Human Molecular Genetics, 2015, Vol. 24, No. 9 2528–2538

doi: 10.1093/hmg/ddv017
Advance Access Publication Date: 22 January 2015
Original Article

ORIGINAL ARTICLE

The mitochondrial protein BNIP3L is the substrate of PARK2 and mediates mitophagy in PINK1/PARK2 pathway

Feng Gao[1,2], Dong Chen[1], Jianmin Si[1,2], Qingsong Hu[1], Zhenghong Qin[1], Ming Fang[3] and Guanghui Wang[1,*]

[1]Laboratory of Molecular Neuropathology, Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases and College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu 215021, China, [2]Laboratory of Molecular Neuropathology, Key Laboratory of Brain Function and Diseases and School of Life Sciences, University of Science & Technology of China, Chinese Academy of Sciences, Hefei, Anhui 230027, China and [3]Institute of Life Sciences, MOE Key Laboratory of Developmental Genes and Human Diseases, Southeast University, Nanjing 210096, China

*To whom correspondence should be addressed at: Laboratory of Molecular Neuropathology, Department of Pharmacology, Soochow University College of Pharmaceutical Sciences, Suzhou, Jiangsu 215123, China, or Laboratory of Molecular Neuropathology, Key Laboratory of Brain Function and Diseases and School of Life Sciences, University of Science & Technology of China, Chinese Academy of Sciences. Hefei, Anhui 230027, China. Tel: +86 512658848458; Fax: +86 51265884845; Email: wanggh@suda.edu.cn

Abstract

Mitochondrial dysfunction plays important roles in Parkinson's disease (PD) and the degradation of the damaged mitochondria by the mitochondria quality control system is important for dopaminergic (DA) neuronal survival. BNIP3L/Nix is a mitochondrial outer membrane protein that is required for the selective clearance of mitochondria. Here, we found that the mitochondrial protein BNIP3L acts downstream of the PINK1/PARK2 pathway to induce mitophagy. BNIP3L is a substrate of PARK2 to drive PARK2-mediated mitophagy. The ubiquitination of BNIP3L by PARK2 recruits NBR1 to mitochondria, thereby targeting mitochondria for degradation. BNIP3L rescues mitochondrial defects in *pink1* mutant Drosophila but not in *park* mutant Drosophila, indicating that the clearance of mitochondria induced by BNIP3L depends on the presence of PARK2. In cells intoxicated with mitochondrial complex I inhibitors rotenone, 6-OHDA or MPP^+, the disrupted mitochondria are not appropriately eliminated by mitophagy due to the improper degradation of BNIP3L. Thus, our study demonstrates that BNIP3L, as a substrate of PARK2, promotes mitophagy in the PINK1/PARK2 pathway associated with PD pathogenesis.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. The pathology of PD is characterized by a specific loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) (1–3), however, the etiology of PD is still not fully understood. Data from multiple approaches indicate that mitochondrial dysfunction may be a central cause of both sporadic and familial PD (4). The first study that implicated mitochondrial dysfunction in PD showed that DA neurons in the SNpc are deficient for mitochondrial complex I in PD patients (5–8). Moreover, 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) that inhibits the activity of mitochondrial complex I results in an acute parkinsonian syndrome in drug abusers, further suggesting that mitochondrial dysfunction is involved in PD (9).

Received: August 23, 2014. **Revised:** December 27, 2014. **Accepted:** January 19, 2015

Similarly, rotenone and 6-hydroxydopamine hydrochloride (6-OHDA), the other complex I inhibitors, also induce parkinsonian syndrome (10). Meanwhile, these inhibitors induce many deleterious biochemical changes, including an induction of oxidative stress and excitotoxicity (1,11), which further affect mitochondrial function. Those studies highlight the role of mitochondrial dysfunction in the pathogenesis of PD.

In genetic studies, mutations in several genes have been identified to be associated with familial early-onset PD. Importantly, most of their encoding proteins, such as SCNA/α-synuclein, PARK2/Parkin, DJ-1 and PINK1 are also reported to be critical for the functional regulation of mitochondria (11). Both the *pink1* (Drosophila homologue to *PINK1* gene) and *park* (Drosophila homolog to *PARK2* gene) mutant Drosophila exhibit mitochondrial dysfunction, whereas park overexpression ameliorates the mitochondrial dysfunction of pink1-mutated fly (12–16). Thus, mitochondrial dysfunction plays an important role in both environmental and genetic factors in association with PD.

The autophagic degradation of dysfunctional mitochondria (mitophagy) is a major process for the mitochondrial quality control (MQC) to maintain mitochondrial homeostasis and for cells to escape from oxidative stress and death (17). Recent studies suggest that PINK1/PARK2 play important roles in mitophagy. The stabilization of PINK1 in mitochondria and subsequently recruitment of PARK2 onto mitochondria play important roles in clearance of mitochondria. Under carbonylcyanide-3-chlorophenylhydrazone (CCCP) treatment, the recruitment of PARK2 onto mitochondria induces mitochondrial ubiquitination and promotes mitophagy (16,18–20). Ubiquitination of mitochondria is an initial process to trigger mitophagy. The ubiquitinated mitochondria can be recognized by the adaptors, subsequently to be transferred to autophagosomes (19). Thus, identification of the mitochondrial substrates in the PINK1/PARK2 pathway important for mitophagy is pivotal for understanding the pathogenesis of PD. Interestingly, in reticulocyte maturation, mitochondrial clearance requires the mitochondrial protein BNIP3L/Nix, a protein belonging to Bcl2 family, providing a clue that BNIP3L is involved in mitophagy (21,22).

In the present study, we identified BNIP3L as a substrate of PARK2 on mitochondria. BNIP3L mediates mitophagy dependent on the presence of PARK2 that ubiquitinates BNIP3L on mitochondria, resulting in a recognition of ubiquitinated mitochondria by autophagic adaptors. 1-methyl-4-phenylpyridinium (MPP^+), rotenone or 6-OHDA induces improper degradation of BNIP3L and results in a failure to eliminate the mitochondria with reduced complex I activity. Thus, our data reveal the role of BNIP3L in mitochondrial degradation pathway and highlight the role for the disrupted mitochondrial clearance in PD pathogenesis.

Results

BNIP3L acts at downstream of the PINK1/PARK2 pathway

Given that the mitochondrial protein BNIP3L has a role in the mitochondrial degradation during reticulocyte maturation process (21,22), we therefore examined whether BNIP3L plays roles in the PINK1/PARK2 pathway. We observed that BNIP3L was mainly located on mitochondria and degraded together with mitochondria after CCCP treatment in HEK293A cells (Supplementary Material, Fig. S1A and B). And in the cells over-expressing Flag-BNIP3L, the distribution of EGFP-PARK2 was not changed without CCCP treatmet (Supplementary Material, Fig. S1C and D). However, EGFP-PARK2 was recruited to mitochondria and co-localized with BNIP3L after CCCP treatment (Supplementary Material, Fig. S1C and D). Consistent with the observations that the induction of autophagy is not disrupted in *BNIP3L*$^{-/-}$ reticulocytes (22), we further observed that the turnover of LC3-I to LC3-II, that was indicative of an induction of total autophagy, was still increased in the *BNIP3L* knockdown cells after the CCCP treatment (Fig. 1A). However, the CCCP-induced mitophagy measured by two mitochondrial loading control proteins (TOMM20 and COX4I1) was inhibited by the *BNIP3L* knockdown (Fig. 1A and B). We also examined the effect of BNIP3, other BNIP3 subfamily protein, on the CCCP-induced mitophagy. We found that *BNIP3* knockdown did not block the CCCP induced mitochondrial degradation (Supplementary Material, Fig. S2A). And the BNIP3 protein level was not interfered by *BNIP3L* knockdown (Supplementary Material, Fig. S2B). Thus, in different to BNIP3L, BNIP3 may not act in the CCCP-mediated mitophagy. Moreover, an increased degradation of TOMM20 and COX4I1 was observed in HEK293A cells transfected with Flag-BNIP3L or Flag-PARK2, although there was no difference in the induction of autophagy (Fig. 1C and D), further suggesting that BNIP3L functions in PARK2-mediated mitophagy. However, the degradation of TOMM20 and COX4I1 in HeLa cells, which are deficient for PARK2 (18), was not induced by Flag-BNIP3L but was induced by Flag-PARK2 (Fig. 1E and F), although the CCCP-induced autophagy was similarly increased in both cells (Fig. 1E). Consistently, following the CCCP treatment, we observed a number of Flag-BNIP3L punctas that co-localized with EGFP-LC3 in HEK293A cells, indicating that Flag-BNIP3L promotes the transport of its associated mitochondria to the autophagosomes (Fig. 1G and H). However, Flag-BNIP3L did not co-localize with EGFP-LC3 in HeLa cells (Fig. 1G and H). These data suggest that BNIP3L-driven mitochondrial degradation is dependent on the presence of PARK2 and that BNIP3L may directly act in the PINK1/PARK2 pathway.

