expression of P62 was decreased (Fig. 2, C and D). To further evaluate the autophagic flux (Klionsky et al., 2016), cells were treated for 12 hours with 100 nM bafilomycin A1, which is known to inhibit vacuolar-type ATPases, block the fusion of autophagosome and

lysosome, and ultimately lead to the inhibition of autophagosome degradation. The level of LC3-II was also decreased in Clk1-deficient MN9D cells compared with LV2-NC-transfected MN9D cells in response to bafilomycin A1 treatment (Fig. 2E), indicating that loss of Clk1 decreased autophagosome synthesis. In contrast, overexpression of Clk1 increased expression of LC3-II with bafilomycin A1 treatment (Fig. 2F). In addition, MPP⁺ treatment enhanced expression of LC3-II without altering P62 expression (Fig. 2G). These results indicate that MPP⁺ impairs autophagic flux in MN9D cells. Furthermore, knockdown of Clk1 by transfecting MN9D cells with LV2-shClk1 inhibited MPP⁺-enhanced expression of LC3-II (Fig. 2H). Taken together, Clk1 deficiency regulated dopaminergic autophagy by altering autophagy and lysosomal biogenesis under either basal conditions or in MPP⁺-treated neurons.

Clk1 Regulates the AMPK/mTORC1 Pathway. We reported previously that Clk1 regulated glycolytic metabolism in microglia cells (Gu et al., 2017). Since the ADP/ATP ratio is an important index of cellular energy status that directly affects AMPK activity (Hardie, 2007), we examined the effect of Clk1 on the ADP/ATP ratio and AMPK activity in dopaminergic MN9D cells. The ADP/ATP ratio and phosphorylation of AMPK on threonine 172 were decreased with Clk1 knockdown (Fig. 3A). It is known that mTORC1 activation plays an important role in the regulation of autophagy, while AMPK phosphorylation negatively regulates mTOR activation

Fig. 2. Clk1 regulates autophagy in MN9D cells. (A) MN9D cells were transfected with LV2-shClk1 for 48 hours. Cells were then harvested and protein levels of LC3, P62, LAMP1, and Clk1 were determined by western blot analysis. $*P < 0.001$ vs. LV2-NC. (B) MN9D cells were transfected as in (A), followed by immunofluorescence assay using antibody against LC3 (green). 4',6-Diamidino-2-phenylindole (DAPI) (blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar, 5 μ m), with quantification of LC3 punctation. $*P < 0.001$ vs. LV2-NC. (C) MN9D cells were transfected with mClk1 plasmid for 48 hours. Cells were then harvested and protein levels of LC3, P62, LAMP1, and Clk1 were determined by western blot analysis. $*P < 0.0167$; $**P < 0.01$ vs. control group. (D) MN9D cells were transfected as in (C), followed by immunofluorescence assay using antibody against LC3 (green). DAPI (blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar, 5 μ m), with quantification of LC3 punctation. $*P < 0.05$ vs. control group. (E) MN9D cells were transfected as in (A) and incubated with bafilomycin A1 (Bafi) (100 nM) for 12 hours. Then, cells were harvested and protein levels of LC3 were determined by western blot analysis. $**P < 0.001$ vs. LV2-NC + Bafi. (F) MN9D cells were transfected as in (C), the cells were incubated with Bafi (100 nM) for 12 hours. Then, cells were harvested and protein levels of LC3 were determined by western blot analysis. $*P < 0.05$ vs. Con + Bafi. (G) MN9D cells were treated with 125, 250, 500, and 1000 μ M MPP⁺ for 24 hours. Cells were then harvested and protein levels of LC3 and P62 were determined by western blot analysis. $*P < 0.025$; $**P < 0.001$ vs. control group. (H) MN9D cells with LV2-shClk1 transfection were treated with MPP⁺ for 24 hours and protein levels of LC3 and P62 were determined by western blot analysis. $*P < 0.01$ vs. LV2-NC + MPP⁺. Data are presented as mean $\pm$ S.E.M. from at least three independent experiments.

