Chronic treatment with the complex I inhibitor MPP+ depletes endogenous PTEN-induced kinase 1 (PINK1) via upregulation of Bcl-2-associated athanogene 6 (BAG6)

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Abstract:

Mitochondrial dysfunction is implicated in sporadic and familial Parkinson’s disease (PD). However, the mechanisms that impair homeostatic responses to mitochondrial dysfunction remain unclear. Previously, we found that chronic, low dose administration of the mitochondrial complex I inhibitor 1-methyl-4-phenylpyridinium (MPP+) dysregulates mitochondrial fission-fusion, mitophagy and mitochondrial biogenesis. Given that PTEN-induced kinase 1 (PINK1) regulates mitochondrial function, dynamics and turnover, we hypothesized that alterations in endogenous PINK1 levels contribute to depletion of mitochondria during chronic complex I injury. Here we found that chronic MPP+ treatment of differentiated SH-SY5Y neuronal cells significantly decreases PINK1 expression prior to reductions in other mitochondrial components. Furthermore, Bcl2 associated athanogene 6 (BAG6, BAT3, or Scythe), a protein involved in protein quality control and degradation, was highly up-regulated during the chronic MPP+ treatment. BAG6 interacted with PINK1, and BAG6 overexpression decreased the half-life of PINK1. Conversely, siRNA-mediated BAG6 knockdown prevented chronic MPP+ stress-induced loss of PINK1, reversed MPP+-provoked mitochondrial changes, increased cell viability and prevented MPP+-induced dendrite shrinkage in primary neurons. These results indicate that BAG6 up-regulation during chronic complex I inhibition contributes to mitochondrial pathology by decreasing the levels of endogenous PINK1. Given that recessive mutations in PINK1 cause familial Parkinson’s disease, the findings suggest that BAG6 could serve as a potential therapeutic target for the treatment of PD.
PD, the finding of accelerated PINK1 degradation in the chronic MPP+ model suggests that PINK1 loss-of-function represents a point of convergence between neurotoxic and genetic causes of PD.

Parkinson's disease (PD) is a chronic, progressive movement disorder, characterized by degeneration of nigrostriatal dopaminergic neurons. Cells and brain tissue from PD patients exhibit reduced mitochondrial complex I activity (1), and exposure to complex I inhibitors such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and the pesticide rotenone cause parkinsonian neurodegeneration and may contribute to risk of PD (2,3). Although the majority of PD cases are sporadic, about 10% show a familial etiology. Genetic models based upon mutations in α-synuclein, Parkin, PINK1, LRRK2 and DJ-1 have further emphasized the role of mitochondrial dyshomeostasis in the pathogenesis of PD (1,4,5). Hence, understanding the mechanisms by which genetic and environmental susceptibilities may interact would provide valuable insights into PD pathogenesis.

The PTEN-induced putative kinase 1 (PINK1) is a ubiquitous serine–threonine kinase, localized in both cytosol and mitochondria (6-9). A normal mitochondrial membrane potential allows full length PINK1 to be imported into mitochondria where it is cleaved by several mitochondrial proteases, including mitochondrial inner membrane presenilin associated rhomboid-like protein (PARL), matrix processing peptidase (MPP), or ATP-dependent Clp protease ATP-binding subunit (ClpX) (10,11). Processed PINK1 may regulate mitochondrial respiration, or it is exported to the cytosol (12), where its stability is regulated by complexing with chaperone proteins (7). PINK1 is implicated in maintaining mitochondrial function, reducing mitochondrial oxidative stress, regulating mitochondrial transport, fission–fusion and autophagy (10,13-16). Loss of PINK1 function alters mitochondrial clearance, increases the production of reactive oxygen species (14,17,18), and renders cells more susceptible to neurotoxicity (19,20). Recently, PINK1 has been shown to regulate mitochondrial bioenergetics by phosphorylating a subunit of complex I, providing a possible link between the complex I dysfunction observed in PD and loss of PINK1 function (21). In addition to these mitochondrial functions, the cytosolic pool of PINK1 regulates neurite outgrowth and dendritic complexity (15) and is sufficient for protecting neurons against MPTP intoxication (8,15).

The Bcl-2 associated athanogene (BAG) proteins, BAG1-6, were initially identified due to their ability to interact with Bcl-2 (22). In recent years, it has been shown that BAG family members regulate not only cell death, but also protein quality control by functioning as co-chaperones. BAG family members can interact with various chaperones including CHIP and the Hsp70/Hsc70 complex (23) through conserved C-terminal BAG domains. Members of the BAG family have been implicated in PD (24-27), and overexpression of BAG2 (25,28) and BAG5 act to stabilize PINK1 (27). Other members of the BAG family can interact with various chaperones including CHIP and the Hsp70/Hsc70 complex (23) through conserved C-terminal BAG domains. Members of the BAG family have been implicated in PD (24-27), and overexpression of BAG2 (25,28) and BAG5 act to stabilize PINK1 (27). Other members of the BAG family act to negatively regulate HSP70 function (29,30).

While it has been shown that engineered knockdown of PINK1 increases sensitivity to acute MPP+ and rotenone elicited apoptosis (8,18), chronic, low dose treatment with complex I inhibitors elicit different mechanisms of injury (31). Here, we show that chronic MPP+ treatment elicits decreased expression of full-length and processed forms of endogenous PINK1 in both cytosolic and mitochondrial fractions. Mechanistically, there was a marked
upregulation of BAG6, which acts to accelerate PINK1 degradation. Furthermore, either restoration of PINK1 or knockdown of BAG6 conferred neuroprotection, reversing the pathological effects of MPP⁺ on cell viability, mitochondrial morphology and neurite shortening/dendritic retraction.