To explore the physiological role of BNIP3L in the PINK1/PARK2 pathway further, we performed a genetic analysis and expressed *hs-GAL4*-driven BNIP3L (Supplementary Material, Fig. S3) in either a *pink1* or *park* mutant Drosophila strain. Unlike the age-matched *pink1* mutant fly (*pink1*^{B9}) that showed fragmented cristae, the *pink1* mutant fly in which BNIP3L was overexpressed recovered the mitochondrial morphology of densely packed cristae (Fig. 2A). In contrast, BNIP3L overexpression in the *park* mutant fly (*park*^{25}) did not suppress the mitochondrial damage (Fig. 2A). These data showed that the BNIP3L-mediated suppression of the pink1 loss-of-function induced phenotypes is not the result of its general protective effect but rather results from its specific protective role against the mitochondrial dysfunction induced by the loss of pink1. Consistently, we found that Flag-BNIP3L could not induce mitophagy, indicated by TOMM20 levels, in the *PARK2* knockdown HEK293A cells, even with the CCCP treatment (Fig. 2B and C). These data indicate that BNIP3L depends on PARK2 to function in mitochondrial clearance.

BNIP3L acts as a mitochondrial substrate of PARK2

As we have shown that BNIP3L depends on PARK2 to promote mitophagy, we investigated whether BNIP3L is a substrate of PARK2. We observed that minimal amounts of PARK2 co-immunoprecipitated with BNIP3L, however, the interactions were significantly increased after PARK2 was recruited to the mitochondria after the CCCP treatment (Fig. 3A). The direct interactions were further confirmed in a GST-pulldown assay, which showed that the N-terminus of PARK2 interacted with BNIP3L (Fig. 3B). In addition, we found that BNIP3L ubiquitination was significantly increased in cells transfected with Flag-PARK2 but not with PARK2 mutants that E3 ligase activity is disrupted (Fig. 3C and Supplementary Material, Fig. S4).

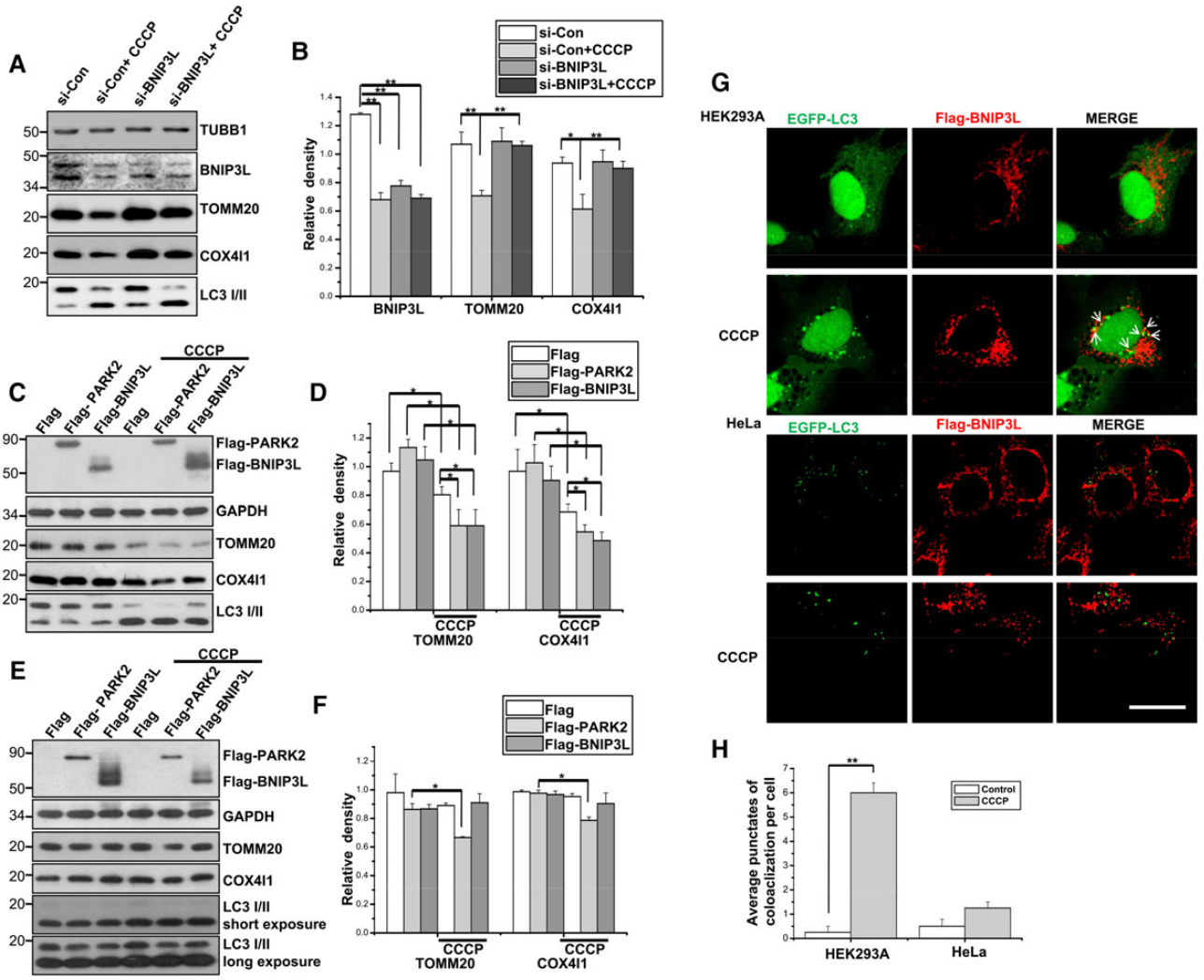

Figure 1. BNIP3L promotes PARK2-dependent mitochondrial degradation. (**A**) HEK293A cells were transfected with small interfering RNAs against *BNIP3L* (si-BNIP3L) or the negative control (si-Con) for 72 h and then treated with or without CCCP for 8 h. The total cell lysates were subjected to immunoblot analysis with the indicated antibodies. (**B**) The quantitative analysis of the relative density of BNIP3L (the lower band), COX4I1and TOMM20 compared with the loading control (TUBB1) from (A) is shown. The data are represented as the mean ± SEM. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA. Western blot extracts from HEK293A cells (**C**) or HeLa cells (**E**) that were transfected with Flag, Flag-PARK2 or Flag-BNIP3L and treated with or without CCCP (10 μM) for 12 h. The quantitative data (**D**) from (C) and (**F**) from (**E**) are shown as relative density compared with the loading control (GAPDH). The data are represented as the mean ± SEM. $n = 3$, $^{*}P < 0.05$, one-way ANOVA. (**G**) HEK293A or HeLa cell pool stably expressing Flag-BNIP3L was transfected with EGFP-LC3 and subjected to immunocytochemical staining after a treatment with or without CCCP for 6 h. Scale bar, 10 μm. (**H**) The average number, per cell, of punctas foci that colocalized with Flag-BNIP3L and EGFP-LC3 was quantified blindly in HEK293A and HeLa cells. The data are represented as the mean ± S.E.M. $n = 20$, $^{**}P < 0.01$, one-way ANOVA.

Similarly, we observed that the BNIP3L ubiquitination was further increased by CCCP (Fig. 3D), suggesting that the translocation of PARK2 to the mitochondria by CCCP enhances BNIP3L ubiquitination. These results were also obtained using immunofluorescent assays, showing a co-localization of BNIP3L, PARK2 on ubiquitin-positive punctas that were labeled with ubiquitin antibody FK2 in the cells co-transfected with BNIP3L and PARK2 after the CCCP treatment (Fig. 3E). Thus, these data suggest that the mitochondrial BNIP3L is a substrate of PARK2.

The autophagy adaptor NBR1 promotes the BNIP3L-mediated mitophagy

The selective autophagic degradation of cytoplasmic substrates with poly-ubiquitination signal is dependent on adaptor molecules, like SQSTM1 or NBR1, to target them to the autophagosomes (23,24). As BNIP3L promotes mitophagy (Fig. 2) and it is ubiquitinated by PARK2 (Fig. 3), we investigated which adaptor(s) is responsible for the BNIP3L-mediated mitophagy. SQSTM1/p62 has been suggested as an adaptor protein for PINK1/PARK2-mediated mitophagy (19). However, mitochondrial degradation is still induced by CCCP in SQSTM1$^{-/-}$ cell (25). We hypothesized that there may be an alternative adaptor involved in the BNIP3L-mediated mitophagy.

