

BAG2 Gene-mediated Regulation of PINK1 Protein Is Critical for Mitochondrial Translocation of PARKIN and Neuronal Survival*

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Emerging evidence has demonstrated a growing genetic component in Parkinson disease (PD). For instance, loss-of-function mutations in *PINK1* or *PARKIN* can cause autosomal recessive PD. Recently, *PINK1* and *PARKIN* have been implicated in the same signaling pathway to regulate mitochondrial clearance through recruitment of *PARKIN* by stabilization of *PINK1* on the outer membrane of depolarized mitochondria. The precise mechanisms that govern this process remain enigmatic. In this study, we identify Bcl2-associated athanogene 2 (*BAG2*) as a factor that promotes mitophagy. *BAG2* inhibits *PINK1* degradation by blocking the ubiquitination pathway. Stabilization of *PINK1* by *BAG2* triggers *PARKIN*-mediated mitophagy and protects neurons against 1-methyl-4-phenylpyridinium-induced oxidative stress in an *in vitro* cell model of PD. Collectively, our findings support the notion that *BAG2* is an upstream regulator of the *PINK1/PARKIN* signaling pathway.

Parkinson disease (PD)² is the second most common neurodegenerative disorder, characterized by selective loss of the pigmented dopaminergic neurons of the substantia nigra pars compacta (1). Although most PD cases are sporadic in nature (2), mutations in several genes have been linked to familial forms of PD (reviewed in Ref. 3). Indeed, these familial genes serve as important vehicles to study the potential mechanisms of pathogenesis in PD. In this regard, increasing evidence suggests that mitochondrial dysfunction may play a critical role in both the inherited and sporadic forms of PD, although the precise role of mitochondrial dysfunction in PD is unclear (4). Recently, two familial recessive PD genes, PTEN-induced putative kinase 1 (*PINK1*), a mitochondrially localized serine/thre-

onine kinase gene, and *PARKIN*, an E3 ubiquitin ligase gene, have been identified as acting along similar pathways in regulating mitochondrial quality control in mammalian systems (5–8). These findings are supported by genetic studies in *Drosophila* models of PD that show that *PARKIN* and *PINK1* function in a common pathway, with *PARKIN* being a downstream player of *PINK1* (9–11). A third recessive PD gene, *DJ-1*, associated with the regulation of oxidative stress, also regulates *PINK1/PARKIN*-mediated control of mitochondrial health (12).

PINK1 is normally shuttled to the inner mitochondrial membrane where it is processed by multiple proteases (13). The endogenous role of *PINK1* at this site is unknown. However, *PINK1*-deficient cells display altered calcium homeostasis at the mitochondria as well as altered mitochondrial function (14). Processed *PINK1* at the inner mitochondrial membrane has also been proposed to be shuttled to the cytosol, where it is degraded by the proteasome (15). However, under conditions of mitochondrial stress such as treatment with the mitochondrial uncoupler carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) or with the complex I inhibitor 1-methyl-4-phenylpyridinium (MPP⁺), *PINK1* can also regulate mitophagy in a *PARKIN*-dependent fashion (12, 16–19). The framework by which this mitochondrial control pathway occurs has been generally delineated. *PINK1* is stabilized on the outer membranes of damaged/depolarized mitochondria (8). *PINK1* then recruits *PARKIN* to the mitochondria, where it ubiquitinates a number of substrates, including MFN1, MFN2, VDAC1, and MCL1, which, in turn, activates mitophagy (8, 16–22). More recently, recruitment of *PARKIN* by *PINK1* to mitochondria has also been observed in primary neurons in a reactive oxygen species-dependent manner (12, 23). Interestingly, processed cytosolic *PINK1* may also interact with cytosolic *PARKIN*, limiting its translocation to the mitochondria and mitophagy (15). Likewise, in the presence of MG-132, an inhibitor of the proteasome, the level of processed cytosolic *PINK1* is increased significantly (24), indicating that turnover of *PINK1* is through the proteasomal pathway. Consistent with this, *PINK1* has also been shown to be ubiquitinated (25, 26). Interestingly, a recent report has indicated that *BAG2* can regulate the ubiquitination of exogenous *PINK1* in HEK293 cells (26). Taken together, these findings indicate that the regulation of *PINK1* levels and activity is crucial to mitochondrial function and quality at a

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² The abbreviations used are: PD, Parkinson disease; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone; MPP⁺, 1-methyl-4-phenylpyridinium; IP, immunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FL, full-length.