Results:

Chronic treatment with MPP⁺ in neuronally differentiated SH-SY5Y cells causes selective depletion of endogenous PINK1.

In contrast to the mM doses of MPP⁺ commonly used to elicit acute injury in SH-SY5Y cells (32,33), we previously found that chronic, low dose exposure reduces mitochondrial content through suppression of mitochondrial biogenesis in addition to mitophagy (31). Given that the low level complex I deficiency observed in Parkinson’s patient cells is not associated with high levels of acute cell death, we selected a dose of MPP⁺ that was sublethal at 1 week of treatment with ~20-25% toxicity at 2 weeks (31) for further study.

Chronic treatment of differentiated SH-SY5Y cells with 250 µM MPP⁺ resulted in reduced expression of both full length (FL) and processed (dN) forms of PINK1 normalized to GAPDH (Figure 1A and 1B). Loss of PINK1 was not due simply to mitophagy, as it occurred prior to the loss of other mitochondrial proteins (Figure 1B and 1C). Since PINK1 localizes to both mitochondria and cytosol, we further studied its alteration in these subcellular compartments. MPP⁺ elicited decreased PINK1 levels in both fractions, with a more striking % decrease observed in the cytosol (Figure 1D). There were no significant decreases in PINK1 mRNA levels suggesting that the loss of PINK1 is due to enhanced degradation (Figure 1E).

Restoration of PINK1 expression rescues MPP⁺ induced neurodegenerative phenotypes:

To determine whether loss of PINK1 played a pathogenic role in the chronic MPP⁺ model, we transfected neuronally differentiated SH-SY5Y cells with plasmids expressing either GFP or GFP-tagged wild type PINK1. Cells were probed for the human mitochondrial p60 protein after treatment with 250 µM MPP⁺ or vehicle for two weeks and mitochondrial morphology studied as previously described (14). As expected, chronic MPP⁺ treatment elicited mitochondrial fission with loss of interconnectivity (Figure 2B and 2C), and reduced mitochondrial content (Figure 2D). Overexpression of PINK1 significantly restored each of these indices of mitochondrial injury (Figure 2A-D), and protected against chronic MPP⁺ induced cell death (Figure 2E).

In addition to mitochondrial injury, MPP⁺ is known to cause neurite retraction (34). Restoration of PINK1 expression reversed the effects of MPP⁺ on neurite shortening (Figure 2F and 2G). These data suggest that the loss of PINK1 plays a pathogenic role in chronic MPP⁺-induced neurodegeneration.

BCL2-Associated athanogene (BAG6) is pathologically upregulated during chronic MPP⁺ injury:

PINK1 is known to be stabilized by the heat shock protein 90 chaperone system (9,35). However, we found no changes in the expression of HSP90, CDC37 or CHIP (Supporting Figure S1). There were small decreases in BAG5 and BAG2 protein levels (Fig. 3A-C), recently implicated in stabilizing PINK1 levels (25). However, the most striking change elicited by chronic

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MPP+ was a large upregulation of BAG6 protein expression (Figure 3A, 3D), which was negatively correlated with PINK1 protein levels (Figure 3A, 3E; Figure 1B, 1F). The increase in BAG6 protein expression was linked to a small, but statistically significant, increase in mRNA expression (Figure 3F).

To examine whether BAG6 upregulation played a causal role in mediating PINK1 depletion during MPP+ toxicity, we applied siRNA targeting BAG6 2 days after initiating MPP+ intoxication and every 4th day until the end of the experiment. This procedure elicited a stable reduction in BAG6 expression (Supporting Figure S2), which persisted in the presence of MPP+ (Figure 4A). RNAi knockdown of BAG6 significantly reversed the changes in PINK1 expression elicited by 2 weeks of chronic MPP+ toxicity (Figure 4A-C). These results suggest that BAG6 plays an important role in downregulating PINK1 expression under chronic stress.

Blocking the MPP+-induced BAG6 upregulation by siRNA also reversed indices of MPP+ induced pathology (Figure 5A-D), and protected against cell death (Figure 5E). Although knockdown of BAG6 by itself had little basal effect on neurite length, possibly because BAG6 expression is very low under basal conditions (Figure 3A), BAG6 knockdown in MPP+ treated cells protected against neurite shortening (Figure 5F and 5G). Use of a second siRNA sequence against BAG6 showed similar protection (Supporting Figure S3).

**BAG6 interacts with PINK1, increasing PINK1 turnover:**

To determine if PINK1 and BAG6 physically interact, we co-expressed PINK1-GFP with V5-tagged BAG6 in HEK293 cells. Cell extracts were immunoprecipitated using anti-GFP conjugated microbeads to pull down PINK1, resulting in co-immunoprecipitation of BAG6 (Figure 6A). Reciprocal pull-down experiment revealed that BAG6 pulled down both full length and processed forms of PINK1 (Figure 6B).

To determine if BAG6 modulates PINK1 degradation, we co-expressed BAG6 or GFP with Flag-tagged PINK1 in HEK293 cells and examined PINK1-Flag levels after cycloheximide treatment to block new protein synthesis. Cells expressing BAG6 enhanced the degradation of both FL and dN forms of PINK1 compared to GFP (Figure 6C, 6D), causing significant reductions in the half-lives ($t_{1/2}$) of PINK1-FL and PINK1-dN (Figure 6E). Elevating BAG6 expression also decreased steady state levels of PINK1 in the absence of MPP+ (Figure 6F, 6G).