We first examined whether NBR1, another adaptor molecule for selective autophagic degradation of cytosolic substrates (23), also targets the damaged mitochondria to autophagosomes. We found that NBR1 mainly distributes in cytoplasm (Supplementary Material, Fig. S5A). In response to the CCCP treatment, NBR1 was recruited to mitochondria (Supplementary Material, Fig. S5A). In addition, we found that the CCCP-induced mitochondrial degradation was inhibited in the *NBR1* knockdown HEK293A

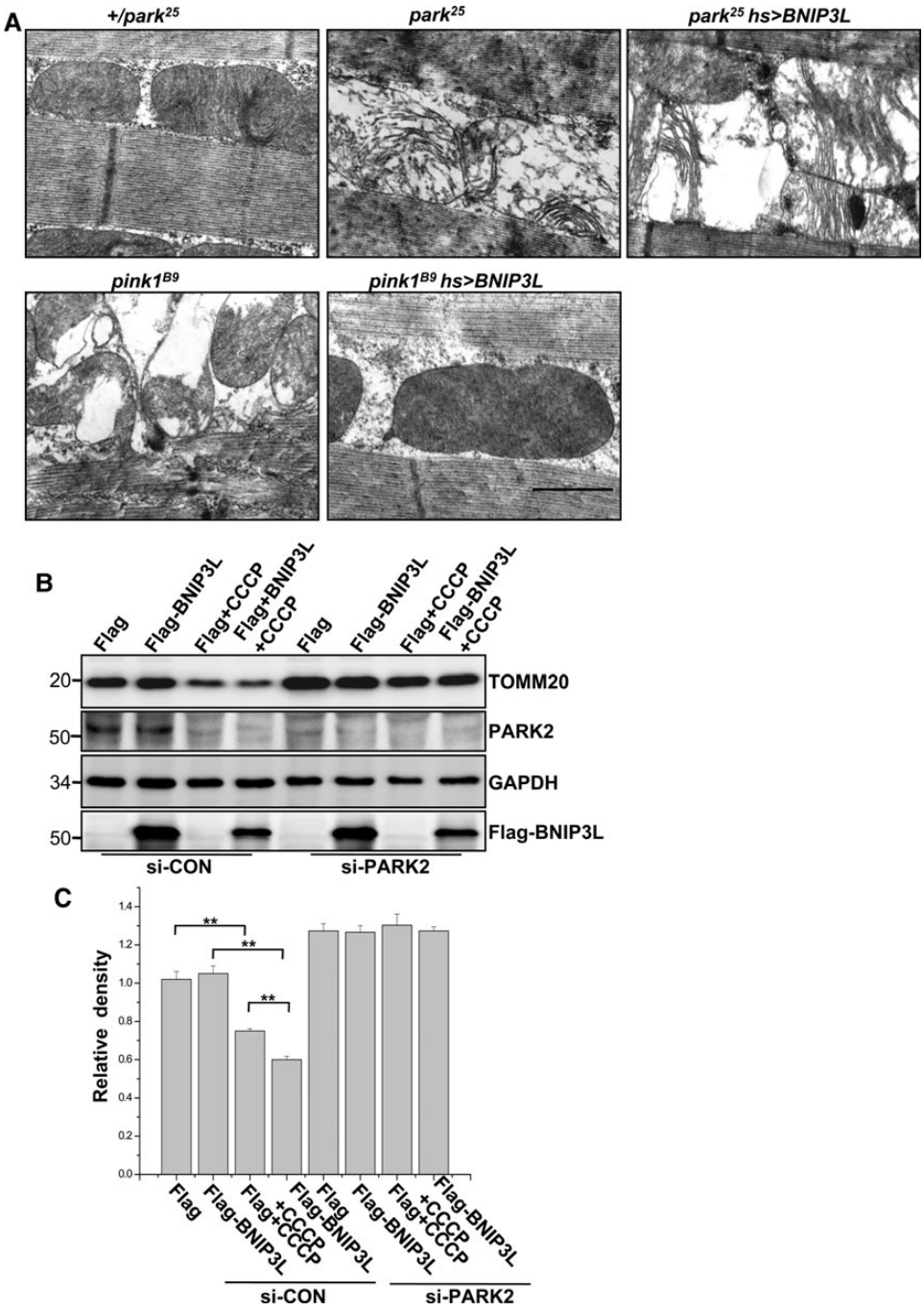

Figure 2. Genetic analysis of the role of BNIP3L in the PINK1/PARK2 pathway. (**A**) The muscle with the indicated fly genotype was examined by TEM assay. The following fly genotypes were used: *park*^{25}/+; *hs-GAL4*, *park*^{25}/*park*^{25}; *hs-GAL4*, *park*^{25}/*park*^{25}; *hs-GAL4/UAS-BNIP3L*, *pink1*^{B9}/Y; *hs-GAL4* and *pink1*^{B9}/Y; *hs-GAL4/UAS-BNIP3L*. Scale bar, 1 μm. (**B**) HEK293A cells were co-transfected with small interfering RNAs against *PARK2* (si-PARK2) or the negative control (si-Con) and EGFP or EGFP-BNIP3L. After 60 h, the cells were treated with or without CCCP for 12 h. (**C**) The quantitative data from (B) is shown as relative density of TOMM20 compared with the loading control (GAPDH). The data are represented as the mean ± S.E.M. $n = 3$, $^{**}P < 0.01$, one-way ANOVA.

cells (Supplementary Material, Fig. S5B). These results imply that NBR1 also participates in the CCCP-mediated mitophagy.

Furthermore, we tested whether HA-NBR1 D50R, a HA-NBR1 mutant that is unable to interact with SQSTM1 (26), could be recruited to mitochondria like the wild-type HA-NBR1. We found that the HA-NBR1 D50R, like the wild-type NBR1, was also properly recruited to the condensed mitochondria upon the CCCP treatment (Supplementary Material, Fig. S6A and B), suggesting that NBR1-promoted mitochondrial degradation is independent of SQSTM1. Conversely, HADC6, another adaptor protein similar to SQSTM1 or NBR1 (Supplementary Material, Fig. S7A) (23), was not recruited to the PARK2-associated mitochondria after the CCCP treatment (Supplementary Material, Fig. S7B). These data suggest that NBR1 can acts independently as an adaptor protein for the CCCP-induced mitophagy.

Then, we examined whether NBR1 is involved in the BNIP3L-mediated mitophagy. In the cells transfected with NBR1, the co-localization of BNIP3L-associated mitochondria and EGFP-LC3