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number of levels, including regulation of mitochondrial health. The exact mechanism through which PINK1 activity/levels is governed is not fully understood. In addition, the biological consequences of dysregulation of PINK1 processing by ubiquitin-mediated pathways are unclear. In this study, we examined the role of BAG2 and provide evidence that it physically interacts with PINK1 *in vivo* and stabilizes PINK1 from proteasomal degradation, facilitates recruitment of PARKIN to depolarized mitochondria, and protects primary cortical neurons under stress conditions in a PINK1-dependent manner.

Experimental Procedures

Mice—Germ line-deleted *pink1* and *parkin* mice have been described previously in detail, respectively (27, 28). CD1 mice were obtained from Charles River Laboratories. All animal procedures were approved by the University of Ottawa Animal Care Committee, and animals were maintained in strict accordance with the Guidelines for the Use and Treatment of Animals put forth by the Animal Care Council of Canada and endorsed by the Canadian Institutes of Health Research.

Antibodies—The following antibodies were used: rabbit anti-BAG2 (Abcam), rabbit anti-PINK1(494) (Novus), mouse anti-FLAG (Sigma), mouse anti-MYC and rabbit anti-RAF1 (Santa Cruz Biotechnology), mouse anti-V5 and anti-complex I (Invitrogen), mouse anti-ACTIN (Sigma), rabbit anti-TOM20 (Santa Cruz Biotechnology), mouse anti-UBIQUITIN (Abcam), and anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibody (eBiosciences) for IP Western blots and anti-mouse and anti-rabbit horse radish peroxidase-conjugated secondary antibodies (Millipore) for regular Western blots. The secondary antibodies labeled by Alexa Fluor 564 for mouse IgG or Alexa Fluor 633 for rabbit IgG were purchased from Life Technologies. All primary antibodies were used at 1:5000 dilution, except anti-ACTIN, which was diluted 1:50,000 for Western blot analyses. All primary antibodies were diluted 1:200 for immunofluorescent staining. The secondary antibodies for IP were diluted 1:5000. The secondary antibodies for Western blots were diluted 1:10,000. The secondary antibodies for immunofluorescent staining were diluted 1:200.

Constructs—*PINK1-V5* was a gift from Dr. Mark Cookson. The *MYC-BAG2* construct was a gift from Dr. Suneil K. Kalia. *GFP-PARKIN* in the pEGFP vector was a gift from Dr. Edward Fon. *PINK1-FLAG* and *PINK1(K219M)-FLAG* were inserted into the pAdTrack vector by using NotI and HindIII. *CHIP* was cloned into the pCMV-3Tag vector by using BamHI and XhoI. The *HA-HSP90* construct was a gift from William Sessa (Addgene, plasmid no. 22487).

Cell Culture—HEK293T cells were cultured with 10% fetal bovine serum (Sigma) in Dulbecco's modified Eagle's medium (Sigma). Cortical neurons were dissected from embryonic days 14.5–15.5 WT CD1, *pink1* (C57BL/6), or *parkin* C57BL/6 mice. The primary cortical neurons were maintained in Neurobasal medium (Invitrogen) supplemented with B27 with antioxidants (Invitrogen), N2 (Invitrogen), 0.5 mM L-glutamine (Sigma), and penicillin/streptomycin (Invitrogen) as described previously (29, 30).

Proteomic Screen—HEK293T cells were transiently transfected with the *PINK1-FLAG* plasmid. The cells were then cul-

tured in fresh medium (DMEM with 10% FBS) for 24 h. The cells were then harvested, lysed by lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 μ g/ml aprotinin, and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (Calbiochem)), and cleared from cell debris by centrifugation at 20,000 \times g for 30 min. The cleared cell lysate was subjected to immunoprecipitation using M2-agarose resin (Sigma-Aldrich) for 1 h. After three washes, the co-precipitated proteins were eluted by 50 mM ammonium bicarbonate containing 400 μ M FLAG peptide. The purified proteins were subjected to SDS-PAGE and detected by colloidal Coomassie staining, and then protein bands from qualified lanes were excised from the gel. These proteins were treated with DTT, iodoacetamide (to alkylate the free sulfhydryl groups), and trypsin, and the digested peptides were then purified from the gel and concentrated and analyzed by mass spectrometry. As reported earlier (31), the data were generated using an LCQ Deca mass spectrometer (Thermo Finnigan). Mascot version 1.9 (Matrix Sciences) was used to analyze the obtained spectra by searching against a human protein sequence database with 122,989 entries. The settings to run the Mascot were as follows: search mode, MS/MS Ion; fixed modification, carbamidomethyl on cysteine; variable modification, oxidation on methionine; peptide mass tolerance, 2 Da; fragment mass tolerance, 0.4 Da; maximum missed cleavages, 2; enzyme, trypsin. The Mascot score is the probability of randomness of the match and is reported as $-10\text{LOG}_{10}(P)$, where P is the absolute probability. In other words, a score of 30 means an absolute probability of 10^{-3} .