Fig. 3. Clk1 regulates the AMPK/mTORC1 pathway. (A) MN9D cells were transfected with LV2-shClk1 for 48 hours. The ADP/ATP ratio was then measured as described in *Materials and Methods*. * $P < 0.01$ vs. LV2-NC. (B) MN9D cells were transfected as in (A). After 48 hours, cells were harvested and protein levels of phosphor-AMPK (p-AMPK), phosphor-mTOR (p-mTOR), phosphor-p70s6k (p-p70s6k), and Clk1 were determined by western blot analysis. * $P < 0.0167$; ** $P < 0.01$ vs. LV2-NC. Data are presented as mean $\pm$ S.E.M. from at least three independent experiments.

(Puente et al., 2016). We found that Clk1 knockdown enhanced phosphorylation of mTOR on serine 2448 and the mTOR substrate p70s6k on threonine 389 in MN9D cells (Fig. 3B). These results indicated that Clk1 is involved in the regulation of the AMPK/mTORC1 signaling pathway in MN9D cells.

Clk1 Mediates Autophagy through the AMPK/mTORC1 Pathway. To identify the roles of the AMPK/mTORC1 pathway in Clk1-mediated autophagy in MN9D cells, Clk1-deficient MN9D cells were incubated with metformin, an AMPK activator, and rapamycin, an mTORC1 inhibitor, with cells then subjected to western blotting assays. As shown in Fig. 4, A and B, metformin and rapamycin enhanced expression of LC3-II, whereas expression of P62 was decreased in Clk1-deficient MN9D cells. Accordingly, we detected increased activation of AMPK and inhibition of mTOR and p70s6k in Clk1-deficient MN9D cells in response to metformin and rapamycin. These results suggest that activation of AMPK or inhibition of mTORC1 reversed the inhibition of autophagy induced by Clk1 deficiency in MN9D cells. Furthermore, metformin prevented the effect of Clk deficiency to sensitize MN9D cell death in response to MPP⁺ (Fig. 4C). Collectively, these results clearly showed that Clk1 inhibited autophagy through the AMPK/mTORC1 signaling pathway and activation of autophagy by metformin protected dopaminergic neurons from MPP⁺ insult in Clk1-deficient MN9D cells.

Clk1 Regulates the Nuclear Translocation of TFEB. TFEB, which is regulated by mTORC1 (Wong and Cuervo, 2010; Peña-Llopis et al., 2011; Settembre et al., 2011; Decressac et al., 2013; Chua et al., 2014), is a transcription factor involved in autophagy and is functionally associated with neurodegenerative disease (Settembre et al., 2012; Martina and Puertollano, 2013). We, therefore, considered whether Clk1 is able to regulate TFEB. Nuclear translocation of TFEB was decreased by Clk1 knockdown after starvation in MN9D cells (Fig. 5A), while overexpression of Clk1 led to increased nuclear translocation of TFEB (Fig. 5B). Moreover, metformin increased nuclear translocation of TFEB in Clk1-deficient MN9D cells (Fig. 5C). These findings indicate that Clk1 regulates the nuclear translocation of TFEB. To further confirm this observation, we next assessed the expression levels of lysosomal and autophagic genes in Clk1-deficient cells. Clk1-deficient cells indeed resulted in a global decrease in mRNA expression levels of a set of TFEB target genes, including genes involved in lysosomal biogenesis and function (such as LAMP1), genes encoding subunits of vacuolar ATPases and cathepsins, and genes involved in autophagy (ATG5 and ATG9B) (Fig. 5D).

Clk1 Mutant Mice Exhibit Impaired Autophagy in Striatum and SNc. To detect autophagy *in vivo*, we employed Clk^{+/-} mutant mice to explore the expression of proteins associated with autophagy. Consistent with observations in MN9D cells, LC3-II and LAMP1 expression were decreased, while P62 expression was increased in striatal and SNc tissue in Clk^{+/-} mice (Fig. 6). Moreover, AMPK phosphorylation was decreased and phosphorylation of mTOR and p70s6k was increased in tissue from Clk^{+/-} mice (Fig. 7). These data further demonstrated that Clk1 deficiency results in impaired autophagy both *in vitro* and *in vivo*.