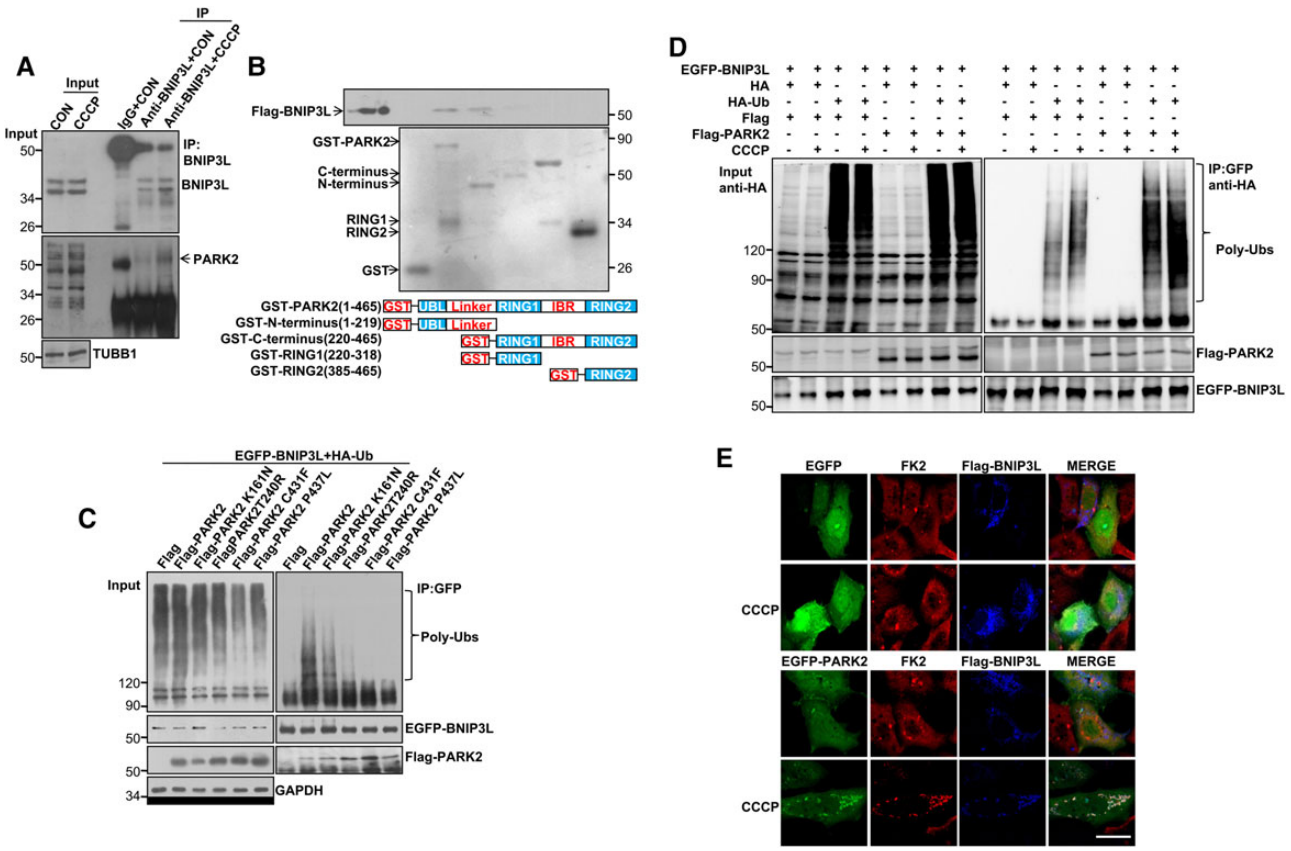

Figure 3. BNIP3L is a specific substrate of PARK2 on mitochondria. (**A**) HEK293A cells were treated with or without CCCP (10 μM) for 4 h and were subsequently lysed and immunoprecipitated using anti-BNIP3L antibodies. The immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies. (**B**) GST, GST-PARK2 or its deletion mutants were coupled to G4B and incubated with the extracts of HEK293A cells that had been transfected with Flag-BNIP3L. The bound proteins were detected with the anti-Flag antibody. (**C**) HEK293A cells were co-transfected with EGFP-BNIP3LHA-ub and Flag or Flag-PARK2 or its mutants. The total cell lysates (input) and immunoprecipitants were subjected to immunoblot analysis with the indicated antibodies. (**D**) HEK293A cells co-transfected with EGFP-BNIP3L, HA or HA-ub and Flag or Flag-PARK2. The cells were treated with DMSO or CCCP (10 μM) for 4 h. The total cell lysates (input) and immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies. (**E**) HEK293A cells were transfected Flag-BNIP3L or co-transfected EGFP-PARK2 and Flag-BNIP3L. The cells were treated with CCCP (10 μM, 6 h) and subjected to immunocytochemical staining with anti-ub (FK2) antibody and anti-Nix antibodies. Scale bar, 10 μm.

punctas was significantly increased (Fig. 4A). We further found that NBR1 targeted more BNIP3L-located mitochondria to autophagosmes after the CCCP treatment (Fig. 4A), suggesting that BNIP3L ubiquitination after PARK2 recruitment to mitochondria by the CCCP treatment can recruit NBR1 to target mitochondria to autophagosomes. These results were also observed in the HEK293A cells transfected with NBR1 D50R (Fig. 4B). The quantitative data for Figure 4A and B were shown in Figure 4C. And with an increased unbiquitination of BNIP3L in cells transfected with PARK2 followed by the CCCP treatment, more co-localization of BNIP3L-associated mitochondria and EGFP-LC3 punctas were observed (Fig. 4D and E). Moreover, the CCCP-induced translocation of NBR1 to mitochondrion was inhibited in the *BNIP3L* knockdown cells (Supplementary Material, Fig. S8A and B). Thus, these data indicate that the ubiquitiniation of BNIP3L on mitochondria by PARK2 provides a signal for NBR1 recognition for BNIP3L-mediated mitochondrial degradation.

Inhibition of mitochondria complex I activity induces BNIP3L degradation and interferes with mitophagy

The autophagic degradation of dysfunctional mitochondria is a major part of the MQC to maintain mitochondrial homeostasis (17). It is well known that mitochondrial complex I inhibition results in mitochondrial damage and plays roles in PD (7,8). We therefore examined whether BNIP3L can promote mitophagy under complex I inhibition.

In contrast to the CCCP treatment that induces PINK1 stabilization that was indicated by the appearance of full length of PINK1 (Fig. 5A, lane 2, upper band), a treatment of mitochondrial complex I inhibitors did not induce a stabilization of PINK1 (Fig. 5A) and a translocation of PARK2 to mitochondria (Supplementary Material, Fig. S9) in SH-SY5Y cells. In SH-SY5Y cells, the CCCP treatment induced mitochondrial degradation indicated by the mitochondrial protein TOMM20, TOMM70 and COX4I1 (Fig. 5B). In contrast, we did not observe the mitochondrial degradation in the cells treated with rotenone, MPP^+ or 6-OHDA (Fig. 5B and C). To further verify this result, we further treated the HEK293A and SH-SY5Y cells with rotenone for 12, 36 or 48 h. As most cells shrank and died 48 h after the rotenone treatment, we could not treat the cells with rotenone more than 48 h. The mitochondrial degradation was not detected even 48 h after treatment (Supplementary Material, Fig. S10A and B). These data suggest a failure of degradation of the mitochondria that are intoxicated with complex I inhibitors.

In the mice midbrain that was treated with MPTP, the BNIP3L, along with tyrosine hydroxylase (TH) was decreased (Fig. 5D and E and Supplementary Material, Fig. S11). Meanwhile, the COX4I1