Assay for Mitochondrial Translocation of PARKIN and Survival—To study PARKIN translocation in HEK293T cells, 2.0×10^5 cells/well were transfected with plasmids by Lipofectamine 2000 according to the instructions of the manufacturer for suspended cells in a 24-well plate. Briefly, *GFP-PARKIN* was transfected with either the empty vector or *MYC-BAG2* at a ratio of 1:3 (0.5 μ g total) with 1 μ l of Lipofectamine 2000 (Invitrogen). 24 h after transfection, the cells were treated with 10 μ M CCCP (Sigma) for up to 2 h to induce oxidative stress as described previously (12). For PARKIN translocation in neurons, the dissected neurons were seeded on poly-D-lysine-coated coverslips in 24-well plates at a density of 1.5×10^5 cells/well. 3 days after plating, the neurons were transfected with plasmids, *GFP-PARKIN* with empty vector or *GFP-PARKIN* with *MYC-BAG2*, at a ratio of 1:3 (0.5 μ g total) with 1 μ l of Lipofectamine 3000 (Invitrogen). 24 h after transfection, the neurons were treated with 10 μ M CCCP (Sigma) for 2 h to depolarize the mitochondrial membrane potential, as described previously (12). After treatment, cells were fixed, permeabilized, and immunostained with the indicated antibodies and visualized by confocal microscopy.

Cell Fractionation—The mitochondrial and cytosolic fractions were isolated as described previously (13). Briefly, collected cells from one 10-cm culture dish were resuspended in 1 ml of BIB buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA, 0.1% bovine serum albumin, and protease inhibitor mixture) and homogenized with 50 strokes using a 2-ml glass homogenizer. The superna-

tant, from two centrifugations at $1000 \times g$ for 10 min at 4°C , was subjected to centrifugation at $12,000 \times g$ for 10 min at 4°C to pellet mitochondria. The supernatant was used as the cytosolic fraction. The mitochondrial pellet was resuspended in 1 ml of BIB buffer for sonication to extract mitochondrial proteins. The extracts were subjected to IP.

Survival Assays—For plasmids transfection, the neurons were transfected 3 days after plating. 24 h after transfection, the neurons were treated with $20 \mu\text{M}$ MPP⁺ for 48 h and assessed for survival as described previously (30). Briefly, following fixation, GFP-positive neurons were assessed for nuclear integrity as determined by Hoechst staining. Neurons with punctate or condensed nuclei were assessed as dead. For siRNA transfection, neurons were transfected with 30 pmol/well (24-well plate) siRNA to mouse *bag2*, *pink1*, or *parkin* plus 30 pmol/well of control siRNA to 60 pmol siRNA in total. For co-transfection of siRNAs, neurons were transfected with 30 pmol/well of each using Lipofectamine 2000 as described previously (32). 24 h after transfection, the neurons were treated with $20 \mu\text{M}$ MPP⁺ for 48 h. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out. Briefly, the neurons were incubated with $10 \mu\text{l}$ of 5 mg/ml MTT for 4 h. After removal of the medium with MTT, $100 \mu\text{l}$ of solubilization buffer was applied for extracting the converted MTT for 2 h. The plate was read at 570 nm by a plate reader (30).