**Increased BAG6 expression is associated with decreased PINK1 levels and dendritic shortening in primary mouse neurons:**

We next studied the effects of chronic, low dose MPP+ exposure in primary mouse cortical neurons. Primary neurons are more sensitive to MPP+, with an LD50 of ~30 µM (36). Under conditions of acute toxicity (25-50 µM), BAG6 levels were decreased at 48 h (Supporting Figure S4). Given that we were able to create a chronic model in SH-SY5Y cells by dropping to ~10% of the LD50, we studied the effects of chronic, repetitive treatment of primary neurons with 2.5-5 µM of MPP+. In contrast to acute toxicity, chronic low-dose exposure to MPP+ increased endogenous BAG6 expression accompanied by significantly decreased PINK1 expression (Fig. 7A-C). As previously reported by others (37), antibodies that recognize mouse PINK1 are not robust, and we could only detect the stronger full-length band.

Using two distinct siRNA sequences (Figure 7D), we studied the effects of mouse
Bag6 knockdown on MPP+ induced neuron injury. Both sequences conferred significant protection against MPP+ in primary neurons (Figure 7E).

Discussion:

While acute treatment of cells with mitochondrial uncouplers results in accumulation of full-length PINK1 (1,4,16,31,38) the effect of less severe, chronic mitochondrial stress has not been previously delineated. MPP+ is the active metabolite of MPTP, which is recognized as an environmental cause of human parkinsonian injury, and these compounds are widely used in cell culture and rodent models of PD (31,39). Early studies using isolated brain mitochondria demonstrated that MPP+ binds specifically to complex I, inhibiting activity without eliciting irreversible damage (42). It is commonly accepted that MPP+ toxicity results from its ability to inhibit mitochondrial complex I activity, as evidenced by the resistance of rho zero (ρ0) cells to MPP+-mediated cell death (32). Nevertheless, cytotoxic mechanisms that appear to be independent of complex I inhibitory effects have also been reported for MPP+, rotenone and paraquat (40,41). Indeed, metabolomic, transcriptomic and proteomic studies demonstrate multiple effects of MPP+ treatment that may result directly or indirectly from complex I inhibition or occur independently of electron transport dysfunction (43,44). While these studies confirm primarily mitochondrial or oxidative changes, other mechanisms may also contribute to MPP+ toxicity in acute or chronic settings.

Using a low dose MPP+ model that elicits no cell death at 1 week and ~20% cell death at 2 weeks (31), we found that chronic complex I inhibition resulted in a significant decrease of endogenous PINK1 from both cytosolic and mitochondrial fractions of differentiated neuronal cells, with a more robust depletion noted in the cytosol. We also discovered that chronic MPP+ treatment robustly increased the expression of BAG6 protein. Similar effects of low dose, chronic treatment were also observed in primary mouse neurons. Knockdown of BAG6 not only prevented PINK1 loss, but also reduced the neurite shortening, mitochondrial fragmentation and cell death induced by chronic MPP+ treatment, conferring protection against MPP+ induced injury in primary neurons. Additionally, we found that BAG6 physically interacts with PINK1 and regulates PINK1 stability by increasing its turnover.

The biological effects of PINK1 are associated with its protein level, kinase activity, localization, and protein-protein interactions. In mitochondria, PINK1 may regulate the phosphorylation of TRAP1, OMM-anchored Parkin, NCLX or the mitochondrial protease HtrA2 to regulate oxidative-stress-induced apoptosis, mitophagy, mitochondrial calcium efflux or mitochondrial dynamics (5-7,45-48). While full-length mitochondrial PINK1 triggers Parkin-dependent mitophagy of depolarized mitochondria (8,9,49-51), recent studies indicate that cytosolic PINK1 may have distinct functions compared to outer mitochondrial membrane-stabilized PINK1 (15). Indeed, N-terminally truncated, cytosolic PINK1 has been reported to suppress autophagy/mitophagy (14), to confer neuroprotection (8), and to promote dendrite outgrowth and neuron differentiation (15,52). These effects may be mediated through activation of Akt (53) or through activation of PKA (52), thereby promoting PKA phosphorylation of p47 (52), DRP1 (54), or LC3 (34,55) to affect neuronal growth or differentiation (52) mitochondrial fission (14) or LC3-mediated cargo targeting (56), respectively. Alternatively, PINK1 may directly bind parkin in the cytosol, inhibiting
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its ability to translocate to the outer mitochondrial membrane for initiating FCCP-dependent mitophagy (57). Taken together, these studies indicate that PINK1 plays multiple roles in promoting neuronal health and function. The MPP⁺-induced decreases of both processed and full-length PINK1 would be predicted to impair both its pro-growth and mitochondrial quality control functions to impact both mitochondrial and synaptodendritic aspects of neuronal health.

In healthy cells, PINK1 is continuously turned over with a half-life that varies from 0.45 to 2.3 h depending on the cell type (7,26,35,58-60). Uncoupling the mitochondria prevents its import, resulting in stabilization of full length PINK1 at the mitochondrial surface; upon washout of CCCP, accumulated FL-PINK1 reverts to its original half-life (49). The variability in PINK1 half-lives may relate to cell-type and context-dependent differences in expression of chaperone proteins, as PINK1 mutants that are unable to bind HSP90 or CDC37 show a 2-4 fold increase in turnover (35,60).

Indeed, the PINK1 protein is known to be stabilized by chaperone proteins such as HSP90α, CDC37, and TRAPs (9,35), as well as BAG2 and BAG5 (25,27,28). Interestingly, there was also a mild decrease in expression of both BAG2 and BAG5. It is possible that loss of these chaperones also contributed to loss of PINK1 in the chronic MPP⁺ model. The most striking change elicited by chronic, low level complex I impairment was a large upregulation of BAG6 expression. Given effects on both full length and processed PINK1, it is unclear whether BAG6 interacts with PINK1 at the mitochondrial surface or in the cytosol, as equilibrium changes in one compartment are likely to affect the other.