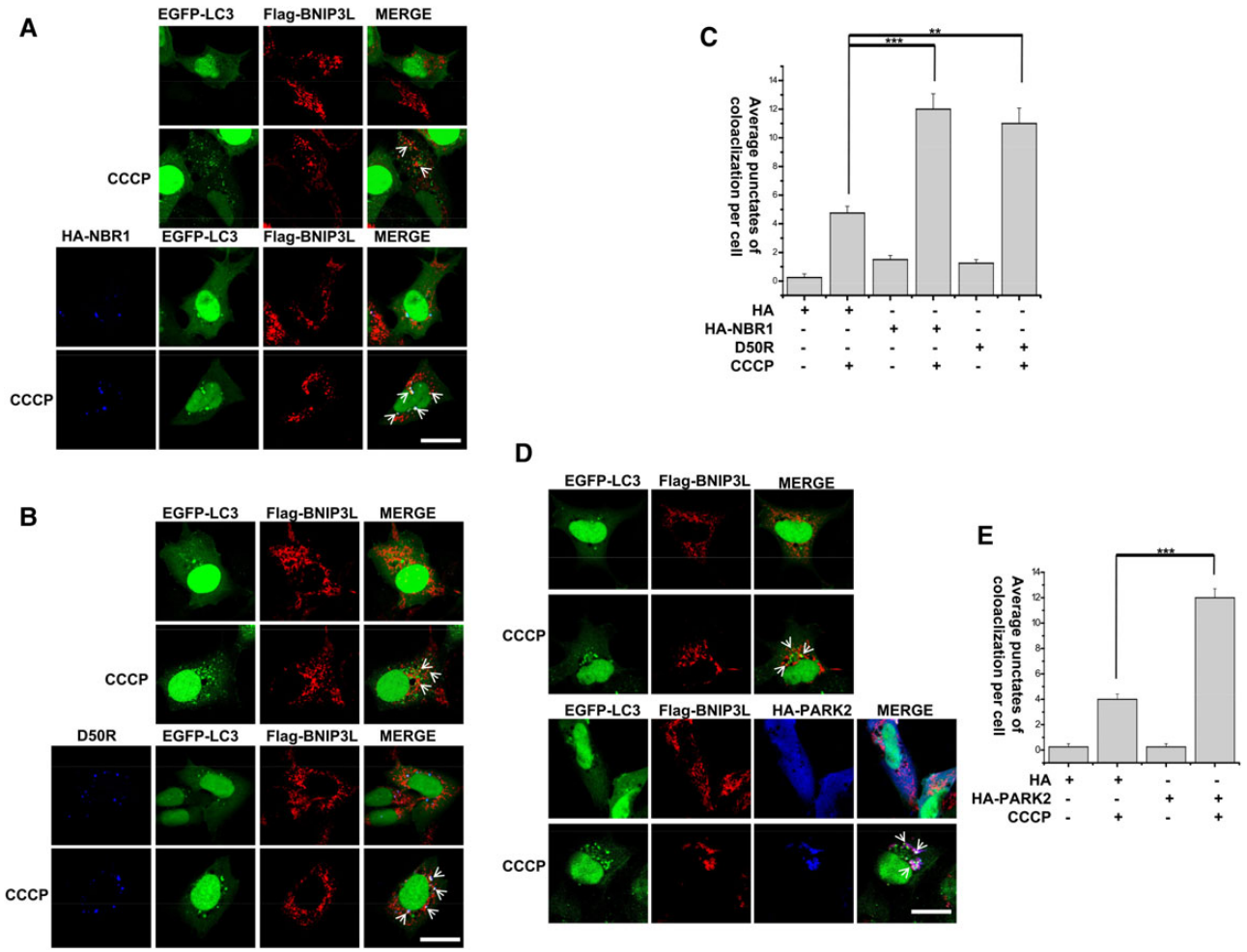

Figure 4. BNIP3L recruits the autophagy adaptor, NBR1, to promote mitophagy. (**A**) HEK293A cells were co-transfected with EGFP-LC3, Flag-BNIP3L and HA or HA-NBR1. HEK293A cells treated with CCCP (10 μM) for 6 h were immunoncytochemically stained with anti-HA antibodies (blue) or anti-Flag antibody (red). (**B**) HEK293A cells were co-transfected with EGFP-LC3, Flag-BNIP3L and HA or HA-NBR1 D50R. HEK293A cells treated with CCCP (10 μM) for 6 h were immunoncytochemically stained with anti-HA antibodies (blue) or anti-Flag antibody (red). (**C**) The average number, per cell, of punctas foci that co-localized with Flag-BNIP3L and EGFP-LC3 was quantified (blindly) in HEK293A that transfected with HA-NBR1 or HA-NBR1 D50R. The data are represented as the mean ± SEM. $n = 15$, $^{**}P < 0.01$, one-way ANOVA. (**D**) HEK293A cell pool stably expressing Flag-BNIP3L was co-transfected with EGFP-LC3 and HA or HA-PARK2. The cells treated with CCCP (10 μM) for 6 h were immunoncytochemically stained with anti-HA antibodies (blue) or anti-Flag antibody (red). Scale bar, 10 μm. (**E**) The average number, per cell, of punctas foci that colocalized with Flag-BNIP3L and EGFP-LC3 was quantified (blindly) in HEK293A that transfected with HA or HA-PARK2. The data are represented as the mean ± SEM. $n = 15$, $^{***}P < 0.001$, one-way ANOVA.

level was not decreased (Fig. 5D), suggesting that the mitochondrial complex I inhibitor MPTP do not induce mitophagy in mice. Moreover, in the SH-SY5Y cells that were pretreated with rotenone, MPP^+ or 6-OHDA, the BNIP3L level was also decreased, although the other mitochondrial proteins were not degraded (Fig. 5F and Supplementary Material, Fig. S12). Thus, the mitochondrial complex I inhibition-induced BNIP3L degradation may not through the mitophagy pathway.

It was reported that the CCCP-induced mitophagy can be blocked by the lysosomal inhibitor, bafilomycin A1 or the proteasomal inhibitor, MG132 (27). Although 12 h after CCCP treatment, the PARK2, BNIP3L and TOMM20 were degraded (Supplementary Material, Fig. S13), which suggests an occurrence of mitophagy, the degradation of mitochondrial proteins BNIP3L and COX4I1 was inhibited if cells were treated with CCCP along with bafilomycin A1 and MG132 (Supplementary Material, Fig. S14A), suggesting CCCP induces a lysosomal degradation of BNIP3L under mitophagy process. However, the rotenone-induced BNIP3L degradation could not be inhibited by bafilomycin A1 or MG132 (Supplementary Material, Fig. S14B), suggesting that mitochondrial complex I inhibitor-induced BNIP3L degradation was not via mitophagy or the proteasome. And the CCCP-induced mitochondrial degradation were also inhibited if the cells were pretreated with the mitochondrial complex I inhibitors (Fig. 5F and Supplementary Material, Fig. S15). Taken together, these data further support that BNIP3L fails to promote the clearance of the damaged mitochondria due to its improper degradation under mitochondrial complex I inhibition. (Fig. 6).

Discussion

Mitochondrial dysfunction is associated with the pathogenesis of PD (1). In autophagic degradation system, ubiquitination of the substrates provides a signal recognized by autophagic adaptors (23). As PARK2 is an E3 ligase and it ubiquitinates multiple substrates, its mitochondrial substrate is important for mitochondrial ubiquitination, a process critical for mitophagy (28,29). It has been shown that PARK2 mediates ubiquitination of some