Determination of PINK1 Levels and Ubiquitination—For determination of levels of overexpressed PINK1, $0.1 \mu\text{g}$ of *PINK1-FLAG* was transfected with $0.4 \mu\text{g}$ of empty vector or *MYC-BAG2* using $1 \mu\text{l}$ of Lipofectamine 2000 (Invitrogen) onto 2.0×10^5 HEK293T cells/well in 24-well plates. 2 days after transfection, the total cell lysates were collected and subjected to Western blot analysis with anti-FLAG antibody for PINK1 and anti-MYC antibody for MYC-BAG2. For endogenous PINK1, empty vector or *MYC-BAG2* was transfected into HEK293T cells for 2 days, and the total cell lysates were analyzed by Western blot for PINK1. For the ubiquitination assay of overexpressed PINK1, 3.0×10^6 HEK293T cells in a 35-mm dish were transfected with $1 \mu\text{g}$ of *PINK1-FLAG* in the presence of $3 \mu\text{g}$ of empty vector or *MYC-BAG2* utilizing $8 \mu\text{l}$ of Lipofectamine 2000. 2 days after transfection, the total proteins were extracted in $200 \mu\text{l}$ of radioimmune precipitation assay (RIPA) buffer. After 10 min of boiling and 20 min of centrifugation ($20,000 \times g$) at 4°C , the supernatants were diluted twice with water for IP using $1 \mu\text{g}$ of anti-FLAG antibody and $30 \mu\text{l}$ of anti-mouse IgG beads (eBiosciences) for 2 h at 4°C . The beads were washed four times using a wash buffer containing 20 mM Tris (pH 7.4), 300 mM NaCl, 0.1% SDS, and 1 mM EDTA. Proteins were eluted in $2 \times$ SDS sample buffer for separation by 4–15% SDS-PAGE gel (Bio-Rad) and Western blot analyses utilizing an anti-UBIQUITIN antibody. The ubiquitination assay of endogenous PINK1 was performed similar to that described above, except that HEK293T cells were transfected with empty vector or *MYC-BAG2*, and the IP was carried out with anti-PINK1 antibody.

siRNA—All siRNAs were purchased from Santa Cruz Biotechnology. For siRNA transfection, 30 pmol of siRNA/well was transfected with $1 \mu\text{l}$ of Lipofectamine 2000 in 24 wells, and 150 pmol of siRNA was transfected with $8 \mu\text{l}$ of Lipofectamine 2000

in a 35-mm dish. Twenty-four hours after siRNA transfection, plasmids were transfected as above for the protein level assay or the ubiquitination level assay.

Statistical Analysis—Statistical significance was determined using paired Student's *t* test for related samples unless stated otherwise. Two-way analysis of variance was used in PARKIN translocation and BAG2-regulated survival to determine the significance between WT and KO under control and treated conditions. All data are presented as mean \pm S.E. Significance is denoted as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. No significance is denoted by N.S.

Results

Physical Interaction between PINK1 and BAG2—PINK1 plays an important role in regulating dopaminergic loss (5) and in a number of critical biological processes such as mitochondrial quality control. However, the potentially diverse set of processes that regulate PINK1 function or its downstream effectors are not completely known. In particular, the mechanisms that control PINK1 levels and stability, likely important components of PINK1 regulation, are not completely clear. To identify these regulatory mechanisms, we performed a PINK1 interactomic screen to search for proteins associated with PINK1 on the basis of mass spectrometric strategy (31). One particular target of interest in the regulation of PINK1 levels was BAG2, which was identified as an interactor of PINK1 with a Mascot protein score of 99.

BAG2 has been identified previously as a chaperone protein involved in the proteasomal process (33), and we hypothesized that it might be critical for the regulation of PINK1 stability. Accordingly, we pursued this target in more detail. To confirm the physical interaction, we first co-expressed V5-tagged *PINK1* (*PINK1-V5*) with MYC-tagged *BAG2* (*MYC-BAG2*) in HEK293T cells. Cell extracts were then immunoprecipitated with IgG control or MYC antibody and probed by Western blot analyses for V5 to assess BAG2 interaction. As shown in Fig. 1A, *left panel*, interaction was observed with MYC pulldown but not the IgG control. We also performed the reverse interaction studies by immunoprecipitating for V5 and probing by Western blot analyses for MYC. Consistent with our initial results, an interaction between expressed BAG2 and PINK1 was also observed (Fig. 1A, *right panel*). As shown in Fig. 1A, BAG2 interacts not only with full-length PINK1 but also with processed PINK1. Previous studies have indicated that cleavage of PINK1 is processed in mitochondria (13). Therefore, the mitochondrial and cytosolic fractions from HEK293T cells transfected with *PINK1-FLAG* and *MYC-BAG2* were subjected to immunoprecipitation with MYC antibody to check where the interaction of PINK1 and BAG2 takes place. As shown in Fig. 1B, the interaction of PINK1 and BAG2 was observed in both fractions but predominantly in the mitochondrial fraction. Finally, to ensure that this interaction could also be observed with endogenous PINK1 and BAG2, we probed non-transfected HEK 293T cells extract by immunoprecipitating with PINK1 antibody or IgG control and immunoblotting with BAG2 antibody. HEK293T cells were utilized to study endogenous interaction because we can readily detect endogenous PINK1 with available antibodies. This interaction could not be