Metformin Promotes Autophagy and Ameliorates MPTP Neurotoxicity on Dopaminergic Neurons in Clk^{+/-} Mice. To determine if inhibition of autophagy by Clk1 deficiency is associated with enhanced dopaminergic neuronal death in Clk^{+/-} mice (Gu et al., 2017), we investigated if activation of AMPK and autophagy by metformin administration to Clk^{+/-} mice could reverse sensitization to dopaminergic neuronal toxicity induced by MPTP. A schematic diagram of drug administration is given in Fig. 8A. As expected, MPTP-treated Clk^{+/-} mice showed enhanced behavioral impairment compared with wild-type mice; pretreatment with metformin attenuated the behavioral impairments induced by MPTP treatment (Fig. 8, B and C). Furthermore, metformin pretreatment attenuated the decrease in number of TH-positive neurons in SNc, as indicated by immunostaining or TH protein levels by western blot, in mice treated with MPTP (Fig. 8D). In agreement with the data observed in MN9D cells, LC3-II expression was upregulated and P62 expression was downregulated in SNc of Clk-deficient MPTP-treated mice pretreated with metformin (Fig. 8E). Taken together, the present data indicate that metformin enhanced autophagy and ameliorated MPTP neurotoxicity in dopaminergic neurons in Clk^{+/-} mice.

Discussion

In this study, we provide the first evidence that Clk1 is an important regulator in the autophagy pathway, and that maintaining adequate Clk1 activity may be critical for

Fig. 4. Clk1 mediates autophagy through the AMPK/mTORC1 pathway. (A) MN9D cells with LV2-shClk1 transfection were treated with metformin for 24 hours and protein levels of LC3, P62, phosphor-AMPK (p-AMPK), phosphor-mTOR (p-mTOR), and phosphor-p70s6k (p-p70s6k) were then analyzed by western blot analysis. $^{*}P < 0.025$ vs. LV2-shClk1. (B) MN9D cells with LV2-shClk1 transfection were treated with rapamycin for 4 hours and protein levels of LC3, P62, p-AMPK, p-mTOR, and p-p70s6k were determined by western blot analysis. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. LV2-shClk1. (C) MN9D cells with LV2-shClk1 transfection were treated with MPP⁺ or metformin before MPP⁺ treatment. Four groups of cell viabilities were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. $^{*}P < 0.05$ vs. LV2-shClk1 + MPP⁺. Data are presented as mean $\pm$ S.E.M. from at least three independent experiments.

Fig. 5. Clk1 regulates the nuclear translocation of TFEB. (A) MN9D cells were transfected with LV2-shClk1 for 48 hours, and then retransfected with TFEB-EGFP 24 hours after transfection and the cells were fixed. 4',6-Diamidino-2-phenylindole (DAPI) (blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar, 5 μ m). * P < 0.05 vs. LV2-NC. (B) MN9D cells were transfected with mClk1 plasmid for 48 hours, and then retransfected with TFEB-EGFP 24 hours after transfection and the cells were fixed. DAPI (blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar, 5 μ m). ** P < 0.01 vs. control group. (C) Clk1-deficient MN9D cells were transfected with TFEB-EGFP and treated with metformin for 24 hours. The cells were then fixed. DAPI (blue) was used for nuclear staining (scale bar, 5 μ m). ** P < 0.01 vs. LV2-shClk1. (D) Similar transfection as in (A) was performed, and transfected cells were processed for quantitative PCR analysis. The mRNA levels of lysosomal and autophagic genes were quantified and normalized relative to GAPDH. * P < 0.05; ** P < 0.01 vs. LV2-NC. Data are presented as mean $\pm$ S.E.M. from at least three independent experiments.

A

B

Fig. 6. Striatum and SNc of Clk1 mutant mice exhibit impaired autophagy. (A and B) Wild-type (WT) and Clk1^{+/-} mouse striatum and SNc were dissected and processed as described in *Materials and Methods*. Protein levels of LC3, P62, LAMP1, TH, and Clk1 were determined by western blot analysis. *n* = 4 mice per group. **P* < 0.0167; ***P* < 0.01; ****P* < 0.001 vs. wild type.

