Up-regulation of autophagy-related gene 5 (ATG5) protects dopaminergic neurons in a zebrafish model of Parkinson’s disease

Zhan-ying Hu, Bo Chen, Jing-pu Zhang, and Yuan-yuan Ma
From the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

Received for publication, October 26, 2016, and in revised form, August 27, 2017
Published, Papers in Press, September 19, 2017, DOI 10.1074/jbc.M116.764795

Parkinson’s disease (PD) is one of the most prevalent motor-related neurodegenerative diseases, which is diagnosed clinically by static tremor, progressive rigidity, bradykinesia, and postural abnormality, and is characterized by dopaminergic neuron loss and Lewy body (LB) accumulation in the substantia nigra neurons of PD patients. Lewy bodies (LBs) are inclusion bodies of some protein aggregates within neurons in PD, dementia with LB, Alzheimer’s disease, and also in other synucleinopathies (1). Typical LBs arise in the brainstem as cytoplasmic, round to elongated inclusions of 8–30 μm in diameter, with a dense eosinophilic core and a narrow pale-stained rim (2). In 1997, Spillantini et al. (3) reported that α-synuclein (α-syn) is the main component of LBs. Physiologically, α-syn is a presynaptic neuronal protein, and as a down-regulator of tyrosine hydroxylase (TH) activity, it can modulate production of dopamine (DA) and control DA cellular levels (4). DA is an important transmitter between the substantia nigra and corpus striatum for human movement. Reduction of DA levels is the direct cause for motor dysfunction of PD patients, and TH is a key enzyme for DA biosynthesis. Thus, the TH level generally is regarded as a positive biomarker for dopaminergic neuron survival.

α-syn protein is a critical protein for neuron survival, but dysregulation of α-syn via aggregation, mutation, or misfolding of overexpressed protein can lead to neurodegeneration and DA neuron death in PD (4–6). Recently, numerous groups reported the pathological mechanism of α-syn. The main pathological action of α-syn resulted from its oligomeric state or fibrillation, and this format can cause LB formation, mitochondrial dysfunction, endoplasmic reticulum stress, oxidative stress, proteasome impairment, disruption of plasma membrane and pore formation, and finally DA neuron death (7, 8). Another important pathological action of α-syn fibrils is its transmission among neurons via endocytosis, plasma membrane penetration, or exosome pathways, thus propagating the LB pathology to other brain regions thereby contributing to the progressiveness of PD (3, 9–11). So, a putative conclusion is converged that PD therapy may be facilitated by inhibition of α-syn aberrant species formation or acceleration of α-syn aggregation elimination.

Additionally, PTEN-induced putative kinase 1 (PINK1) was detected within a subset (5–10%) of LBs and Parkin co-localized with α-syn in LBs, which suggests that PINK1 and Parkin are also components of LBs (12–14). Mutations in Parkin and PINK1 were found in PD, which led to their deficient interaction and preventing the clearance of damaged mitochondria via...
autophagy (15–17). Normally, Parkin, as an E3 ubiquitin ligase, and PINK1, as a mitochondrially targeted kinase, function together in a common pathway to remove dysfunctional mitochondria by autophagy (18, 19). Thus, defects of Parkin or PINK1 can cause not only accumulation of dysfunctional mitochondria and release of reactive oxygen species, but also LB accumulation, including α-syn species (11).

Recently, several studies and reviews have shown that abnormal autophagy contributes to neurodegenerative diseases that manifest abnormal protein accumulation. The autophagy pathway plays an important role in the pathological process of neurodegenerative disease and therapeutic modulation (20–23). Conversely, PD-related proteins, such as α-syn, PINK1, and Parkin, also participate in regulating autophagy (24–26). Klucken and co-workers (27) demonstrated that the impaired autophagy–lysosomal pathway by bafilomycin A1 (BAF) (an inhibitor of autophagolysosomes) in the diseased brain not only limited intracellular degradation of misfolded proteins, but also led to a detrimental microenvironmental response, including uptake, inflammation, and cellular damage due to enhanced α-syn protein secretion. Stefanis and co-workers (28) reported that lysosomal dysfunction contributed to α-syn accumulation and the related toxicity. The results indicate the correlation of autophagy with synucleinopathies.

Furthermore, protective roles of autophagy-related gene 5 (atg5) on neurons in synucleinopathies were reported. atg5 deficiency in mice led to progressive deficits in motor function with accumulation of cytoplasmic inclusion bodies in neurons in mice (20). ATG5-dependent autophagy acted as a protective mechanism during apoptotic cell death induced by MPP⁺ toxicity in a cell model (29). In patients with PD, ATG5 protein levels were altered, which suggests that ATG5 deficit is a risk factor that may serve as a PD-related marker gene (30). Therefore, ATG5 may play a key role in autophagy-regulated neurodegenerative disease. In the basic autophagy process, ATG5 conjugates to ATG12 and forms a ubiquitin-conjugating complex, which plays a crucial role in autophagic vacuole formation (31, 32). In addition, ATG5 functions in regulation of embryonic nervous system development and differentiation in zebrafish (33, 34). Our earlier study showed that ATG5 was expressed mainly in the zebrafish embryonic brain region and affected transcription of pink1 and β-syn (33). However, the molecular mechanism of ATG5’s protective action on DA survival remains unclear.