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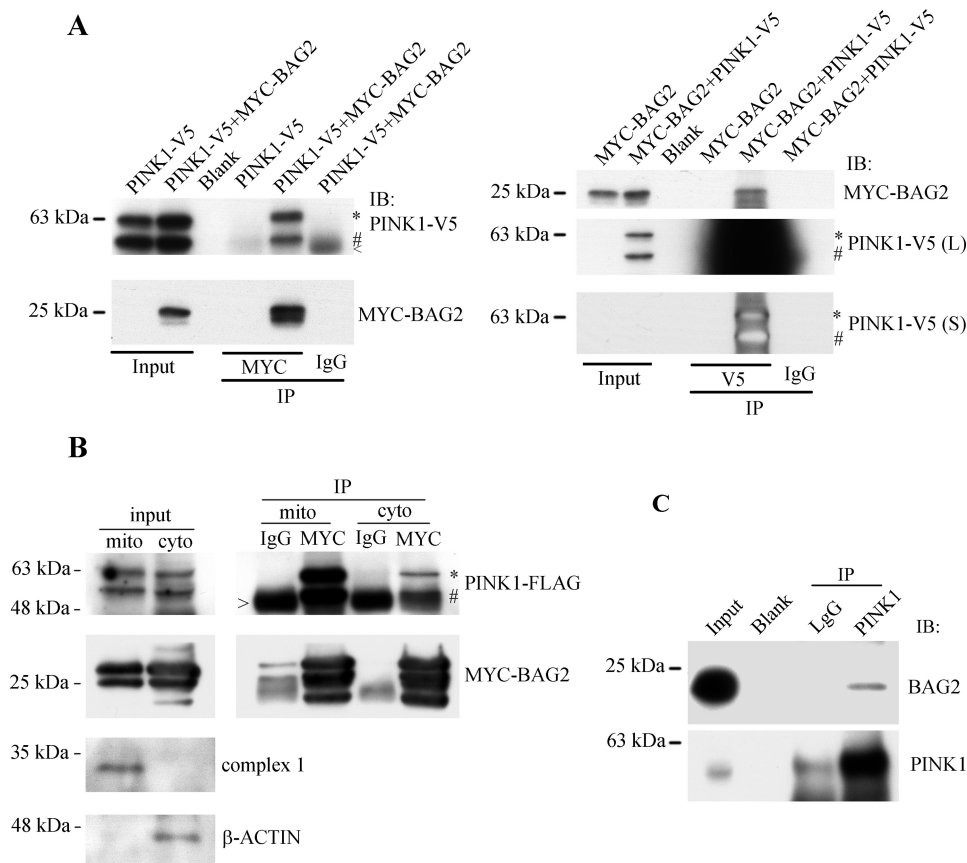


FIGURE 1. BAG2 forms a complex with PINK1. A, BAG2 interacts with PINK1 upon expression of PINK1 and BAG2 in HEK293T cells by co-immunoprecipitation. The plasmids *PINK1-V5* and *MYC-BAG2* were co-transfected into HEK293T cells. For IP of MYC-BAG2 (left panel), transfection of an empty vector and *PINK1-V5* was as one of the controls. For reverse IP of PINK1-V5 (right panel), transfection of an empty vector and *MYC-BAG2* was as one of the control. IB, immunoblot. B, mitochondrial (mito) and cytosolic (cyto) fractions from HEK293T cells transfected with *PINK1-FLAG* and *MYC-BAG2* were subjected to IP using MYC antibody. The precipitated proteins were separated by SDS-PAGE for Western blots. C, the total cell lysate from HEK293T was used for IP of endogenous PINK1. The endogenous BAG2 was detected from IP of PINK1. Asterisks, full-length PINK1; #, processed PINK1; > and <, the heavy chain of IgG.

studied in primary neurons because PINK1 antibodies do not readily recognize PINK1 from murine or rat sources. In agreement with our tagged expression interaction studies, we detected endogenous interaction between PINK1 and BAG2, confirming the validity of this interaction (Fig. 1C).