dopaminergic neuronal survival. We demonstrated that Clk1 deficiency inhibited dopaminergic neuronal autophagy in cultured MN9D dopaminergic neurons and in SNc of Clk1^{+/-} mutant mice. We found that Clk1 regulated the AMPK/mTORC1 pathway, which in turn impaired the ALP and TFEB nuclear translocation, and consequentially promoted dopaminergic neuronal damage in vivo and in vitro; this ultimately contributed to sensitization to MPTP-induced dopaminergic neuronal death and behavioral impairments in Clk1-deficient mice. Moreover, we found that activation of autophagy by metformin treatment increased dopaminergic neuronal survival in vitro and in the MPTP model of PD. These results reveal that Clk1 plays an important role in dopaminergic neuronal survival via regulation of ALP pathways that may contribute to the pathologic development of PD. Thus, modulation of Clk1 activity may represent a potential therapeutic target for PD.

Clk1 (coq7) is essential for electron transference in the mitochondrial respiratory chain (Nakai et al., 2001; Lapointe and Hekimi, 2008). Deficiency in Clk1 function is known to alter mitochondrial metabolism, including reduced mitochondrial oxygen consumption, impaired electron transport, and mitochondrial ATP synthesis (Hekimi, 2013). Our previous study found that loss of Clk1 in microglia promotes neuroinflammation through regulation of microglial metabolic reprogramming, and consequently enhances dopaminergic neuronal death (Gu et al., 2017). However, a direct role of Clk1 in the regulation of dopaminergic neuronal survival has not been explored. In the present study, we found that silencing Clk1 sensitized MPP⁺-induced neurotoxicity toward

dopaminergic neurons in cultured MN9D dopaminergic cells. These findings indicate that Clk1 is indeed involved in the regulation of dopaminergic neuronal survival. To explore the underlying mechanism, we found that silencing Clk1 inhibited autophagy in MN9D cells, as evidenced by downregulation of LC3-II and LAMP1 in MN9D cells and in SNc of Clk1^{+/-} mice (Fig. 6). Importantly, activation of autophagy by metformin treatment protected dopaminergic neurons from the neurotoxic effects of MPTP administration in both Clk1-deficient and MPTP-treated wild-type animals (Fig. 8, B and C). Although it has been reported that inhibition of autophagy favors cell survival (Feng et al., 2014), here we showed that Clk1 deficiency resulted in impairment of autophagy that sensitized cells to MPTP-induced neurotoxicity. In agreement with other reports (Lee et al., 2015; Hwang et al., 2017), our data indicate that induction of autophagy is protective on cell survival in our system. Although a role for the ALP pathway in the development of PD may appear complex, it may be explained by the following points. 1) In the early stage of PD, when the lysosome is not severely impaired, enhancement of autophagy may be a complementary response to sustain normal functions by degrading dysfunctional cellular components and organelles; hence, the ALP pathway could play differing roles according to the stage of PD development. 2) We found that Clk1 deficiency inhibited autophagy under basal conditions and in MPP⁺-treated neurons (Fig. 2, G and H), and impaired autophagy contributed to cell death (Fig. 4C).

The serine/threonine kinase mTOR regulates cell survival and proliferation and is also known to be a major negative regulator of autophagy. Upstream of mTORC1, AMPK is a

Fig. 7. Striatum and SNc of Clk1 mutant mice exhibit impaired expression of AMPK/mTORC1 signal pathway protein. (A and B) Wild-type (WT) and Clk1^{+/-} mouse striatum and SNc were dissected and processed as described in *Materials and Methods*. Protein levels of phosphor-AMPK (p-AMPK), phosphor-mTOR (p-mTOR), phosphor-p70s6k (p-p70s6k), and Clk1 were determined by western blot analysis. *n* = 4 mice per group. **P* < 0.0167; ***P* < 0.01 vs. wild type.