Compound MPTP is a mitochondrial toxin and primarily causes damage to the nigrostriatal DA pathway with a profound loss of DA neurons in the striatum and substantia nigra in mice and primates. MPTP as a standard compound used for establishing PD models in vivo or in vitro has been accepted widely (35, 36). MPTP treatment on zebrafish also has been shown to cause loss of diencephalic dopaminergic neurons and behavioral defects in swimming responses (37, 38). Mutations in Parkin and PINK1 made zebrafish vulnerable to sub-effective doses of MPTP (39, 40). In zebrafish, the molecular markers TH and DAT are available for identification of dopaminergic neurons at 3 days post-fertilization (dpf) (41). At this stage, th expression patterns are similar to those observed in the adult (42, 43). Therefore, in this study we used MPTP to establish a PD model in zebrafish, and we examined the effects of regulating ATG5 on PD progression. We found that down-regulation or up-regulation of ATG5 notably impacted levels of the PD-related markers and the autophagy process. An appropriate level of ATG5 could restore expression levels of PD-marker genes disrupted by MPTP and reverse the pathological progress of PD.

Results

PD-like symptoms are accompanied by impaired autophagic flux in the MPTP-induced zebrafish larvae model

A PD-like model was established by exposing zebrafish larvae to MPTP. A locomotion assay showed that swimming behavior of the larvae was disturbed after exposure to MPTP, in which zebrafish swimming velocity and distance were significantly suppressed, and their activity duration was also moderately reduced by MPTP treatment (Fig. 1A). The detailed assay of the locomotion was investigated by stratification in a low active, active, and high active status according to the speed of the larvae. The swimming parameters (including swimming distance, duration, and velocity) in the “active state” (speed between 0.2 and 4 cm/s) were significantly inhibited, whereas the “low active” (speed below 0.2 cm/s) swimming was moderately reduced by MPTP, and the “high active” (speed over 4 cm/s) movement was sporadic and is included in the “overall” data (supplemental Fig. S1). This suggests that MPTP mainly affected larval “active” swimming behavior. The data indicated significant locomotor defects caused by MPTP. In addition, expression of the dopaminergic neuron marker gene th was down-regulated at both transcription and translation levels (Fig. 1B), which suggests that MPTP resulted in a decrease in dopaminergic neuron number. The key component of LBs, α-syn protein in human PD, is not found in zebrafish, but in zebrafish β- and γ-synucleins do exist, and mainly the β-syn exerts a similar role to the human α-syn protein (44, 45). Furthermore, the PD-related proteins β-syn, PINK1, and Parkin were detected and up-regulated by MPTP in a dose-dependent manner, but their mRNA levels were not changed (Fig. 1, C–E). These results indicate that MPTP-induced PD-like symptoms of motor dysfunction, loss of dopaminergic neurons, and abnormal increase of the PD proteins were similar to those in the mouse MPTP-PD model (46).

Based on our previous results that ATG5 regulated expression of nervous system developmental genes, including β-syn and pink1 (33), we explored autophagy mechanisms in MPTP-induced neuronal toxicity by examining expression of the autophagy-related gene atg5 in MPTP-treated larvae. The results showed that ATG5 mRNA and protein levels were clearly down-regulated by MPTP in a dose-dependent manner (Fig. 2A), which suggests that ATG5 is involved in MPTP-induced PD pathology. In addition, levels of autophagic flux markers, selective receptor p62 and LC3B II proteins, were measured and increased in an MPTP dose-dependent manner (Fig. 2, B and C). However, transcription of the lc3 gene did not change in zebrafish larvae (Fig. 2, B and C). Generally, an increase of LC3B II protein presents in a normal autophagy process. To prove an increase of LC3B II protein also occurring
in MPTP-impaired autophagy, a BAF experiment was performed, and the result shows that BAF caused an apparent elevation of the LC3B II protein whether MPTP was added or not added (supplemental Fig. S2), indicating an impaired autophagy flux with elevation of LC3B II levels in the MPTP-PD model. These data showed that MPTP impaired autophagic flux in zebrafish larvae. Furthermore, to check whether the ATG5 decrease was related to oxidative stress, we tested an antioxidant N-acetylcysteine (NAC) effect on MPTP-dependent atg5 mRNA. The results show that NAC did not recover the level of MPTP-dependent atg5 mRNA under NAC concentration below 200 μmol but rescued atg5 mRNA by NAC at 500 μmol (Fig. 2D), which may hint that at least partly oxidative stress restricted ATG5 transcription under MPTP treatment.

**Figure 1. MPTP induced low movement and dysregulation of PD-related protein in zebrafish larvae.** A, larvae were exposed to 50 μM MPTP at 5 dpf. Movement data (distance, duration, and velocity) of the larvae were recorded for 20 min in the dark at 6 dpf. A representative swimming trajectory (5 min) is presented. Scatter diagrams show the behavior changes that were derived from 12 larvae. Statistics comparison (p value) was performed between WT and MPTP groups. B–E, larvae were exposed to 100 and 200 μM MPTP at 2 dpf for 1 day and collected to assess mRNA and protein levels of TH (B), β-syn (C), Parkin (D), and PINK1 (E). D and E, RT-PCR results of pink1, parkin, and β-actin were performed at the same gel; pink1 and parkin shared the β-actin bands, and the samples were collected from one experiment. *, p values <0.05; **, p values <0.01; ***, p values <0.001 versus untreated groups (one-way ANOVA). The data in B–E were from three independent gels.