BAG2 Modulates PINK1 Levels through Proteasomal Degradation—To explore the functional consequences of this interaction, we first investigated whether the PINK1 level is regulated by BAG2. We initially explored the effects of expression of BAG2 on exogenously expressed tagged PINK1 levels. As shown in Fig. 2A, increasing amounts of *MYC-BAG2* plasmid (balanced for amounts by a control vector) was cotransfected with a constant level of *PINK1* plasmid. Increasing expression of BAG2 was confirmed by Western blot analyses. We showed that increased BAG2 expression correlates with increased levels of FLAG-tagged PINK1. Recent studies have suggested that chaperones such as HSP90 also affect PINK1 stability (34, 35). Accordingly, we checked whether expression of HSP90 may affect PINK1 stability in ways similar to that of BAG2 expression. We found that HSP90 failed to affect PINK1 levels, suggesting that BAG2 acts in ways different from that of HSP90 (Fig. 2B). We next performed the converse experiment, where we examined siRNA-mediated down-regulation of endogenous BAG2 on the levels of exogenous PINK1. As shown

in Fig. 2A, right panel, *siBAG2*, compared with scrambled siRNA controls, led to significantly reduced levels of FLAG-tagged PINK1. siRNA mediated down-regulation of BAG2 was confirmed by Western blot analyses. Finally, we examined whether overexpression of BAG2 or siRNA-mediated knock-down of BAG2 affects the endogenous PINK1 level in HEK293T cells. Consistent with the effect of BAG2 on exogenous PINK1, expression of BAG2 increased levels of PINK1, whereas siRNA-mediated down-regulation of BAG2 reduced endogenous PINK1 levels (Fig. 2C).

Next we explored whether the effect of BAG2 on PINK1 protein levels is mediated via a proteasomal pathway. HEK293T cells were cotransfected with FLAG-tagged *PINK1* or FLAG-tagged *PINK1(K219M)*, a kinase-dead mutant of *PINK1*, along with *MYC-BAG2* or *PINK1* and siRNA for BAG2 and treated with the proteasomal inhibitor MG132 to stabilize ubiquitinated PINK1. After immunoprecipitation of PINK1 with anti-FLAG antibody, ubiquitinated PINK1 was detected by Western blot analyses using an anti-ubiquitin antibody. As shown in Fig. 3A, left panel, overexpression of BAG2 dramatically reduced the level of ubiquitinated PINK1. In this experiment, it is interesting to note that MG132 treatment only slightly elevated the levels of ubiquitinated PINK1 (mostly higher molecular weight species). This may be due to the dif-