Fig. 8. Metformin promotes autophagy and ameliorates MPTP neurotoxicity on dopaminergic neurons in $Clk^{+/−}$ mice. (A–C) Schematic diagram of drug administration as in (A). Wild-type (WT) and $Clk^{+/−}$ mice were treated with saline, metformin, and MPTP as described in *Materials and Methods*. The pole-climbing test and rotarod test for bradykinesia were performed on day 8, after which mice were sacrificed and brains collected, $n = 10$ mice per group. * $P < 0.05$; ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. $Clk^{+/−}$ treated with MPTP. (D) Immunostaining for TH in SNc, $n = 6$ mice per group (scale bar, 200 μ m). WT and $Clk^{+/−}$ mouse SNc were dissected and processed as described in *Materials and Methods*. * $P < 0.05$; ** $P < 0.01$ vs. control group; # $P < 0.05$ vs. $Clk^{+/−}$ treated with MPTP. (E) Protein levels of LC3, P62, TH, phosphor-AMPK (p-AMPK), and Clk1 were determined by western blot analysis. $n = 4$ mice per group, ** $P < 0.01$ vs. control group, # $P < 0.05$ vs. $Clk^{+/−}$ treated with MPTP.

metabolic sensor, such that activation of AMPK protects neuronal survival through regulating mitochondrial biogenesis and maintaining energy balance (Egan et al., 2011). Activation of AMPK inhibits mTOR and induces autophagy (Li et al., 2013; Guo et al., 2014). We have previously shown

that $Clk1$ modulates AMPK activity and energy metabolism in microglia cells; therefore, we investigated the role of the AMPK/mTORC1 signaling pathway in inhibition of autophagy by $Clk1$ deficiency in dopaminergic neurons. We found knockdown of $Clk1$ in MN9D dopaminergic neurons resulted

in suppression of AMPK activation and elevation of phosphorylation of mTOR and the mTOR substrate p70s6k (Fig. 3B). Furthermore, we found that Clk1 regulated transcription factor TFEB nuclear translocation and TFEB-targeted genes, which was dependent on the AMPK/mTORC1 signaling pathway. Mechanistic studies demonstrated that Clk1 regulated TFEB nuclear translocation through the AMPK/mTORC1 signaling pathway.

Our previous study has demonstrated that increased dopaminergic neuronal loss may be associated with enhanced microglia activation in Clk1^{+/-} mice treated with MPTP (Gu et al., 2017). The present study provides evidence that Clk1 is directly involved in the regulation of dopaminergic neuronal survival. This appears to be mediated via changes in autophagy consequent to alterations in the AMPK/mTORC1 pathway. Indeed, pretreatment with metformin ameliorated MPTP-induced behavioral impairment and dopaminergic neuronal survival in Clk1^{+/-} mice (Fig. 8, B–D). The underlying mechanism may be attributed to enhanced autophagy by AMPK/mTORC1 in dopaminergic neurons. Our data thus indicate that, in addition to modulating microglia-mediated inflammation, Clk1 exerts neuroprotection by directly regulating autophagic activity of dopaminergic cells.

In conclusion, we found that Clk1 is directly involved in the regulation of dopaminergic neuronal survival by mediating autophagy via the AMPK/mTORC1 pathway. Modulation of Clk1 activity may represent a potential novel therapeutic approach for PD.

Authorship Contributions

Participated in research design: Yan, Zhen.

Conducted experiments: Yan.

Performed data analysis: Yan, Han, Wang, Zheng.

Wrote or contributed to the writing of the manuscript: Yan, Waddington, Zhen.