BAG6 also known as Bat3/Scythe is a multifunctional protein involved in gene regulation, cell cycle, apoptosis and protein quality control (61). Bag6 acts as a co-chaperone, negatively regulating HSP70’s protein folding capacity (29), in opposition to BAG2 and BAG5. BAG6 acts to target misfolded proteins for proteasomal degradation (62), regulates the distribution of damaged mitochondria (63), and stabilizes the apoptosis inducing factor (AIF) (64), which has been implicated in MPP⁺ toxicity (65). BAG6 knockout neurons are resistant to ER-stress induced cell death (66). Our current data demonstrate that accelerated degradation of the neuroprotective protein PINK1 also contributes to the detrimental effects of BAG6.

PINK1 plays an important role in the regulation of mitochondrial dynamics and function (14,67-70). There was increased mitochondrial fragmentation and swelling caused by chronic MPP⁺ treatment, accompanied by reduced mitochondrial content. Interestingly, siBAG6 transfection not only suppressed BAG6 upregulation but also rescued the loss of PINK1 and prevented each of these chronic MPP⁺ induced mitochondrial changes. These data suggest that the upregulation of BAG6 expression plays a central role in chronic MPP⁺-induced mitochondrial pathology.

Neuritic shortening is a common pathological change in neurodegenerative diseases and has been reported in multiple PD models to include 6-OHDA, PINK1 knockdown, and mutant LRRK2 (15,30,71-73). Here, we found that overexpression of PINK1 reversed the MPP⁺-induced neurite shortening, consistent with the previously reported role of PINK1 in promoting dendrite outgrowth (15,52). These pro-differentiation effects of PINK1 may be mediated through indirect activation of Akt (53) or by the ability of PINK1 to phosphorylate and activate PKA (52). In addition, PINK1 may influence neurite outgrowth through its effects on mitochondrial trafficking (15), as the ability to deliver mitochondria plays a limiting role in synaptogenesis (74,75).
Interestingly, siRNA against BAG6 not only prevented MPP+ induced loss of endogenous PINK1, but also protected against neurite shortening in differentiated SH-SY5Y cells and dendritic shrinkage in primary mouse neurons.

In conclusion, our data indicate a novel role of BAG6 in regulating PINK1 levels under stress conditions. Under basal conditions, BAG6 is maintained at low levels. However, upon chronic MPP+ induced stress, BAG6 levels are elevated resulting in accelerated PINK1 degradation. Restoration of PINK1 levels through either overexpression or via BAG6 RNAi protected against chronic MPP+-induced changes in mitochondrial structure, neurite shortening, and cell death. These data suggest a possible point of mechanistic convergence between neurotoxic/environmental and genetic causes of PD, which centers on the loss of PINK1 function.

Experimental procedures:

Cell lines, primary neuron culture and treatments. SH-SY5Y cells (ATCC, Manassas, VA, USA) were maintained in antibiotic-free Advanced Dulbecco’s modified Eagle’s medium with 5% heat-inactivated fetal calf serum (BioWhittaker, Walkersville, MD, USA), 2mM glutamine and 10mM HEPES. HEK293 cells (ATCC, Manassas, VA, USA) were maintained in antibiotic free DMEM (BioWhittaker, Walkersville, MD, USA) supplemented with 10% FBS (Mediatech), 2mM L-glutamine, and 10mM HEPES in a humidified incubator at 37 °C and 5% CO2. Timed pregnant female C57BL/6 mice were purchased from Charles Rivers Laboratories. All procedures for derivation of primary neuron cultures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Primary cortical neurons from E14-16 male or female pups were isolated from cerebral cortices as described previously (15,30). Neurons were plated at 150,000 cells/cm² in LabTek II coverglass chamber slides coated with poly-L-lysine (0.1 mg/ml). They were maintained in antibiotic-free Neurobasal medium supplemented with 2% B27 and 2mM glutamax (Gibco, Bethesda, Maryland). Half of the media was replaced with fresh media every other day.

SH-SY5Y cells were plated in 6 well plates or LabTek II coverglass chamber slides (Thermo Fisher, Pittsburgh, PA, USA) and neuronally differentiated with 10 μM retinoic acid (RA) (Sigma Aldrich, St Louis, MO) for 72 h prior to and during each experiment. SH-SY5Y cells were treated with 250 μM MPP⁺ three times per week for up to 2 weeks. Mouse primary cortical neurons were treated with 2.5 μM or 5 μM MPP⁺ from DIV7 (days in vitro) for 1 week with half media change containing fresh toxin every 2 days. For cycloheximide experiments, HEK293 cells were transfected with the indicated plasmids for 48h. Transfected cells were treated with 100 μg/ml cycloheximide (Sigma Aldrich, St Louis, MO), diluted in fresh DMEM from a 100 mg/ml stock. Cells were treated at staggered intervals every 30 minutes, and all time points were quickly harvested and lysed after 2 h (30 min after the last set of cells was treated). Untreated transfected cells were used as the zero-time point.

RNA interference and DNA transfection. Small interfering RNAs (siRNAs) targeting different portions of the BAG6 mRNA sequence were employed: Assay ID: HSS111844 (siBAG6#1), which targets human, mouse and rat sequences, and assay ID: s15467 (siBAG6#2), which targets human and mouse sequences (ThermoFisher, Pittsburgh, PA). SH-SY5Y cells were transfected with the siRNAs 2 days after the first dose of MPP treatment and again every
fourth day, which results in persistent knockdown (Supporting Figure S2). For DNA transfection in differentiated SH-SY5Y cells, cells were transfected with 750 ng/well of either GFP-tagged wild type PINK1, or control vector (Genecopoeia, Rockville, MD, USA) 2 days before MPP+ treatment. HEK293 cells were co-transfected with either 2 µg/well PINK1-FLAG and GFP or PINK1-FLAG and 1 µg/well BAG6-V5 (HsCD00442162, DNASU plasmid repository, AZ, USA) in 6-well plates at two days prior to cycloheximide chase experiments. For co-immunoprecipitation experiments, cells were transfected with 2 µg/well of PINK1-GFP or control vector and 1 µg/well BAG6-V5 in a 6-well plate for 48 hours. For immunoblotting experiments, mouse cortical neurons were transfected with 25 nM of the two siRNAs targeting different regions of Bag6 mRNA, at one day after the start of MPP+ treatment and again at DIV 11. Mouse primary cortical neurons were transfected with 500 ng/well GFP expressing plasmid 48h before fixing the cells for total dendrite length analysis.