**Level of ATG5 correlates with autophagy flux and key PD-related proteins in zebrafish larvae**

To examine the effects of ATG5 on PD-related genes and autophagy flux, ATG5 capped mRNA and an ATG5 morpholino oligonucleotide (SMO) that binds to the initiation site of
ATG5 mRNA and inhibits translation were injected separately into zebrafish embryos to up-regulate or down-regulate ATG5. LC3B II, p62 protein, and PD-related proteins TH, β-syn, PINK1, and Parkin were detected by Western blotting. The results showed that compared with wild-type (WT) and lacZ-injected larvae, overexpression of ATG5 decreased p62 protein and increased LC3B II protein, which suggests that ATG5 mRNA injection elevated the level of autophagy. In contrast, ATG5 knockdown increased p62 protein and decreased the level of LC3B II (Fig. 3, A–C), which suggests that autophagic flux was blocked and might have resulted from inhibition of LC3B I to LC3B II conversion. These data imply that ATG5 is a positive regulator of autophagy flux.

Further experiments showed that when zebrafish larvae were injected with ATG5 mRNA, the level of TH protein was similar to the WT group, and β-syn, Parkin, and PINK1 proteins were moderately suppressed or had no obvious change compared with the WT group and lacZ group. When the atg5 gene was knocked down, the TH protein was significantly down-regulated, whereas β-syn, PINK1, and Parkin proteins were up-regulated (Fig. 3D). These data indicate that ATG5 decrease can promote dopaminergic neuron loss with an increase of the PD-related proteins. Furthermore, the level of parkin mRNA was tested using RT-PCR, and the result showed that atg5 down-regulation really had an active effect on parkin mRNA levels, but atg5 overexpression did not apparently affect parkin transcription levels (see data shown in supplemental Fig. S3). So, the function of ATG5 probably is involved in some PD-related gene transcription and autophagosome formation.

Figure 2. atg5 gene was suppressed, and autophagic flux was impaired in zebrafish larvae exposed to MPTP. A–C, larvae were exposed to 100 and 200 μM MPTP at 2 dpf for 1 day and collected to measure mRNA and protein levels of ATG5 (A), LC3B (B), and p62 (C). D, antioxidant NAC effect on MPTP-dependent atg5 mRNA tested by RT-PCR. Statistics comparison was carried out between MPTP-treated and untreated groups. *, p values < 0.05; **, p values < 0.01; ***, p values < 0.001 versus the untreated groups (one-way ANOVA). The data were from three independent gels.
ATG5 levels negatively correlate with MPTP toxicity in dopaminergic neurons

Loss of dopaminergic neurons in the diencephalon is key evidence for PD pathology. We measured and compared the number of dopaminergic neurons in the diencephalon using whole-mount in situ hybridization (WISH) and whole-mount immunofluorescent assay (WIFA) among five groups as follows: MPTP; MPTP plus ATG5 mRNA; MPTP plus lacZ; MPTP plus 5MO; and MPTP plus MisMO. In the MPTP groups, the number of dopaminergic neurons, integral optical density (IOD), and distribution of the \( \text{th} \) gene signal was significantly reduced in the larval diencephalon; the IOD in some dopaminergic neurons in the diencephalon was clearly enhanced (Fig. 4). In the MPTP plus ATG5-mRNA groups, the number of dopaminergic neurons was clearly more than in the MPTP groups; the IOD per neuron still showed dose-dependent changes, but the changes were gradual (Fig. 4). In the MPTP plus lacZ control groups, the number of dopaminergic neurons in the diencephalon was similar with the MPTP-treated groups, but the IOD and IOD per neuron were slightly reduced. In contrast, compared with the MPTP groups, the MPTP plus ATG5-MO groups showed dose-dependent decreases in the number of dopaminergic neurons and IOD (Fig. 4). In the MPTP plus MisMO control groups, there were no obvious changes compared with the MPTP-treated groups. These results were confirmed using WIFA (Fig. 5) with an anti-TH antibody and using detection of the \( \text{dat} \) gene by WISH (Fig. 6), which showed the same changes as those in Fig. 4. These results indicated that the sensitivity of the larvae to MPTP-induced neurotoxicity was notably impacted by the level of ATG5, in that ATG5 prevented loss of DA neurons during MPTP exposure.

ATG5 levels negatively correlate with MPTP toxicity in dopaminergic neurons

Figure 3. Autophagy marker proteins and PD-related proteins were regulated by the autophagy-related gene atg5 in zebrafish larvae. ATG5 mRNA, lacZ, 5MO, or MisMO was injected into embryos at the 1–4-cell stage, respectively. The larvae were collected at 3 dpf for protein detection by Western blotting. lacZ was as a control of atg5 up-regulation, and MisMO was as a control of atg5 gene down-regulation. A, ATG5 protein level was regulated by atg5 mRNA and 5MO injection, respectively. B, in contrast, p62 expression was down-regulated or up-regulated by ATG5 mRNA and 5MO. C, level of LC3 II was positively up-regulated by ATG5 mRNA and negatively by 5MO, which suggests that the level of autophagy was increasing and decreasing, accordingly. D, PD-related proteins, \( \beta \)-syn, Parkin, and PINK1 were elevated by ATG5 knockdown and descended by ATG5 overexpression; DA marker protein, TH, was reversely changed by ATG5 regulation. Here, as a loading control, \( \beta \)-ACTIN images were reused, because the protein samples were obtained from one experiment, \( \beta \)-syn, PINK1, PARKIN, and TH shared the \( \beta \)-ACTIN bands. The means and S.D. were derived from three independent gels. *, \( p \) values <0.05; **, \( p \) values <0.01 versus WT group (one-way ANOVA).
MPTP group and MPTP plus lacZ group (Fig. 7A). These results suggest that MPTP-induced locomotor aberration can be rescued by up-regulation of ATG5 in zebrafish larvae. In contrast, in the MPTP plus 5MO group, the swimming distance, duration, and trajectory were more impaired compared with the MPTP group and MPTP plus MisMO group (Fig. 7B).