References

- Bar-Peled L and Sabatini DM (2014) Regulation of mTORC1 by amino acids. *Trends Cell Biol* **24**:400–406.
- Camilleri A and Vassallo N (2014) The centrality of mitochondria in the pathogenesis and treatment of Parkinson's disease. *CNS Neurosci Ther* **20**:591–602.
- Choi HK, Won L, Roback JD, Wainer BH, and Heller A (1992) Specific modulation of dopamine expression in neuronal hybrid cells by primary cells from different brain regions. *Proc Natl Acad Sci USA* **89**:8943–8947.
- Choi JS, Park C, and Jeong JW (2010) AMP-activated protein kinase is activated in Parkinson's disease models mediated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Biochem Biophys Res Commun* **391**:147–151.
- Chua JP, Reddy SL, Merry DE, Adachi H, Katsuno M, Sobue G, Robins DM, and Lieberman AP (2014) Transcriptional activation of TFEB/ZKSCAN3 target genes underlies enhanced autophagy in spinobulbar muscular atrophy. *Hum Mol Genet* **23**:1376–1386.
- Cortes CJ, Miranda HC, Frankowski H, Batlevi Y, Young JE, Le A, Ivanov N, Sopher BL, Carroumeu C, Muotri AR, et al. (2014) Polyglutamine-expanded androgen receptor interferes with TFEB to elicit autophagy defects in SBMA. *Nat Neurosci* **17**:1180–1189.
- Decressac M, Mattsson B, Weikop P, Lundblad M, Jakobsson J, and Björklund A (2013) TFEB-mediated autophagy rescues midbrain dopamine neurons from α -synuclein toxicity. *Proc Natl Acad Sci USA* **110**:E1817–E1826.
- Dehay B, Bové J, Rodríguez-Muela N, Perier C, Recasens A, Boya P, and Vila M (2010) Pathogenic lysosomal depletion in Parkinson's disease. *J Neurosci* **30**:12535–12544.
- Dias V, Junn E, and Mouradian MM (2013) The role of oxidative stress in Parkinson's disease. *J Parkinsons Dis* **3**:461–491.
- Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohn RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, et al. (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science* **331**:456–461.
- Feng L, Ma Y, Sun J, Shen Q, Liu L, Lu H, Wang F, Yue Y, Li J, Zhang S, et al. (2014) YY1-MIR372-SQSTM1 regulatory axis in autophagy. *Autophagy* **10**:1442–1453.
- Gu R, Zhang F, Chen G, Han C, Liu J, Ren Z, Zhu Y, Waddington JL, Zheng LT, and Zhen X (2017) Clk1 deficiency promotes neuroinflammation and subsequent dopaminergic cell death through regulation of microglial metabolic reprogramming. *Brain Behav Immun* **60**:206–219.
- Guo Z, Cao G, Yang H, Zhou H, Li L, Cao Z, Yu B, and Kou J (2014) A combination of four active compounds alleviates cerebral ischemia-reperfusion injury in correlation with inhibition of autophagy and modulation of AMPK/mTOR and JNK pathways. *J Neurosci Res* **92**:1295–1306.
- Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8**:774–785.
- Hekimi S (2013) Enhanced immunity in slowly aging mutant mice with high mitochondrial oxidative stress. *Oncol Immunology* **2**:e23793.
- Hwang CJ, Kim YE, Son DJ, Park MH, Choi DY, Park PH, Hellström M, Han SB, Oh KW, Park EK, et al. (2017) Parkin deficiency exacerbate ethanol-induced dopaminergic neurodegeneration by P38 pathway dependent inhibition of autophagy and mitochondrial function. *Redox Biol* **11**:456–468.
- Kalia LV and Lang AE (2015) Parkinson's disease. *Lancet* **386**:896–912.
- Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, et al. (2016) Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12**:1–222.
- Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, et al. (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* **441**:880–884.
- Lapointe J and Hekimi S (2008) Early mitochondrial dysfunction in long-lived *Mclk1*^{+/-} mice. *J Biol Chem* **283**:26217–26227.
- Lee KI, Kim MJ, Koh H, Lee JI, Namkoong S, Oh WK, and Park J (2015) The anti-hypertensive drug reserpine induces neuronal cell death through inhibition of autophagic flux. *Biochem Biophys Res Commun* **462**:402–408.
- Li L, Chen Y, and Gibson SB (2013) Starvation-induced autophagy is regulated by mitochondrial reactive oxygen species leading to AMPK activation. *Cell Signal* **25**:50–65.
- Maiuri MC, Zalckvar E, Kimchi A, and Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* **8**:741–752.
- Malagelada C, Ryu EJ, Biswas SC, Jackson-Lewis V, and Greene LA (2006) RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation. *J Neurosci* **26**:9996–10005.