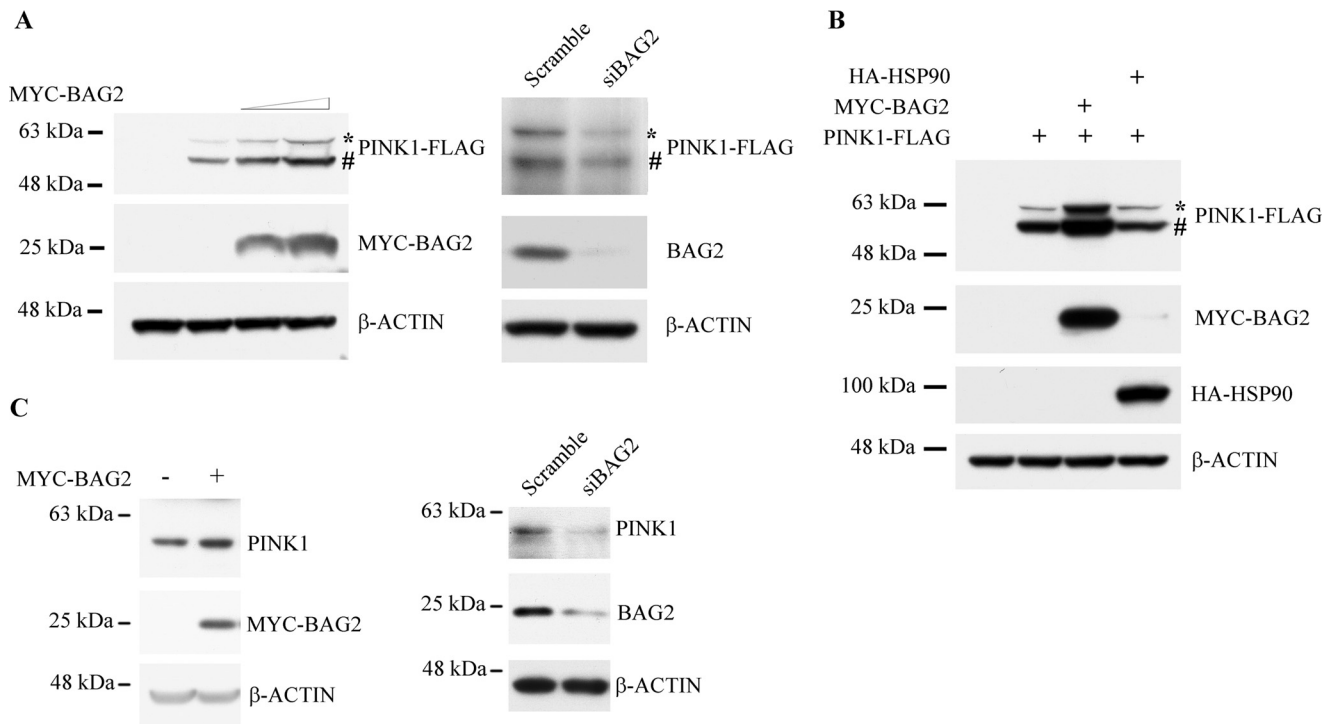


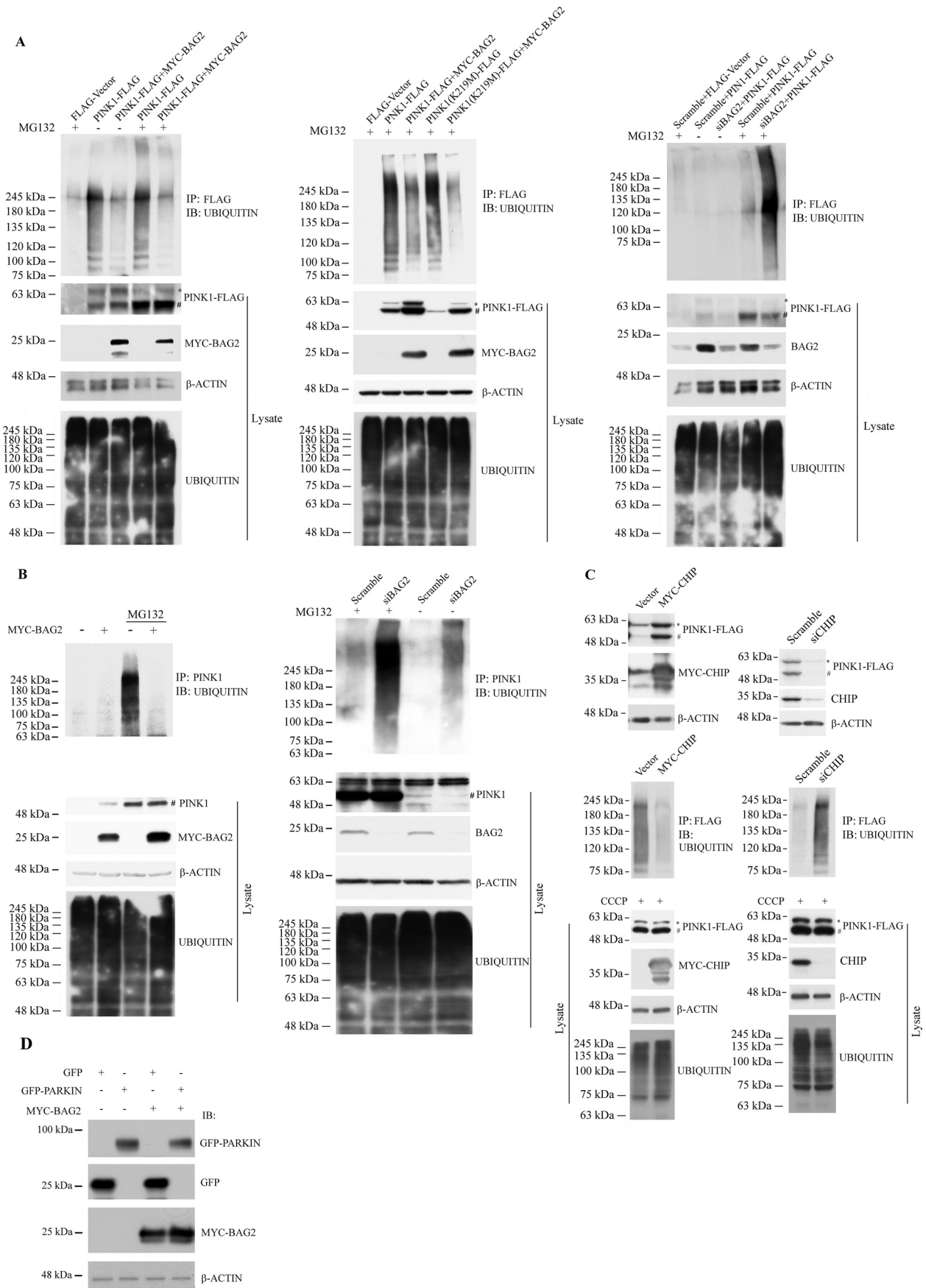
FIGURE 2. BAG2 regulates PINK1 stability. *A*, PINK1-FLAG and MYC-BAG2 were coexpressed in HEK293T cells. Expression of PINK1-FLAG or MYC-BAG2 was observed from total cell lysates (*left panel*). PINK1 was expressed in BAG2 knockdown HEK293T cells. Overexpressed PINK1-FLAG and MYC-BAG2 (*left panel*) or endogenous BAG2 (*right panel*) were probed from total cell lysates. *B*, PINK1-FLAG alone or with HA-HSP90 or MYC-BAG2 was transfected into HEK293T cells. 24 h after transfection, the total cell lysates were analyzed by Western blot analysis using the indicated antibodies. *C*, endogenous PINK1 was detected in HEK293T cells with expression of MYC-BAG2 (*left panel*) or knockdown of BAG2 (*right panel*). Asterisks, full-length PINK1; #, processed PINK1.