The locomotor data suggested that ATG5 deficiency aggravated MPTP-induced behavioral defects and dopaminergic neuron loss. Within the no-MPTP groups, in the group of 5MO-injected larvae the mean values of locomotion duration were moderately greater and the speed was slower than those in the WT group (Fig. 7B). These differential effects were more clearly expressed in the active state (supplemental Fig. S4).

Human ATG5 antagonize MPTP-induced dopaminergic neuronal toxicity in zebrafish

To validate functional conservation of ATG5 protection on DA neurons between humans and zebrafish, we tested the effect of human ATG5 on the zebrafish PD model. The validity of hATG5 was assessed by transfecting SH-SY5Y cells with human ATG5 5′-capped mRNA (hmRNA). The human ATG5-ATG12 protein complex was up-regulated (Fig. 8A). hmRNA and lacZ were then injected into zebrafish embryos at the 1–4-cell stage, and the expression pattern of the th gene in the larval brain was detected by WISH. The results showed that the number of DA neurons was increased or recovered in the larval ventral diencephalon as indicated by the th signal in the MPTP plus hATG5 mRNA groups compared with MPTP groups without ATG5 mRNA and lacZ plus MPTP groups (Fig. 8, B and C).

Thus, the effect of human ATG5 mRNA on dopaminergic neurons was very similar to that of zebrafish ATG5 mRNA. These results suggest that both sequence (protein identity of 82%) (33) and function of zebrafish ATG5 are highly homologous to human ATG5. Both ATG5 proteins antagonized MPTP-induced dopaminergic neuronal toxicity in zebrafish.

Discussion

The key component of LBs, α-syn protein in human PD, is not found in zebrafish, but β- and γ-synucleins exist in zebrafish brain. Previous studies have indicated that roles of human α- and β-syn proteins are discrepant on LBs formation; human β-syn is considered a potent inhibitor of α-syn aggregation by forming α/β-syn hetero-oligomers up to a tetramer that do not further propagate (47, 48). However, there are conserved functions between human α-syn and zebrafish β-syn in neural dopaminergic homeostasis and PD pathology (42, 43). Zebrafish and human β-syn are 71% identical (42). In this study, levels of β-syn increased following MPTP treatment or ATG5 knockdown. Based on previous studies showing MPTP as a typical neurotoxin inducing a PD model in mice (49, 50), and dien-
cephalic DA neuron loss and behavioral defects in zebrafish larvae (24, 25), we established MPTP-induced PD model in zebrafish and found behavioral symptoms and DA neuron loss similar to the mouse PD model. Moreover, the PD model presented not only accumulated /H9252-syn, PINK1, and Parkin proteins but impaired autophagy processes as well. We conclude that MPTP induces a zebrafish PD model with characteristics similar to the mouse PD model.

Currently, many studies indicated that PINK1 and Parkin can join into LBs together with /H9251-syn in PD. Normally, PINK1 and Parkin are regarded as neuroprotective proteins. PINK1 can recognize and anchor to the outer membrane of impaired mitochondria, then recruit Parkin on the impaired mitochondria; they function together in a common pathway to remove dysfunctional mitochondria by autophagy (18, 19). However, upon exposure to mitochondrial toxins, Parkin binds /H9251-syn and PINK1 on the surface of damaged mitochondria, and overexpression of /H9251-syn induces mitochondrial fragmentation and dysfunction along with neurodegeneration (51, 52). Also deg-
More and more data suggest a central role of autophagy in the molecular mechanisms of PD pathogenesis (54). Dysfunction of some autophagy-related proteins, including ULK1, ULK2, beclin1, VPS34, and AMBRA1, which are involved in autophagy initiation and autophagosome formation, are associated with the disease process of PD (55). Mice with an \( \text{atg5} \) defect developed pathological features of PD such as defects in movement and accumulation of cytoplasmic LBs in neurons (20). In this study, we found that \( \text{atg5} \) morphants were vulnerable to sub-effective doses of MPTP, and MPTP-treated larvae presented aberrant swimming behavior (movement distance, velocity, and swimming duration) and loss of DA neurons. In addition, under MPTP treatment, the ATG5 protein level was significantly decreased, which suggested that an \( \text{atg5} \) deficiency might be important in PD pathological processes. These data demonstrated that ATG5 dysfunction enhanced the protein accumulation of PD related proteins /H9252/-syn, Parkin, and PINK1.

In contrast, ATG5 up-regulation preserved DA neuron number and locomotor behavior and maintained the proteins at near-physiological levels, indicating that ATG5 can exert protection against MPTP-induced PD. Despite a few reports on anti-PD effects of ATG5, several studies have implicated \( \alpha \)-syn, Parkin, and PINK1 in autophagy and have shown that \( \text{atg5} \) knockdown significantly increased \( \alpha \)-syn levels in vitro (56–59). Our previous study demonstrated that in zebrafish, ATG5 down-regulation enhanced transcription of \( \text{pin1} \) and \( \beta \)-syn; conversely, ATG5 up-regulation moderately suppressed the two gene transcription (33). ATG5 probably is a protein with multiple functions; except for its pro-autophagy role, ATG5 can down-regulate expression of neural development- and function-related genes for homeostasis of the neural development. In the PD condition, elevation of \( \text{parkin} \) and \( \text{pink1} \) mRNA levels cannot only promote the autophagy process but can aggravate accumulation of excess \( \text{parkin} \) and \( \text{pink1} \) proteins due to an impaired autophagy pathway by ATG5 deficiency.