**Immunoprecipitation.** Forty-eight hours after plasmid transfection, HEK293 cells were lysed in buffer containing 1% triton X-100 and protease/phosphatase inhibitors. Protein concentration was determined by Coomassie Blue Protein Assay (Pierce, Rockford, IL). Equal amounts of protein (1 mg total protein) were used for IP. For IP of GFP tagged PINK1, the uMACS GFP Isolation kit (130-091-125, Miltenyi Biotec, Gaithersburg, MD, USA) was used according to manufacturer recommended protocol. For reverse IP, protein lysates were incubated with 2 µg of mouse anti-V5 antibody (Santa Cruz), 50 µl of Protein G agarose beads (16-266, EMD Millipore) overnight at 4 ºC. The Beads were pelleted at 5000 x g for 5 minutes, the supernatant was removed, and the beads were washed 3X with wash buffer 1 and 2X with wash buffer 2 from GFP Isolation kit. IPed complexes were eluted by boiling the beads in 50 µl of elution buffer followed by centrifugation at 10000 xg for 5 minutes. The supernatant was used for SDS-PAGE.

**Quantitative RT-PCR.** Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA). Total of 1 µg of RNA was used for reverse transcription reaction using the SuperScript IV One-Step RT-PCR system (Life Technologies, Grand Island, NY). The cDNA was quantified by Q-PCR using PINK1 TaqMan probes (Hs00260868_m1, Life Technologies, Grand Island, NY), BAG6 (Hs00190383_m1, Life technologies) and normalized to GAPDH mRNA (4333764F).

**Western blot analysis and densitometry.** SH-SY5Y, mouse cortical neurons and HEK293 cells were lysed in buffer containing the following (in mM): 150 NaCl, 5 EDTA, 25 HEPES, 10% glycerol and 1% Triton X-100 supplemented with protease inhibitors as described previously (33). Equal amounts of protein as determined by Coomassie Plus Protein Assay (Thermo Scientific) were resolved on a 10% gel by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% nonfat milk and probed with antibodies as listed in Table 1 overnight with gentle agitation at 4 ºC. Immunoreactive bands were detected using anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (GE Healthcare) followed by exposure to ECL solution. Images were acquired using the Odyssey Fc imaging system (Li-Cor) for densitometry using Image Studio software (Li-Cor). Densitometry data was normalized to loading control, and the half-life of PINK1 isoforms estimated by fitting a one-phase exponential decay curve using Prism 8 software (GraphPad, San Diego, CA).
**Fluorescence microscopy.** SH-SY5Y cells and mouse cortical neurons were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked with Superblock Buffer (catalog #37515, Thermo Scientific). GFP or PINK1-GFP cells were stained with mouse anti-human mitochondrial antigen 60KD (1:400) and rabbit anti-human GFP (1:1000) for mitochondrial morphology analysis, or GFP for neurite length or total dendrite length analysis as previously described (71). Cells were then incubated with Alexa 488-donkey anti-rabbit (Molecular Probes, Eugene, CA) or Cy3-donkey anti-mouse secondary antibodies (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA). Labeled cells were imaged using an IDX71 Olympus fluorescence microscope (Olympus America Inc., Melville, NY; excitation/emission filter, 490/520 nm; 541/572 nm) or an inverted Fluoview 1000 laser-scanning confocal microscope (Olympus America).

**Quantification of mitochondrial morphology and neurite length.** The parameters of mitochondrial morphology including mitochondrial area, perimeter and mitochondrial connectivity were analyzed using Image J software (NIH, Bethesda, MD). Mitochondrial connectivity was calculated as the square root of the perimeter/circularity ratio normalized by the minor elliptical axis of mitochondria as described previously with slight modification (12). The inverse circularity was used as a measure of mitochondrial elongation. Differentiated SH-SY5Y cells typically exhibit a small cell body and one major neurite projecting from the cell body. The longest neurite from the cell body was measured using intensity-tracing algorithm in ImageJ (34). For neurite length and total dendritic length analysis, color images were extracted to 8-bit grayscale images for quantification using NIH Image J supplemented with the Neuron J plug-in.

**Cell death assay and quantification.** After two weeks of chronic MPP+ treatment, SH-SY5Y cells were gently washed with once warm DMEM and replaced with DMEM containing Hoechst (Thermo Scientific; 1:1000) and propidium iodide (Molecular Probes, Eugene, OR, USA; 1:1000) and incubated for 20 minutes at 37 °C. After that the medium was replaced with dye free medium and incubated for 30 minutes at 37 °C to remove extra dye. Before imaging, the cells were washed once with warm DMEM and imaged at room temperature using Olympus IX71 microscope using 10X objective. 7-9 random fields were imaged per condition. For quantification, the color images were background subtracted and converted to grayscale using ImageJ. After thresholding, Particle Counting plugin was used to count total cells (Hoechst) and PI positive cells. Percent dead cells was determined using the formula [(total PI positive cells)/ (total number of cells)] * 100.

**Statistics.** All graphed data represent mean ± SD from independent, replicate experiments. Two group data were analyzed using two-tailed Student’s t test. Multigroup data were analyzed using one-way or two-way ANOVA. If the null hypothesis was rejected, post-hoc comparisons using Bonferroni-corrected two-tailed t-tests were performed to test specific hypotheses in the context of each experiment. Values of p<0.05 were considered significant.