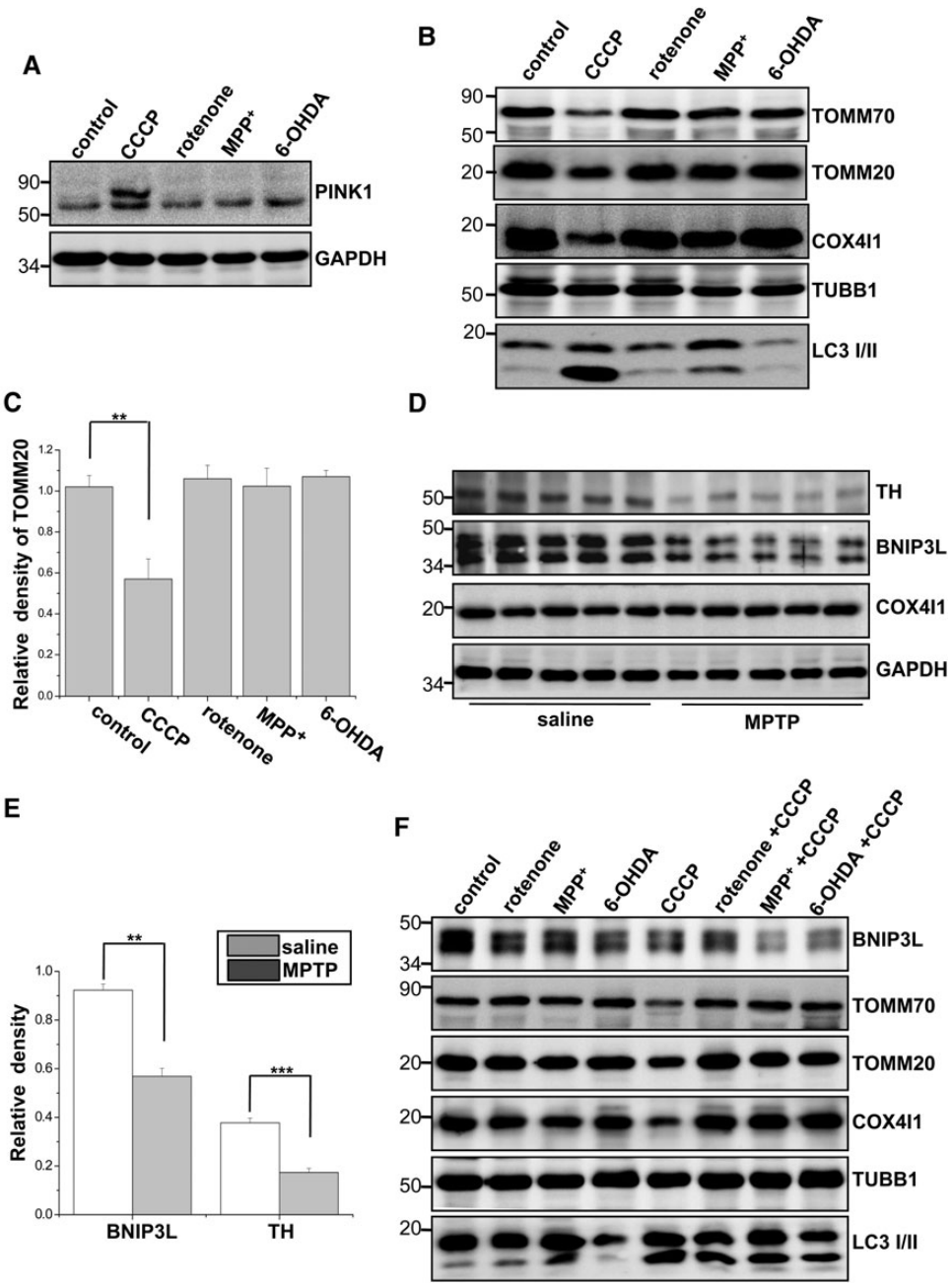

Figure 5. Mitochondria complex I inhibition induces an improper degradation of BNIP3L. (**A**) SH-SY5Y cells were treated with CCCP (5 μM), rotenone (1 μM), MPP^+ (0.5 mM) or 6-OHDA (20 μM) for 6 h. Then the cells were lysed for immunoblot assay. (**B**) SH-SY5Y cells were treated with CCCP (10 μM), rotenone (1 μM), 6-OHDA (20 μM) or MPP^+ (0.5 mM) for 12 h. The cells were lysed, and the lysates were subjected to immunoblot analysis with the indicated antibodies. (**C**) The quantitative data from (B) is shown as relative density of TOMM20 compared with the TUBB1. The data are represented as the mean ± SEM. $n = 3$, $^{**}P < 0.01$, one-way ANOVA. (**D**) Mice (C57BL/6J) were injected s.c. with saline or MPTP (18 mg/kg body weight) once daily for 5 consecutive days. The midbrain were isolated and lysed for immmunoblot analysis. (**E**) The quantitative data from (D) is shown as relative density of TH (tyrosine hydroxylase) and BNIP3L (the down band) compared with the loading control (TUBB1). The data are represented as the mean ± S.E.M. $n = 8$, $^{**}P < 0.01$,$^{***}P < 0.01$, one-way ANOVA. (**F**) SH-SY5Y cells were treated with rotenone (1 μM), MPP^+ (0.5 mM) or 6-OHDA (20 μM) for 12 h. Then the cells were treated with CCCP (5 μM) for another 12 h. Then the cells were lysed for immunoblot analysis.

mitochondrial proteins. Most of them are associated with mitochondrial fusion–fission cycle, such as mitofusin 1/2 (Mfn1/2), dynamin-related protein 1 (Drp1), Miro and voltage-dependent anion channel 1 (VDAC1) (19,27,30–36). Mfn1 and Mfn2 are important proteins involved in mitochondrial fusion–fission cycle. As the substrates of PARK2, the degradation of Mfn1 and Mfn2 are driven by PARK2, to prevent the damaged mitochondria to be fused to healthy mitochondria, and to promote mitochondrial fission at mitophagy initiation step (30,31). Recently, Mfn2 was identified as a PARK2 receptor to mediate PARK2 recruitment to damaged mitochondria (35), but as a receptor, its mediated substrates are still unclear. Miro is also a mitochondrial out membrane protein for axonal transport of mitochondria and identified as a mitochondrial substrate of PARK2 for the proteasome degradation (34). The Miro degradation prevents mitochondrial movement and may initiate mitophagy

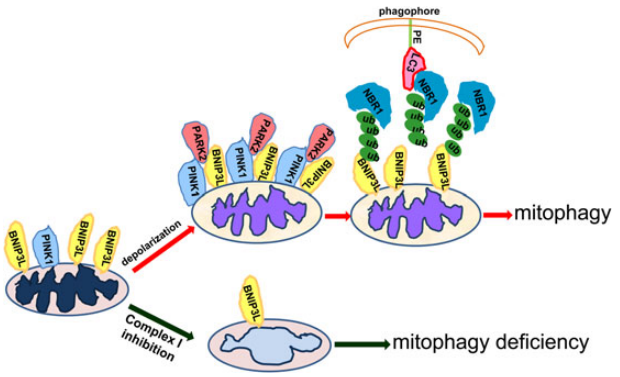

Figure 6. Proposed pathway in mitochondrial complex I inhibition-induced PD. Under the CCCP treatment, PARK2 is recruited to mitochondria, then ubiquitinates the mitochondrial protein BNIP3L. The ubiquitination of BNIP3L provides an ubiquitinated signal on mitochondria, which is recognized by autophagic adaptor, thereby inducing mitophagy. BNIP3L is degraded together with mitochondrion as other mitochondrial protein when mitophagy occurs. However, the mitochondrial complex I inhibitors induce an improper degradation of BNIP3L on mitochondria, leading to a failure of mitophagy initiation and an accumulation of damaged mitochondria.

induction. VDAC1 is a PARK2 substrate that may be recognized by p62 to mediate mitophagy, and it was also reported that the major role of VDAC1 in mitophagy is to mediate PARK2 recruitment onto mitochondria (25,37).

BNIP3L is a mitochondrial protein important for a selective autophagic degradation of mitochondria during reticulocytes maturation, and *BNIP3L*$^{-/-}$ mice exhibits mitochondrial retention in their reticulocytes (18,21,22). Using genetic assays in Drosophila, we found that overexpression of BNIP3L can rescue the phenotype of mitochondrial dysfunction in *pink1* mutant fly, but not in *park* mutant fly. In cultured cells, BNIP3L induces mitophagy in *PARK2* wild-type cells but not in *PARK2*-deficient HeLa cells. Importantly, the direct interactions between PARK2 and BNIP3L and the enhancement of BNIP3L ubiquitiniation by PARK2 were observed. Moreover, the interactions between BNIP3L and PARK2, and the ubiquitination of BNIP3L are significantly increased when PARK2 is translocated to mitochondria, suggesting that BNIP3L is a substrate of PARK2 on mitochondria. These findings are of help to understand the mitochondrial phenotype rescued in Drosophila in genetic assays. As PARK2 acts downstream of PINK1 and overexpression of PARK2 rescues the phenotype of *pink1* mutant fly, it suggests that some other factors may also affect PARK2 recruitment to damaged mitochondria *in vivo* but the unknown factors are not effective as PINK1 so that the phenotype of *pink1* mutant fly is only rescued with the presence of extensive PARK2. In our present study, we found that BNIP3L is a substrate of PARK2 and its function definitely depends on PAKR2. Thus, overexpression of BNIP3L rescues the phenotype of *pink1* mutant fly because of the presence of PARK2 but overexpression of BNIP3L fails to rescue the phenotype in the absence of PARK2. These findings provide evidence that BNIP3L is a downstream factor of the PINK1/PARK2 pathway and that BNIP3L strictly depends on PARK2 to induce mitophagy.

The ubiquitination of mitochondria is an important signal for autophagic adaptors to mediate mitophagy. NBR1 is an autophagic adaptor protein that targets cytosolic substrates to autophagosome (26). In our present study, we found that a co-localization of BNIP3L, PARK2, NBR1 on LC3 punctas, along with mitochondria, suggesting that NBR1 is an adaptor for the PARK2/BNIP3L-mediated mitophagy. It was reported that the autophagic protein, SQSTM1, can target the mitochondria to autophagosomes. Interestingly, the mitochondria are still degraded in SQSTM1-deficient cells (25). In our observation, NBR1 D50R mutant that does not interact with SQSTM1 is still recruited to the condensed mitochondria. Taken together, these findings suggest that NBR1 functions independently in mitophagy and can compensate for SQSTM1 in targeting the mitochondria to autophagosomes for degradation. Thus, ubiquitination of BNIP3L by PARK2 may provide a signal on mitochondria for the recognition by the autophagic adaptor NBR1, leading to the occurrence of mitophagy.

Although DA neurons possess an intact PINK1/PARK2/BNIP3L pathway to cope with the disrupted mitochondria in most sporadic PD patients, increased levels of disrupted mitochondria with reduced complex I activity have been detected in PD brains (4,38). Rotenone and MPTP that inhibit complex I activity are causative factors for PD. In the present study, we observed that cells with reduced mitochondrial complex I activity induced by rotenone, MPP^+ or 6-OHDA present a significant degradation of BNIP3L and that the BNIP3L-mediated mitochondrial degradation pathway is disrupted, thereby resulting in a retention of the damaged mitochondria. Interestingly, BNIP3L is degraded after the usage of mitochondrial complex I inhibitors, which will not be blocked by both lysosomal and protease inhibitors. As the lysosomal inhibitor blocks mitophagy; and the proteasomal inhibitor blocks the proteasomal system as well as mitophagy because of an inhibition of mitofusin I and II degradation, a process necessary for the initiation of mitophagy (27), the degradation of BNIP3L is unlikely caused by the proteasome or mitophagy. It is highly possible that it is processed by unknown proteases that are activated under mitochondrial complex I inhibitor treatment. Together with previous findings by other investigators, showing that PINK1 or PARK2 mutants interfere with mitophagy (16,19), our study suggests that the degradation of BNIP3L caused by complex I inhibition factors results in BNIP3L-inability to clear the damaged mitochondria. Thus, our findings also provide a mechanistic explanation why the existed PINK1/PARK2 pathway fails to clear the damaged mitochondria caused by complex I inhibitors in PD.