ATG5 is a highly conserved key factor for regulating formation of autophagic vacuoles and autophagy level (33, 60). ATG5 functions in elongation of the isolation membrane, \( \text{LC3B} \) lipidation, and its correct localization (61). In this study, ATG5 knockdown inhibited conversion of \( \text{LC3B I} \) into \( \text{LC3B II} \), and ATG5 overexpression increased the level of \( \text{LC3B II} \) and decreased the \( \text{p62} \) level (Fig. 3 A). This may be one of reasons that ATG5 deficiency enhanced sensitivity of larvae to MPTP-induced neurotoxicity by suppression of \( \text{LC3B II} \) formation. As we know, \( \text{LC3B II} \) is derived from \( \text{LC3B I} \) that becomes conjugated to phosphatidylethanolamine, which is mediated orderly by \( \text{Atg7} \), \( \text{Atg3} \), and the \( \text{ATG12-ATG5-Atg16L} \) complex (62–64). Therefore, we hypothesize that ATG5 dysfunction plays a key role as an accelerating factor in the pathological processes of PD by causing deficient autophagosomes and increasing expression of PD-related genes in the zebrafish PD model.

In summary, MPTP causes PD symptoms, which may be partially mediated by ATG5 deficiency. Adequate ATG5 levels can inhibit accumulation of PD-related proteins in DA neurons by modulation of gene transcription and promote autophagosome formation, which facilitates DA neuron survival and resists PD damage. We conclude that ATG5 modulation may be a potential therapeutic target for screening new drugs against parkinsonism.

Figure 7. Larval locomotor behavior was modulated by ATG5 level in MPTP-induced PD model. Embryos were injected with SMO, MisMO, ATG5 mRNA, or \( \text{lacZ} \) at the 1–4-cell stage and exposed to 50 \( \mu \text{M} \) MPTP at 5 dpf. At 6 dpf, for both low active and active states, larval swimming distance, movement duration, and swimming velocity in the different groups were recorded for 20 min and compared with WT control. Spontaneous movement trajectory during 5 min is also presented. \( \text{lacZ} \) was as a control of \( \text{atg5} \) mRNA, and MisMO was as a control of \( \text{atg5} \) MO injection. A, larval movement defects were rescued in the ATG5 mRNA-injected larvae exposed to MPTP compared with MPTP-treated groups without mRNA injection and WT groups. B, larval swimming behavioral disorders were exacerbated in the SMO-injected larvae exposed to MPTP compared with MPTP-treated groups without SMO injection and WT groups. Scatter diagrams were used to show the changes; the data were derived from 24 larvae for \( \text{A} \) and 12 larvae for \( \text{B} \). A multiple variable ANOVA test was used in comparison with WT, \( \text{atg5 mRNA}, \text{MPTP}, \text{MPTP plus atg5 mRNA}, \text{lacZ}, \text{MPTP plus lacZ}, \text{and among WT, SMO, MisMO, MPTP plus SMO, and MPTP plus MisMO groups.} \)
Experimental procedures

Fish husbandry and embryo and larvae preparation

Zebrafish (Danio rerio) wild-type AB strain was originally obtained from the College of Life Sciences and Technology, Tsinghua University. Fish feeding, breeding, maintenance, and staging were performed as described previously (65). Briefly, embryos and larvae were incubated in 280 mg/liter Tropical Marine Artificial Seawater crystal (CNSIC Marine Biotechnology Co., Ltd., China) at 28.5 °C. The embryos were obtained by natural mating, and synchronous embryos at the appropriate stage were collected. They were fixed with 4% paraformaldehyde in phosphate-buffered saline for in situ hybridization and whole-mount immunohistochemistry or immersed in TRIzol reagent for mRNA isolation.

Synthesis and microinjection of morpholino oligonucleotides (MOs) and capped mRNAs

5MO and MisMO were designed by and purchased from Gene Tools LLC; their sequences were described previously (33). 5MO was used to inhibit ATG5 expression by binding to ATG5 initiation codon sites, and MisMO was used as a control. The injection of 0.5 nl of 50 μM 5MO or MisMO was conducted at the 1–4-cell embryo stage. Clone of a full-length cDNA encoding ATG5, capped mRNA synthesis, and injection were performed as described in a previous study (33). Human ATG5 cDNA was cloned from the SH-SY5Y cell line based on its published sequence (GenBank™ JQ924061.1). lacZ cDNA fragment according to pEASY-T1 Simple (TransGen Biotech, CB111-01) was subcloned into pBluescript II SK™ plasmid by Sangon Biotech (Shanghai) Co., Ltd. Capped mRNA of human atg5 and lacZ was prepared as described above.

Chemical treatment

MPTP was purchased from Sigma (M0896). Stock solutions of MPTP (100 mM) were made using double distilled H2O and were stored at −20 °C. MPTP was added to the embryo media at final concentrations of 50, 100, 200, and 500 μM at 2 and 5 dpf. One day later, after washing three times with normal rearing solution, larvae were used for neurobehavioral detection, fixed in 4% paraformaldehyde for in situ hybridization and whole-mount immunohistochemistry assay, or collected for extraction of total protein or RNA.