**Data availability statement:** All data described are contained within the manuscript.
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Table 1: List of antibodies used in the study.

| Antibody   | Species | Catalog number | Source                | Dilution |
|------------|---------|----------------|-----------------------|----------|
| PINK1      | Rabbit  | BC100-494      | Novus Biological      | 1:1000   |
| TOM20      | Rabbit  | Sc11415        | Santa Cruz            | 1:10,000 |
| B-ACTIN    | Mouse   | A5316          | Sigma                 | 1:2000   |
| HSP60      | Mouse   | 611563         | BD Transduction       | 1:1000   |
| BAG6       | Mouse   | Sc365928       | Santa Cruz            | 1:1000   |
| BAG5       | Rabbit  | NB100-56091    | Novus Biological      | 1:1000   |
| BAG2       | Mouse   | Sc101216       | Santa Cruz            | 1:1000   |
| HSP90      | Rabbit  | 4877           | Cell Signaling        | 1:1000   |
| CDC37      | Rabbit  | 3604           | Cell Signaling        | 1:1000   |
| CHIP       | Rabbit  | Sc66830        | Santa Cruz            | 1:1000   |
| GAPDH      | Rabbit  | Ab37168        | Abcam                 | 1:10,000 |
| GFP        | Rabbit  | A6455          | Invitrogen            | 1:10,000 |
| V5         | Mouse   | Sc271944       | Santa Cruz            | 1:1000   |
| FLAG       | Rabbit  | F7425          | Sigma                 | 1:1000   |
| B-Tubulin  | Rabbit  | Ab6046         | Abcam                 | 1:5000   |
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Table II: One-way ANOVA Analysis.

| Figure | Analysis               | F(DFn, DFd) | P     |
|--------|------------------------|-------------|-------|
| 1B – PINK1-FL | F(2,6)= 86.84          | P<0.0001    |
| 1B – PINK1-dN  | F(2,6)= 122.9           | P<0.0001    |
| 1C – HSP60    | F(2,6)= 44.20           | P= 0.0003   |
| 1C – MFN2     | F(2,6)= 31.77           | P= 0.0006   |
| 1F           | F(2,6)= 64.98           | P<0.0001    |
| 7B           | F(2,6)= 15.33           | P= 0.0044   |
| 7C           | F(2,6)= 10.60           | P= 0.0107   |

Table III: Two-way ANOVA Analysis.

| Figure | Analysis               | F(DFn, DFd) | P     |
|--------|------------------------|-------------|-------|
| 2B     | Factor A: Plasmid      | F(1,8)= 75.65 | P<0.0001 |
| Factor B: MPP+ vs Veh | F(1,8)= 110.9 | P<0.0001 |
| A * B: Interaction   | F(1,8)= 28.93 | P= 0.0007 |
| 2C     | Factor A: Plasmid      | F(1,8)= 30.80 | P= 0.0005 |
| Factor B: MPP+ vs Veh | F(1,8)= 55.69 | P<0.0001 |
| A * B: Interaction   | F(1,8)= 6.053  | P= 0.0393  |
| 2D     | Factor A: Plasmid      | F(1,8)= 57.73 | P<0.0001 |
| Factor B: MPP+ vs Veh | F(1,8)= 60.80 | P<0.0001 |
| A * B: Interaction   | F(1,8)= 13.69  | P= 0.0060  |
| 2E     | Factor A: Plasmid      | F(1,8)= 30.68 | P= 0.0001 |
| Factor B: MPP+ vs Veh | F(1,8)= 44.95 | P<0.0001 |
| A * B: Interaction   | F(1,8)= 14.02  | P= 0.0028  |
| 2G     | Factor A: Plasmid      | F(1,8)= 20.69 | P= 0.0019 |
| Factor B: MPP+ vs Veh | F(1,8)= 30.57 | P= 0.0006 |
| A * B: Interaction   | F(1,8)= 14.65  | P= 0.0050  |
| 4B     | Factor A: siRNA        | F(1,8)= 6.336 | P= 0.0360 |
| Factor B: MPP+ vs Veh | F(1,8)= 29.16 | P= 0.0006 |
| A * B: Interaction   | F(1,8)= 15.23  | P= 0.0045  |
| 4C     | Factor A: siRNA        | F(1,8)= 167.0 | P<0.0001 |
| Factor B: MPP+ vs Veh | F(1,8)= 523.5 | P<0.0001 |
| A * B: Interaction   | F(1,8)= 75.21  | P<0.0001  |
| 5B     | Factor A: siRNA        | F(2,12)= 4.832 | P= 0.0289 |
| Factor B: MPP+ vs Veh | F(1,12)= 57.78 | P<0.0001 |
| A * B: Interaction   | F(2,12)= 5.021  | P= 0.0260  |
| 5C     | Factor A: siRNA        | F(2,12)= 8.956 | P= 0.0042 |
| Factor B: MPP+ vs Veh | F(1,12)= 267.0 | P<0.0001 |
| A * B: Interaction   | F(2,12)= 10.19  | P= 0.0026  |
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| 5D   | Factor A: siRNA | F(2,12) = 13.86 | P = 0.0008 |
|------|----------------|-----------------|------------|
|      | Factor B: MPP+ vs Veh | F(1,12) = 104.8 | P < 0.0001 |
|      | A * B: Interaction | F(2,12) = 10.53 | P = 0.0023 |

| 5E   | Factor A: siRNA | F(2,12) = 7.495 | P = 0.0043 |
|------|----------------|-----------------|------------|
|      | Factor B: MPP+ vs Veh | F(1,12) = 160.5 | P < 0.0001 |
|      | A * B: Interaction | F(2,12) = 14.03 | P = 0.0002 |

| 5F   | Factor A: siRNA | F(2,12) = 6.483 | P = 0.0123 |
|------|----------------|-----------------|------------|
|      | Factor B: MPP+ vs Veh | F(1,12) = 38.76 | P < 0.0001 |
|      | A * B: Interaction | F(2,12) = 3.362 | P = 0.0693 |

| 7E   | Factor A: siRNA | F(3,16) = 4.763 | P = 0.0147 |
|------|----------------|-----------------|------------|
|      | Factor B: MPP+ vs Veh | F(1,16) = 100.6 | P < 0.0001 |
|      | A * B: Interaction | F(3,16) = 4.531 | P = 0.0175 |
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Figures and figure legends:

Figure 1. Endogenous PINK1 is depleted in chronic MPP+ treated cells. A) Levels of endogenous PINK1 (full length-FL and processed-dN forms) are decreased by chronic MPP+ exposure (250 µM) (B) Quantification of PINK1-FL and PINK1-dN bands normalized to GAPDH. PINK1-FL, *p=0.0016 for 1-wk MPP+ vs Ctrl and *p<0.0001 for 2-wk MPP+ vs Ctrl. For PINK1-dN, *p= 0.0003 for 1-wk MPP+ vs Ctrl and *p<0.0001 for 2-wk MPP+ vs Ctrl. (C) Quantification of HSP60 and MFN2 bands normalized to GAPDH. For HSP60, *p=0.0017 for 2-wk MPP+ vs Ctrl. For MFN2, *p=0.0044 for 2-wk MPP+ vs Ctrl. (D) Chronic MPP+ treatment depletes PINK1 levels in both cytosolic and mitochondrial fractions. (E) Chronic MPP+ treatment does not affect PINK1 mRNA levels. (F) Quantification of BAG6 expression in chronic MPP+ treated SH-SY5Y cells normalized to GAPDH. *p=0.0013 for 1-wk MPP+ vs Ctrl and *p<0.0001 for 2-wk MPP+ vs Ctrl. Graphs represent mean +/- SD; N=3 independent experiments. Data was analyzed by one-way ANOVA (see table II for details) followed by post-hoc Bonferroni corrected t-tests.
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Figure 2: Restoration of PINK1 rescues mitochondrial fragmentation, cell death and neurite shortening in chronic MPP+ treated SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with either vehicle or 250 µM MPP+ for two weeks. There is a significant interaction between MPP+ treatment and PINK1 overexpression for all dependent variables analyzed (Table III). (A-C) PINK1-GFP prevents mitochondrial fragmentation induced by chronic MPP+ treatment, as analyzed by (B) mitochondrial elongation [*p < 0.0001 for GFP(MPP+ vs Veh); †p < 0.0001 for MPP+(PINK1-GFP vs GFP)], and (C) mitochondrial connectivity [*p=0.0007 for GFP(MPP+ vs Veh); †p=0.0028 for MPP+(PINK1-GFP vs GFP)]. (D) PINK1-GFP rescues the decreased mitochondrial content (% area occupied by mitochondria per cell) caused by chronic MPP+ treatment. *p=0.0002 for GFP(MPP+ vs Veh); †p=0.0003 for MPP+(PINK1-GFP vs GFP). (E) PINK1-GFP prevents chronic MPP+ induced cell death. *p<0.0001 for GFP(MPP+ vs Veh); †p=0.0002 for MPP+(PINK1-GFP vs GFP). (F-G) PINK-GFP prevents chronic MPP+ induced neurite shortening. *p=0.001 for GFP(MPP+ vs Veh); †p=0.0021 for MPP+(PINK1-GFP vs GFP). All graphs represent Mean ±SD; N=3 independent experiments for B-D and G; N=4 independent experiments for E. Data was analyzed by two-way ANOVA with post-hoc t-tests (See Table III for analysis of main factors and interactions). Scale bars: A = 20µm; F = 50µm.
Figure 3: Chronic MPP+ treatment induces BAG6 expression. Differentiated SH-SY5Y cells were either untreated (Ctrl) or treated with 250 µM of MPP+ for two weeks and protein expression analysed. (A) Chronic MPP+ treatment induces BAG6 expression and decreases BAG2, BAG5 and PINK1 levels. (B) BAG5 protein quantification, *p=0.0005. (C) BAG2 protein quantification, *p<0.0001. (D) BAG6 protein quantification, *P<0.0001; see also Figure 1F. (E) PINK1-FL, *p<0.0001 and PINK1-dN protein quantification, *p<0.0001. (F) BAG6 mRNA expression normalized to GAPDH, p=0.0037. Graphs represent Mean ± SD; N = 5 independent experiments for B-E; N=4 independent experiments for F; Student’s two-tailed t-test.
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Figure 4. BAG6 knockdown rescues the loss of PINK1 in chronic MPP+ treated SH-SY5Y cells. (A) siControl (siCtrl) and siBAG6 transfected cells were treated with 250 µM MPP+ for two weeks as described in Experimental Procedures. (B) PINK1-FL quantification. *p=0.001 for siCtrl(MPP+ vs Veh); †p=0.0113 for MPP+(siBAG6 vs siCtrl). (C) PINK1-dN quantification. *p<0.001 for siCtrl(MPP+ vs Veh); †p<0.001 for MPP+(siBAG6 vs siCtrl). Graphs represent mean +/- SD; N=3 independent experiments; two-way ANOVA with post-hoc t-tests. (See Table II for analysis of main factors and interactions).
Stress induced BAG6 modulates PINK1 expression