In summary, our study identified that BNIP3L is a substrate of PARK2 on mitochondria. The BNIP3L ubiquitination induced by mitochondria-located PARK2 recruits the NBR1 to mitochondria to target the mitochondria for degradation. However, the environmental toxins that induce BNIP3L degradation can disrupt the PINK1/PARK2/BNIP3L-mediated mitophagy and cause an accumulation of damaged mitochondria, leading to the injury of DA neurons and the occurrence of disease.

Material and Methods

Animals and treatment

Eight-week-old male C57/bl6 mice (Model Animal Research Center of Nanjing University) were studied. The mice received ($n = 4$–7 each group, four groups) subcutaneous injections of MPTP-HCl (20 mg/kg of free base; Sigma, St Louis, MO, USA) or vehicle (saline) as the control for five consecutive days (39). Five days after the final MPTP-HCl administration, the mice were sacrificed and the midbrains were isolated and lysed for immunoblot assay. All of the mice experiments were performed according to the protocols approved by the Animal Welfare Committee of University of Science and Technology of China.

Fly stocks and procedures

park^{25} mutants were kindly provided by Dr Leo Pallanck (The University of Washington, USA) (40). *pink1*^{B9} mutants were provided

by Dr Alexander J Whitworth (University of Sheffield, UK) (41). The *hs-GAL4* line was obtained from the Bloomington Stock Center. The *BNIP3L* transgenic fly was generated by excising the full-length *BNIP3L* cDNA from EGFP-BNIP3L and inserting the segment into the pUAST vector at the *BglII*/*KpnI* sites and subsequently introducing the construct into the Drosophila germ line. UAS-BNIP3L was driven by *hs-GAL4* in the $park^{25}$ or $pink1^{B9}$ mutant background.

For fly muscle transmission electronic microscope (TEM) assay, 3-day-old male fly thoraces were fixed in 0.1 M cacodylate buffer with 4% glutaraldehyde overnight at 4°C and subsequently in 0.1 M Na-phosphate buffer (pH 7.4) containing 2% glutaraldehyde. The samples were then treated with osmium tetroxide, dehydrated and embedded in Epon. A minimum of three strains of each genotype were observed using TEM (JEOL-1230, Tokyo, Japan).

Plasmids

The EGFP, EGFP-PARK2, Flag, Flag-PARK2, Flag-PARK2 K161N, Flag-PARK2 T240R, Flag-PARK2 C431F, Flag-PARK2 P437L, GST-PARK2, GST-PARK2 C-terminus, GST-PARK2 N-terminus, GST-PARK2 Ring1, GST-PARK2 Ring2, Flag-SQSTM1, Flag-HDAC6, Desred-mito, HA-ub, Flag-ub and EGFP-LC3 constructs were described previously (42–44). The full-length human BNIP3L cDNA was amplified from a human embryo cDNA library by PCR with the primers, 5′-cggaattcatgtcgtcccacctagtcgag-3′ and 5′-acgcgtcgactcagtaggtgctggcaga-3′, and then inserted into pEGFP-C2 at the *EcoRI*/*PstI* sites. Flag-BNIP3L was created by subcloning the PCR product that was amplified with the primers, 5′-cccaagcttaccatgtcgtcccacctagtc-3′ and 5′-acgcgtcgactcagtaggtgctggcaga-3′, from EGFP-BNIP3L into p3xFLAG-myc-CMV-24 vector at the *HindIII*/*SalI* sites. HA-NBR1 and HA-NBR1 D50R were kindly provided by Dr Ivan Dikic (26) (Goethe University, Germany).

Cell culture and transfection

The HeLa and HEK293A cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Los Angeles, CA, USA) containing 10% fetal calf serum (Gibco, Los Angeles, CA, USA). SH-SY5Y cells were cultured in DMEM/F12 medium with 10% fetal calf serum. The HeLa and HEK293A cell pool stably expressing Flag-BNIP3L were constructed by the G418 (Invitrogen, Carlsbad, CA, USA) selection system. In brief, the cells transfected with Flag-BNIP3L were treated with G418 (200 μg/ml) for 1 week. The survival cells were cultured in new medium for other experiments with G418. The cells were transfected with the indicated plasmids using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. CCCP, 6-OHDA, rotenone and MPP^+ were purchased from Sigma. For the RNA interference experiments, the cells were transfected with oligonucleotides that were synthesized by Shanghai GenePharma (GenePharma, Shanghai, China) using Lipofectamine RNAimax (Invitrogen) according to the manufacturer's instructions. The siRNA sequences for *BNIP3L* are AACAGUUCCUGGGUGGAGCUATT, GCAUCUAUAUUGGAAAGCGTT, GCAGCAAUGGCAAUGAUAATT, GGAUGCACAACAUGAAUCATT; for *BNIP3*: CCCAAGGAGUUCCUCUUUATT, GGCAUAUUCUCUGCAGAAUTT, CAGCCUCGGUUUCUAUUUATT; for *NBR1*: GUCCCUCAGAAGUCUCAAUTT; and the si-RNA sequences for *PARK2* were described previously (42).

Immunoprecipitation, *in vitro* binding assays and immunoblotting

The immunoprecipitation, *in vitro* binding assays and immunoblot analyses were carried out as described previously (42). The following antibodies were used for immunoblotting: polyclonal anti-EGFP antibodies (45) and polyclonal anti-BNIP3L antibodies (Enzo Life Science, Plymouth Meeting, PA, USA). The immunoblotting assay was carried out with the following primary antibodies: monoclonal anti-EGFP antibody (Santa Cruz, Santa Cruz, CA, USA); monoclonal anti-Flag antibody (Sigma); monoclonal anti-GAPDH antibody and polyclonal anti-TH antibodies (Millipore, Billerica, MA, USA); and monoclonal anti-HA antibody (Santa Cruz); monoclonal anti-TOMM20 antibody (Santa Cruz); monoclonal anti-TUBB1 antibody (Milipore); polyclonal anti-COX4I1 antibodies and monoclonal anti-PARK2 antibody (Cell Signal, Beverly, MA, USA); polyclonal anti-TOMM70 antibodies and polyclonal anti-LC3 antibodies (Novas, Littleton, CO, USA).

Immunocytochemistry

Cells cultured on cover slides were fixed with 4% paraformaldehyde for 5 min at room temperature and permeabilized in 0.5% Triton X-100/PBS for an additional 5 min. After they were blocked with 3% FBS in PBST, the cells were incubated with primary polyclonal antibodies, anti-Flag (Sigma) or rabbit polyclonal antibodies against HA (Santa Cruz), followed by an incubation with FITC- or Rho-conjugated donkey anti-mouse or -rabbit secondary antibodies (Santa Cruz) or Alexa Fluor-350-labeled goat anti-mouse or -rabbit IgG (Invitrogen). Lastly, the cells were observed using an inverted IX71 microscope system (Olympus, Japan) or Zeiss LSM710 confocal microscope (Germany).

Statistical analysis

Statistical comparison between groups and treatments was performed using one-way analysis of variance (ANOVA). Student's *t*-tests were used when comparing two groups. A *P*-value of <0.05 was considered statistically significant. Data are presented as the mean ± SEM.

Supplementary Material

Supplementary Material is available at *HMG* online.

Acknowledgments

We would like to thank Drs Leo Pallanck and Alexander J. Whitworth for fly stocks and Dr Ivan Dikic for HA-NBR1 plasmids.

Conflict of Interest statement. None declared.

Funding

This work was supported in part by the National Natural Sciences Foundation of China (Nos 31330030 and 81371393), the National High-tech Research and Development program of China 973-projects (2011CB504102), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases (BM2013003) and the Natural Science Foundation of Jiangsu Province (BK20140328).

References

1. Abou-Sleiman, P.M., Muqit, M.M. and Wood, N.W. (2006) Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nat. Rev. Neurosci.*, **7**, 207–219.