NAC (Sigma, A7250) was dissolved in fish water to create a 100 mM stock solution and then diluted to a concentration of...
100, 200, 500 and 1000 μM in fish water (66). Larvae at 2 dpf were exposed to the MPTP mixed with NAC solution for 1 day and then collected for RT-PCR detection of atg5 mRNA. Bafilomycin A1 (Sigma, B1793) was dissolved in DMSO to create a 25 mM stock solution and then diluted to a concentration of 20 nM in fish water (67). Larvae at 3 dpf were exposed to the BAF solution for 6 h and then collected for detection; 0.1% DMSO was used as a solvent control.

**SH-SYSY cell transfection and detection**

SH-SYSY cells were obtained from Dr. Shuying Wang (Xuanwu Hospital, Capital Medical University). SH-SYSY cells were grown in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (Hyclone) and cultured at 37 °C under humidified 5% CO2 atmosphere. SH-SYSY cells were transfected with capped ATG5 mRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 2 days in culture, cells were collected for RT-PCR and Western blotting.

**WISH**

For the preparation of sense and antisense RNA probes, th and dat cDNA fragment sequences were cloned using PCR (the parameters are presented in Table 1) into the pGEM-T plasmid. Antisense and sense RNA probes were synthesized using the DIG RNA labeling kit (Roche Applied Science, 1175041) according to the manufacturer's instructions. Embryos were fixed with 4% paraformaldehyde overnight at 4 °C. Larvae at 3 dpf or longer were pretreated by DNase of 3 units/200 μl at 37 °C for 24 h prior to starting the hybridization to remove genomic DNA pseudo-positive interference. In situ hybridization was performed as described by Whitlock and Westerfield (68).

**WIFA**

Larvae were collected at 3 dpf and pretreated with proteinase K (Tiangen, RT403). The procedure has been described previously (69). Briefly, larvae were incubated in the primary antibody, rabbit anti-mouse TH monoclonal antibody (Minipore, MAB318), at a dilution of 1:500 overnight at 4 °C. They were then incubated with a FITC-conjugated anti-rabbit secondary antibody at a dilution of 1:2000 overnight at 4 °C. Images were taken using an inverted phase-contrast fluorescence microscope (Olympus, IX 51).

**RT-PCR**

Embryos, larvae, or cells at selected stages were collected into TRIzol reagent (Sigma). Total RNA was extracted following the TRIzol reagent RNA extraction kit manual. First-strand cDNAs were synthesized by reverse transcription using the Moloney murine leukemia virus RTase cDNA synthesis kit (Promega). PCR amplification cycles and primer pairs are listed in Table 1. β-Actin was amplified as a template loading control. PCR parameters were 94 °C for 5 min (94 °C for 30 s, 54–55 °C for 30 s, and 72 °C for 1 min) for 22–35 cycles and 72 °C for 10 min.

**Western blotting**

The immunoblotting procedure has been previously described in the literature (70). Briefly, total protein of zebrafish embryos or SH-SYSY cells was extracted with the RIPA lysis kit (Applygen Technologies Inc., C1053) and separated using 10% SDS-PAGE. The following procedure is the same as used in our previous study (33). The membranes were incubated with primary antibody at a dilution of 1:1000 overnight at 4 °C. The primary antibodies were as follows: anti-human ATG5 (Abcam, ab54033); mouse anti-human ACTB antibody (Zhongshan Goldbridge, TA09); mouse anti-GAPDH mAb (Zhongshan Goldbridge, TA08); rabbit anti-mouse TH monoclonal antibody (Minipore, MAB318); p62 rabbit polyclonal antibody (MBL, PM045); LC3 mouse monoclonal antibody (MBL, M186-3); PINK1 (D8G3) rabbit mAb antibody (CST, catalog no. 6946); Parkin mAb antibody (CST, catalog no. 4211); and α/β-syn (Syn205) mouse mAb antibody (CST, catalog no. 6946).

### Table 1

**Primer pairs and parameters of PCR in the study**

| Gene name | Primer sequence | Annealing temperature | Cycles |
|-----------|-----------------|-----------------------|--------|
| th        | F, TACAGCCGAGATGATACATCCCTC | 54 | 35 |
|           | R, TTATGTTCTGAGATGATCTCCAA   | 54 | 35 |
| dat       | F, GCTATGGGAGGATAGAGCTGCTGT | 55 | 28 |
|           | R, AGGAACCTGCCCTGGAGAGTTG   | 55 | 28 |
| lc3b      | F, AAAGGAGACATTGGAGACAG | 52 | 30 |
|           | R, AAGGTCTCCTGGAGAAGCCTA | 52 | 30 |
| parkin    | F, GCTGGCGGCCATCTTTGCTGCTCT | 55 | 30 |
|           | R, CTCGGTGAGATCCGTTGCTCC | 55 | 30 |
| p62       | F, CCTGCGTGCATCTGACTCTGCC | 55 | 28 |
|           | R, TACCTTGGTGCCGCTTTCCC | 55 | 28 |
| atg5      | F, ATGATGATGACGAGATGACAG | 55 | 28 |
|           | R, TACGTCATCGTGACGAGG | 55 | 28 |
| β-actin   | F, AAGGAATCTGCTGATGACATCA | 52 | 30 |
|           | R, ACTGTCATGCTGACGAGGTA | 52 | 30 |
| pink1     | F, GGAGTGGCATCGATGTCGCTC | 55 | 30 |
|           | R, TCTGGCATTGGGTGACCTGCTT | 55 | 30 |
| β-syn     | F, ATGGATGTGTGTATGAGGAGGC | 55 | 30 |
|           | R, TACCTGCTGAGATGATCGG | 55 | 30 |
| htag5     | F, ATGACAGATGACGAAAATG | 55 | 30 |
|           | R, TACATGCTGCTGCTGAGG | 55 | 30 |

- **Actin** was amplified as a template loading control. PCR parameters were 94 °C for 5 min (94 °C for 30 s, 54–55 °C for 30 s, and 72 °C for 1 min) for 22–35 cycles and 72 °C for 10 min.
2644). The membranes were then treated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence using the SuperSignal® West Pico chemiluminescent substrate (Thermo Fisher Scientific, 34080) with the AlphaEase® FC Imaging System.