Figure 5: Knockdown of BAG6 protein prevents neurite shortening and reduces MPP+ induced mitochondrial fragmentation and cell death. Differentiated SH-SY5Y cells were treated with siRNA followed by MPP+ as in Figure 4. (A-D) siRNA mediated knockdown of BAG6 prevents chronic MPP+ treatment-induced mitochondrial changes. (A) Mitochondria were visualized by immunostaining for mitochondrial antigen 60kDa (Mito P60) and nuclei visualized using DAPI. Scale bar = 20 µm. (B) Knockdown of BAG6 with siRNA rescues chronic MPP+ induced changes in mitochondrial elongation [*p=0.0009 for Ctrl(MPP+ vs Veh); *p=0.0027 for siCtrl(MPP+ vs Veh), †p=0.045 for MPP+(siBAG6 vs siCtrl)], and (C) mitochondrial connectivity index [*p<0.0001 for Ctrl(MPP+ vs Veh); *p<0.0001 for siCtrl(MPP+ vs Veh), †p=0.0034 for MPP+(siBAG6 vs siCtrl)]. (D) Knockdown of BAG6 rescues chronic MPP+ induced loss of mitochondrial content (% cellular area occupied by mitochondria). *p<0.0001 for Ctrl(MPP+ vs Veh), *p<0.0001 for siCtrl(MPP+ vs Veh), †p=0.0011 for MPP+(siBAG6 vs siCtrl). (E) Chronic MPP+ treatment induced cell death is partially rescued by down regulation of BAG6 expression using siRNA. *p<0.0001 for Ctrl(MPP+ vs Veh), *p<0.0001 for siCtrl(MPP+ vs Veh), †p=0.0001 for MPP+(siBAG6 vs siCtrl). (F-G) Chronic MPP+ treatment-induced neurite shortening in differentiated SH-SY5Y cells is rescued by siRNA mediated knockdown of BAG6. *p=0.0181 for Ctrl(MPP+ vs Veh), *p=0.0043 for siCtrl(MPP+ vs Veh) and †p=0.0214 for MPP+ (siBAG6 vs siCtrl). Scale bar = 50 µm. Graphs represent Mean ±SD; N=3 independent experiments for B-D and F; N=4 independent experiments for E; two-way ANOVA with post-hoc Bonferroni corrected t-test (See Table III for analysis of main factors and interaction).
Figure 6: BAG6 - PINK1 interaction and PINK1 turn over. (A) Co-immunoprecipitation of BAG6-V5 in PINK-GFP pulldowns from transfected HEK293 cells. (B) Co-immunoprecipitation of PINK1 by V5-tagged BAG6 in transfected HEK293 cells. (C-D) Overexpression of BAG6-V5 accelerated the loss of co-transfected FLAG-tagged PINK1 in cycloheximide treated HEK293 cells compared to cells co-overexpressing GFP and FLAG-tagged PINK1. (E) The half-life of PINK1 isoforms was estimated by fitting a one-phase exponential decay curve for each independent experiment using Prism 8 software (GraphPad, San Diego, CA). PINK1-FL: *p=0.0084 for BAG6-V5 vs GFP; PINK1-dN: *p=0.0430 for BAG6-V5 vs GFP. (F-G) Steady state levels of endogenous PINK1 was determined by transfecting SH-SY5Y cells with 2 µg/well of V5-tagged BAG6 plasmid or vector control. GFP transfection was used to monitor transfection efficiency and normalize densitometry ratios. PINK1-FL: *p=0.0066 for BAG6-V5 vs Ctrl), PINK1-dN: *p=0.0104 for BAG6-V5 vs Ctrl. All graphs represent Mean ±SD, N=3 independent experiments, two-tailed Student’s t-test.
Figure 7. Chronic, low dose MPP+ elevates BAG6 and reduces PINK1 levels in primary neurons, and siBAG6 protects against neuronal injury. (A-C) Mouse primary cortical neurons were plated at 1 x 10^6 cells/well in 6-well plates. At DIV7, cells were treated with 2.5 and 5 µM of MPP+ for an additional 7 days, with half media changes containing fresh toxin on DIV9 and DIV12. Cells were harvested and lysed for western blot analysis at DIV14. BAG6 densitometry: *p=0.01854 for 2.5 µM MPP+ vs Veh; *p=0.0065 for 5.0 µM MPP+ vs Veh. PINK1 densitometry: *p= 0.0184 for 2.5 µM MPP+ vs Veh; *p=0.0287 for 5.0 µM MPP+ vs Veh. Data was analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison test (see Table II for details). (D) Mouse primary cortical neurons were either transfected with control siRNA (siCtrl) or 25nM of siBAG6#1 or siBAG6#2 at DIV7. A second transfection with the same amount of siRNA was done at DIV11. Cells were harvested and lysed at DIV14 for western blot analysis (left panel) and densitometry (right panel) of Bag6 knockdown in mouse primary cortical neurons. *p=0.01442 for siBAG6#1 vs siCtrl; *p=0.0485 for siBAG6#2 vs siCtrl, Bonferroni-corrected two-tailed t-test. (E) Mouse primary neurons plated on LabTek chamber slides were transfected with 25nM of the indicated siRNA as described in (D). The neurons were treated with 5 µM of MPP+ as described in (A). The dendritic arbor of transfected neurons was visualized by co-transfection with GFP (left panels). Summated dendrite length per neuron was quantified (right panel) as described in Experimental Procedures. *p<0.0001 for Ctrl(MPP+ vs Veh); *p=0.0002 for siCtrl(MPP+ vs Veh); †p=0.0170 for MPP+(siBAG6#1 vs siCtrl); †p=0.0354 for MPP+(siBAG6#2 vs siCtrl). Scale bar = 100 µm. Data was analyzed by two-way ANOVA with post-hoc Bonferroni-corrected t-test (See Table III for analysis of main factors and interactions). All graphs represent Mean ± SD from three independent experiments.
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