2. Banerjee, R., Starkov, A.A., Beal, M.F. and Thomas, B. (2009) Mitochondrial dysfunction in the limelight of Parkinson's disease pathogenesis. *Biochim. Biophys. Acta.*, **1792**, 651–663.
3. Bossy-Wetzel, E., Schwarzenbacher, R. and Lipton, S.A. (2004) Molecular pathways to neurodegeneration. *Nat. Med.*, **10** (suppl.), S2–S9.
4. Dawson, T.M. and Dawson, V.L. (2003) Molecular pathways of neurodegeneration in Parkinson's disease. *Science*, **302**, 819–822.
5. Bindoff, L.A., Birch-Machin, M., Cartlidge, N.E., Parker, W.D. Jr. and Turnbull, D.M. (1989) Mitochondrial function in Parkinson's disease. *Lancet*, **2**, 49.
6. Parker, W.D. Jr., Boyson, S.J. and Parks, J.K. (1989) Abnormalities of the electron transport chain in idiopathic Parkinson's disease. *Ann. Neurol.*, **26**, 719–723.
7. Mann, V.M., Cooper, J.M., Daniel, S.E., Srai, K., Jenner, P., Marsden, C.D. and Schapira, A.H. (1994) Complex I, iron, and ferritin in Parkinson's disease substantia nigra. *Ann. Neurol.*, **36**, 876–881.
8. Schapira, A.H., Cooper, J.M., Dexter, D., Jenner, P., Clark, J.B. and Marsden, C.D. (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet*, **1**, 1269.
9. Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*, **219**, 979–980.
10. Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V. and Greenamyre, J.T. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.*, **3**, 1301–1306.
11. Schapira, A.H. (2008) Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol.*, **7**, 97–109.
12. Park, J., Lee, S.B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J.M. *et al.* (2006) Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature*, **441**, 1157–1161.
13. Exner, N., Treske, B., Paquet, D., Holmstrom, K., Schiesling, C., Gispert, S., Carballo-Carbajal, I., Berg, D., Hoepken, H.H., Gasser, T. *et al.* (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. *J. Neurosci.*, **27**, 12413–12418.
14. Clark, I.E., Dodson, M.W., Jiang, C., Cao, J.H., Huh, J.R., Seol, J. H., Yoo, S.J., Hay, B.A. and Guo, M. (2006) Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature*, **441**, 1162–1166.
15. Yang, Y., Gehrke, S., Imai, Y., Huang, Z., Ouyang, Y., Wang, J. W., Yang, L., Beal, M.F., Vogel, H. and Lu, B. (2006) Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. *Proc. Natl Acad. Sci. USA*, **103**, 10793–10798.
16. Narendra, D.P., Jin, S.M., Tanaka, A., Suen, D.F., Gautier, C.A., Shen, J., Cookson, M.R. and Youle, R.J. (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.*, **8**, e1000298.
17. Tatsuta, T. and Langer, T. (2008) Quality control of mitochondria: protection against neurodegeneration and ageing. *EMBO J.*, **27**, 306–314.
18. Narendra, D., Tanaka, A., Suen, D.F. and Youle, R.J. (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J. Cell Biol.*, **183**, 795–803.
19. Geisler, S., Holmstrom, K.M., Skujat, D., Fiesel, F.C., Rothfuss, O.C., Kahle, P.J. and Springer, W. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat. Cell Biol.*, **12**, 119–131.
20. Okatsu, K., Oka, T., Iguchi, M., Imamura, K., Kosako, H., Tani, N., Kimura, M., Go, E., Koyano, F., Funayama, M. *et al.* (2012) PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. *Nat. Commun.*, **3**, 1016.
21. Sandoval, H., Thiagarajan, P., Dasgupta, S.K., Schumacher, A., Prchal, J.T., Chen, M. and Wang, J. (2008) Essential role for Nix in autophagic maturation of erythroid cells. *Nature*, **454**, 232–235.
22. Schweers, R.L., Zhang, J., Randall, M.S., Loyd, M.R., Li, W., Dorsey, F.C., Kundu, M., Opferman, J.T., Cleveland, J.L., Miller, J.L. *et al.* (2007) NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc. Natl Acad. Sci. USA*, **104**, 19500–19505.
23. Kirkin, V., McEwan, D.G., Novak, I. and Dikic, I. (2009) A role for ubiquitin in selective autophagy. *Mol. Cell*, **34**, 259–269.
24. Matsumoto, G., Wada, K., Okuno, M., Kurosawa, M. and Nukina, N. (2011) Serine 403 Phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Mol. Cell*, **44**, 279–289.
25. Narendra, D., Kane, L.A., Hauser, D.N., Fearnley, I.M. and Youle, R.J. (2010) p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. *Autophagy*, **6**, 1090–1106.
26. Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P. *et al.* (2009) A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol. Cell*, **33**, 505–516.
27. Tanaka, A., Cleland, M.M., Xu, S., Narendra, D.P., Suen, D.F., Karbowski, M. and Youle, R.J. (2010) Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J. Cell Biol.*, **191**, 1367–1380.
28. Yang, Y., Nishimura, I., Imai, Y., Takahashi, R. and Lu, B. (2003) Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. *Neuron*, **37**, 911–924.
29. Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L. and Dawson, T.M. (2001) Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat. Med.*, **7**, 1144–1150.
30. Gegg, M.E., Cooper, J.M., Chau, K.Y., Rojo, M., Schapira, A.H. and Taanman, J.W. (2010) Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. *Hum. Mol. Genet.*, **19**, 4861–4870.
31. Ziviani, E., Tao, R.N. and Whitworth, A.J. (2010) Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc. Natl Acad. Sci. USA*, **107**, 5018–5023.
32. Sarraf, S.A., Raman, M., Guarani-Pereira, V., Sowa, M.E., Huttlin, E.L., Gygi, S.P. and Harper, J.W. (2013) Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature*, **496**, 372–376.
33. Chan, N.C., Salazar, A.M., Pham, A.H., Sweredoski, M.J., Kolawa, N.J., Graham, R.L., Hess, S. and Chan, D.C. (2011) Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Hum. Mol. Genet.*, **20**, 1726–1737.
34. Wang, X., Winter, D., Ashrafi, G., Schlehe, J., Wong, Y.L., Selkoe, D., Rice, S., Steen, J., LaVoie, M.J. and Schwarz, T.L. (2011) PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell*, **147**, 893–906.
35. Chen, Y. and Dorn, G.W. II. (2013) PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. *Science*, **340**, 471–475.

36. Wang, H., Song, P., Du, L., Tian, W., Yue, W., Liu, M., Li, D., Wang, B., Zhu, Y., Cao, C. *et al.* (2011) Parkin ubiquitinates Drp1 for proteasome-dependent degradation: implication of dysregulated mitochondrial dynamics in Parkinson disease. *J. Biol. Chem.*, **286**, 11649–11658.
37. Sun, Y., Vashisht, A.A., Tchieu, J., Wohlschlegel, J.A. and Dreier, L. (2012) Voltage-dependent anion channels (VDACs) recruit Parkin to defective mitochondria to promote mitochondrial autophagy. *J. Biol. Chem.*, **287**, 40652–40660.
38. Navarro, A., Boveris, A., Bandez, M.J., Sanchez-Pino, M.J., Gomez, C., Muntane, G. and Ferrer, I. (2009) Human brain cortex: mitochondrial oxidative damage and adaptive response in Parkinson disease and in dementia with Lewy bodies. *Free. Radic. Biol. Med.*, **46**, 1574–1580.
39. Jackson-Lewis, V. and Przedborski, S. (2007) Protocol for the MPTP mouse model of Parkinson's disease. *Nat. Protoc.*, **2**, 141–151.
40. Greene, J.C., Whitworth, A.J., Kuo, I., Andrews, L.A., Feany, M. B. and Pallanck, L.J. (2003) Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. *Proc. Natl Acad. Sci. USA*, **100**, 4078–4083.
41. Tain, L.S., Mortiboys, H., Tao, R.N., Ziviani, E., Bandmann, O. and Whitworth, A.J. (2009) Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. *Nat. Neurosci.*, **12**, 1129–1135.
42. Chen, D., Gao, F., Li, B., Wang, H., Xu, Y., Zhu, C. and Wang, G. (2010) Parkin mono-ubiquitinates Bcl-2 and regulates autophagy. *J. Biol. Chem.*, **285**, 38214–38223.
43. Li, B., Hu, Q., Wang, H., Man, N., Ren, H., Wen, L., Nukina, N., Fei, E. and Wang, G. (2010) Omi/HtrA2 is a positive regulator of autophagy that facilitates the degradation of mutant proteins involved in neurodegenerative diseases. *Cell Death Differ.*, **17**, 1773–1784.
44. Liu, C., Fei, E., Jia, N., Wang, H., Tao, R., Iwata, A., Nukina, N., Zhou, J. and Wang, G. (2007) Assembly of lysine 63-linked ubiquitin conjugates by phosphorylated alpha-synuclein implies Lewy body biogenesis. *J. Biol. Chem.*, **282**, 14558–14566.
45. Ying, Z., Wang, H., Fan, H., Zhu, X., Zhou, J., Fei, E. and Wang, G. (2009) Gp78, an ER associated E3, promotes SOD1 and ataxin-3 degradation. *Hum. Mol. Genet.*, **18**, 4268–4281.