**Behavioral experiment**

Spontaneous movement of larvae was analyzed by ZebraLab (Viewpoint, ZebraLab 3.3) instrument. The larvae were placed separately in the wells of a 96-well plate (1 fish/well). Locomotor distance, swimming duration, velocity, and swimming track were recorded in darkness. Velocity lower than 0.2 cm/s was defined as low active movement, velocity higher than 4 cm/s was defined as high active movement, and velocity between 0.2 and 4 cm/s was defined as active movement. The velocity threshold values were set based on our preliminary experiment, by which zebrafish swim behavior can be better sorted into normal status or MPTP-induced inactive status. Larval behaviors were recorded for 20 min, and data were collected once per 5 min. In this study, high active movement was rare, so we mainly analyzed low active and active movement; but the data of high active movement was included in the overall data.

**Data analysis**

In statistical analyses of larvae, data were shown by scatter plots. Statistical analyses were performed using t test in comparison with the WT group and the MPTP group (Fig. 1A), using multiple variable ANOVA test in comparison with WT, mRNA, MPTP, lacZ, MPTP plus mRNA, lacZ plus MPTP, and with WT, MO, MisMO, MPTP plus MO, and MPTP plus MisMO groups in Fig. 7.

Data from Western blotting and RT-PCR were relative values versus wild types or versus the untreated groups, in which the mean values of wild types or the untreated groups are defined as 1. The means and standard deviations were derived from independent triplicates and are presented as mean ± S.D. in histograms.

Scatter diagram was used to show and compare the changes of dopaminergic neuron number among the groups treated differently; line charts showed average values of IOD per group and average IOD per dopaminergic neuron in the larval ventral diencephalon, respectively. Standard deviations were shown in all graphs (n >7).

Statistical analyses for RT-PCR, Western blotting, WISH, and WIFA were performed using one-way ANOVA tests, and p values <0.05 were considered as significant. */#, p values <0.05; **/##, p values <0.01; and ***/###, p values <0.001.

**Author contributions**—J. P. Z. conceived and designed the project. Z. Y. H. and B. C. performed the experiments and data analysis. Y. Y. M. finished the RT-PCR experiments in Fig. 2D. Z. Y. H. and J. P. Z. wrote the manuscript.

**Acknowledgments**—We thank Professor Anming Meng (Tsinghua University) for providing zebrafish AB stain seedlings, and we thank Jie Meng for fish husbandry.