- Martina JA, Chen Y, Gucsek M, and Puertollano R (2012) MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy* **8**:903–914.
- Martina JA and Puertollano R (2013) Rag GTPases mediate amino acid-dependent recruitment of TFEB and MITF to lysosomes. *J Cell Biol* **200**:475–491.
- Menzies FM, Fleming A, and Rubinstein DC (2015) Compromised autophagy and neurodegenerative diseases. *Nat Rev Neurosci* **16**:345–357.
- Moon HE and Paek SH (2015) Mitochondrial dysfunction in Parkinson's disease. *Exp Neurol* **24**:103–116.
- Morris G and Berk M (2015) The many roads to mitochondrial dysfunction in neuroimmune and neuropsychiatric disorders. *BMC Med* **13**:68.
- Nakai D, Yuasa S, Takahashi M, Shimizu T, Asaumi S, Isono K, Takao T, Suzuki Y, Kuroyanagi H, Hirokawa K, et al. (2001) Mouse homologue of *coq7/clk-1*, longevity gene in *Caenorhabditis elegans*, is essential for coenzyme Q synthesis, maintenance of mitochondrial integrity, and neurogenesis. *Biochem Biophys Res Commun* **289**:463–471.
- Peña-Llopis S, Vega-Rubin-de-Celis S, Schwartz JC, Wolff NC, Tran TA, Zou L, Xie XJ, Corey DR, and Brugarolas J (2011) Regulation of TFEB and V-ATPases by mTORC1. *EMBO J* **30**:3242–3258.
- Puente C, Hendrickson RC, and Jiang X (2016) Nutrient-regulated phosphorylation of ATG13 inhibits starvation-induced autophagy. *J Biol Chem* **291**:6026–6035.
- Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, et al. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet* **36**:585–595.
- Ren ZX, Zhao YF, Cao T, and Zhen XC (2016) Dihydromyricetin protects neurons in an MPTP-induced model of Parkinson's disease by suppressing glycogen synthase kinase-3 beta activity. *Acta Pharmacol Sin* **37**:1315–1324.
- Roczniak-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, Walther TC, and Ferguson SM (2012) The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. *Sci Signal* **5**:ra42.
- Santini E, Heiman M, Greengard P, Valjent E, and Fisone G (2009) Inhibition of mTOR signaling in Parkinson's disease prevents L-DOPA-induced dyskinesia. *Sci Signal* **2**:ra36.
- Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, Di Malta C, Donaudy F, Embrione V, Polishchuk RS, et al. (2009) A gene network regulating lysosomal biogenesis and function. *Science* **325**:473–477.
- Savitt JM, Dawson VL, and Dawson TM (2006) Diagnosis and treatment of Parkinson disease: molecules to medicine. *J Clin Invest* **116**:1744–1754.
- Segura-Aguilar J and Kostorz RM (2015) Neurotoxin mechanisms and processes relevant to Parkinson's disease: an update. *Neurotox Res* **27**:328–354.
- Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, Erdin SU, Huynh T, Medina D, Colella P, et al. (2011) TFEB links autophagy to lysosomal biogenesis. *Science* **332**:1429–1433.
- Settembre C, Zoncu R, Medina DL, Vetrini F, Erdin S, Erdin S, Huynh T, Ferron M, Karsenty G, Vellard MC, et al. (2012) A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J* **31**:1095–1108.
- Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, Gindi R, Adame A, Wyss-Coray T, and Masliah E (2009) Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in α -synuclein models of Parkinson's and Lewy body diseases. *J Neurosci* **29**:13578–13588.
- Tain LS, Mortiboys H, Tao RN, Ziviani E, Bandmann O, and Whitworth AJ (2009) Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. *Nat Neurosci* **12**:1129–1135.

- Takahashi M, Shimizu T, Moriizumi E, and Shirasawa T (2008) *Clk-1* deficiency induces apoptosis associated with mitochondrial dysfunction in mouse embryos. *Mech Ageing Dev* **129**:291–298.
- Wong E and Cuervo AM (2010) Autophagy gone awry in neurodegenerative diseases. *Nat Neurosci* **13**:805–811.
- Wu Y, Li X, Zhu JX, Xie W, Le W, Fan Z, Jankovic J, and Pan T (2011) Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease. *Neurosignals* **19**:163–174.
- Ye N, Neumeyer JL, Baldessarini RJ, Zhen X, and Zhang A (2013) Update 1 of: recent progress in development of dopamine receptor subtype-selective agents:

potential therapeutics for neurological and psychiatric disorders. *Chem Rev* **113**:PR123–PR178.

Address correspondence to: Xuechu Zhen, Jiangsu Key Laboratory of Translational Research and Therapy for Neuropsychiatric Diseases and College of Pharmaceutical Sciences, Soochow University, 199# Ren-Ai Road, Suzhou Industrial Park, Suzhou, Jiangsu 215021, China. E-mail: zhenxuechu@suda.edu.cn