**References**

1. Ma, M. R., Hu, Z. W., Zhao, Y. F., Chen, Y. X., and Li, Y. M. (2016) Phosphorylation induces distinct α-synuclein strain formation. *Sci. Rep.* 6, 37130
2. Giráldez-Pérez, R., Antolin-Vallespin, M., Muñoz, M., and Sánchez-Capelo, A. (2014) Models of α-synuclein aggregation in Parkinson’s disease. *Acta Neuropathol. Commun.* 2, 176
3. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) α-Synuclein in Lewy bodies. *Nature* 388, 839–840
4. Emamzadeh, F. N. (2016) α-Synuclein structure, functions, and interactions. *J. Res. Med. Sci.* 21, 29
5. Kanaan, N. M., and Manfredsson, F. P. (2012) Loss of functional α-synuclein: a toxic event in Parkinson’s disease? *J. Parkinsons Dis.* 2, 249–267
6. Peng, X., Peng, X. M., Tehrani, R., Dietrich, P., Stefanis, L., and Perez, R. G. (2005) α-Synuclein activation of protein phosphatase 2A reduces tyrosine hydroxylase phosphorylation in dopaminergic cells. *J. Cell Sci.* 118, 3523–3530
7. Longhena, F., Faustini, G., Missale, C., Pizzi, M., Spano, P., and Bellucci, A. (2017) The contribution of α-synuclein spreading to Parkinson’s disease synaptopathy. *Neural Plast.* 2017, 501219
8. Jellinger, K. A. (2010) Basic mechanisms of neurodegeneration: a critical update. *J. Cell. Mol. Med.* 14, 457–487
9. Gallegos, S., Pacheco, C., Peters, C., Opazo, C. M., and Aguayo, L. G. (2015) Features of α-synuclein that could explain the progression and irreversibility of Parkinson’s disease. *Front. Neurosci.* 9, 59
10. Wong, Y. C., and Krainc, D. (2017) α-synuclein toxicity in neurodegeneration: mechanism and therapeutic strategies. *Nat. Med.* 23, 1–13
11. Lopes da Fonseca, T., Villar-Piqué, A., and Outeiro, T. F. (2015) The interplay between α-synuclein clearance and spreading. *Biomolecules* 5, 435–471
12. Gandhi, S., Muqit, M. M., Stanyer, L., Healy, D. G., About-Sleiman, P. M., Hargreaves, I., Heales, S., Ganguly, M., Parsons, L., Lees, A. J., Latchman, D. S., Holton, J. L., Wood, N. W., and Revesz, T. (2006) PINK1 protein in normal human brain and Parkinson’s disease. *Brain* 129, 1720–1731
13. Um, I. W., Stichel-Gunkel, C., Lübbert, H., Lee, G., and Chung, K. C. (2009) Molecular interaction between parkin and PINK1 in mammalian neuronal cells. *Mol. Cell. Neurosci.* 40, 421–432
14. Murakami, T., Moriwicki, Y., Kawarabayashi, T., Nagai, M., Ohta, Y., Deguchi, K., Kurata, T., Morimoto, N., Takehisa, Y., Matsubara, E., Ikeda, M., Harigaya, Y., Shoji, M., Takahashi, R., and Abe, K. (2007) PINK1, a gene product of PARK6, accumulates in α-synucleinopathy brains. *J. Neurol. Neurosurg. Psychiatry* 78, 653–654
15. Corti, O., Lesage, S., and Brice, A. (2011) What genetics tells us about the causes and mechanisms of Parkinson’s disease. *Physiol. Rev.* 91, 1161–1218
16. Geisler, S., Holmström, K. M., Treis, A., Skujat, D., Weber, S. S., Fiesel, F. C., Kahle, P. J., and Springer, W. (2010) The PINK1/Parkin-mediated mitophagy is compromised by PD-associated mutations. *Autophagy* 6, 871–878
17. Nguyen, T. N., Padman, B. S., and Lazaro, M. (2016) Deciphering the signals of molecular PINK1/Parkin mitophagy. *Trends Cell Biol.* 26, 733–744
18. Durcan, T. M., and Fon, E. A. (2015) The three ‘P’s of mitophagy: PARKIN, PINK1, and post-translational modifications. *Genes Dev.* 29, 989–999
19. Barodia, S. K., Creed, R. B., and Goldberg, M. S. (2017) Parkin and PINK1 functions in oxidative stress and neurodegeneration. *Brain Res. Bull.* 133, 51–59
20. Hará, T., Nakamura, K., Matsu, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441, 885–889
21. García-Arencibia, M., Hochfeld, W. E., Toh, P. P., and Rubinstein, D. C. (2010) Autophagy, a guardian against neurodegeneration. *Semin. Cell Dev. Biol.* 21, 691–698
61. Walsh, C. M., and Edinger, A. L. (2010) The complex interplay between autophagy, apoptosis, and necrotic signals promotes T-cell homeostasis. *Immunol. Rev.* 236, 95–109
62. Klionsky, D. J., Baehrecke, E. H., Brumell, J. H., Chu, C. T., Codogno, P., Cuervo, A. M., Debnath, J., Deretic, V., Elazar, Z., Eskelinen, E. L., Finkbeiner, S., Fueyo-Margareto, J., Gewirtz, D., Jäättelä, M., Kroemer, G., et al. (2011) A comprehensive glossary of autophagy-related molecules and processes (2nd Ed.). *Autophagy* 7, 1273–1294
63. Wirawan, E., Vanden Berghe, T., Lippens, S., Agostinis, P., and Vandenberghe, P. (2012) Autophagy: for better or for worse. *Cell Res.* 22, 43–61
64. Xu, M., Zhang, Q., Li, P. L., Nguyen, T., Li, X., and Zhang, Y. (2016) Regulation of dynein-mediated autophagosomes trafficking by ASM in CASMCs. *Front. Biosci.* 21, 696–706
65. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995) Stages of embryonic development of the zebrafish. *Dev. Dyn.* 203, 253–310
66. Arnold, M. C., Forte, J. E., Osterberg, J. S., and Di Giulio, R. T. (2016) Antioxidant rescue of selenomethionine-induced teratogenesis in zebrafish embryos. *Arch. Environ. Contam. Toxicol.* 70, 311–320
67. Varga, M., Sass, M., Papp, D., Takács-Vellai, K., Kobolak, J., Dinnyés, A., Klionsky, D. J., and Vellai, T. (2014) Autophagy is required for zebrafish caudal fin regeneration. *Cell Death Differ.* 21, 547–556
68. Whitlock, K. E., and Westerfield, M. (2000) The olfactory placodes of the zebrafish form by convergence of cellular fields at the edge of the neural plate. *Development* 127, 3645–3653
69. Higashijima, S., Hotta, Y., and Okamoto, H. (2000) Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J. Neurosci.* 20, 206–218
70. Yousefi, S., Perozzo, R., Schmid, I., Ziemiecki, A., Schaffner, T., Scapozza, L., Brunner, T., and Simon, H. U. (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat. Cell Biol.* 8, 1124–1132

**Dopaminergic neuron guardian in zebrafish-PD model**

66. Arnold, M. C., Forte, J. E., Osterberg, J. S., and Di Giulio, R. T. (2016) Antioxidant rescue of selenomethionine-induced teratogenesis in zebrafish embryos. *Arch. Environ. Contam. Toxicol.* 70, 311–320
67. Varga, M., Sass, M., Papp, D., Takács-Vellai, K., Kobolak, J., Dinnyés, A., Klionsky, D. J., and Vellai, T. (2014) Autophagy is required for zebrafish caudal fin regeneration. *Cell Death Differ.* 21, 547–556
68. Whitlock, K. E., and Westerfield, M. (2000) The olfactory placodes of the zebrafish form by convergence of cellular fields at the edge of the neural plate. *Development* 127, 3645–3653
69. Higashijima, S., Hotta, Y., and Okamoto, H. (2000) Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J. Neurosci.* 20, 206–218
70. Yousefi, S., Perozzo, R., Schmid, I., Ziemiecki, A., Schaffner, T., Scapozza, L., Brunner, T., and Simon, H. U. (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat. Cell Biol.* 8, 1124–1132