ferential kinetics of turnover of ubiquitinated PINK1 species. Similarly, we also observed that BAG2 dramatically affects the level of ubiquitinated PINK1(K219M) in HEK293T cells along with overexpression of BAG2, as shown in Fig. 3*A*, *center panel*. In contrast, knockdown of BAG2 led to the converse and increased PINK1 ubiquitination, as shown in Fig. 3*A*, *right panel*. In agreement with the effect of BAG2 on exogenously expressed PINK1, overexpression or knockdown of BAG2 decreased or increased the ubiquitinated level of endogenous PINK1, respectively (Fig. 3*B*). Importantly, BAG2 expression did not significantly affect levels of total ubiquitination. BAG2 has been associated with CHIP (C terminus of HSC70-interacting protein) E3 ubiquitination activity. Previous results have shown that BAG2 inhibits CHIP E3 ligase activity (36). Accordingly, we examined whether CHIP may be involved in the BAG2-mediated effects on ubiquitination of PINK1. Expression of CHIP with exogenous PINK1 led to an unexpected increase in total PINK1 and a decrease in ubiquitinated PINK1 (Fig. 3*C*, *left panels*). Conversely, knockdown of CHIP resulted in a decrease in total PINK1 but an increase in ubiquitinated PINK1 (Fig. 3*C*, *right panels*). Although not completely conclusive, this suggests a CHIP independent pathway of regulation of PINK1 by BAG2. Increasing evidence suggests that PINK1 and PARKIN function in the same pathway in regulating mitochondrial functions (9–11, 37, 38). Therefore, we examined whether BAG2 affects the level of PARKIN. Expression of BAG2 did not cause an increase in PARKIN in HEK293T cells (Fig. 3*D*), indicating that BAG2 may regulate PARKIN mitochondrial translocation through PINK1.

BAG2 Regulates PARKIN Translocation to Mitochondria in a PINK1-dependent Manner—We next examined whether BAG2-mediated regulation of PINK1 affects known PINK1-relevant biological outcomes. Loss of mitochondrial membrane potential promotes PINK1/PARKIN-mediated mitophagy in different cell lines as well as primary cultured neurons (8, 12, 16, 18, 19). To test whether the stabilizing effect of BAG2 exerted on PINK1 translates into a functional outcome, we determined whether BAG2 influences PARKIN translocation in a PINK1-dependent manner. To this end, HEK293T cells were cotransfected with GFP or GFP-PARKIN along with MYC-BAG2 or siRNA for BAG2. 1–2 days after transfection, cells were treated with CCCP to induce mitochondrial depolarization. As shown in Fig. 4, *A* and *B*, expression of exogenous BAG2 significantly increased mitochondrial translocation of PARKIN compared with controls transfected with control vector. Importantly, down-regulation of BAG2 almost significantly diminished PARKIN translocation to mitochondria (Fig. 4, *C* and *D*). In agreement with the results from the immunofluorescence assay, the biochemical fractionation studies confirmed that BAG2 significantly affects the mitochondrial translocation levels of overexpressed or endogenous PARKIN (Fig. 4, *E* and *F*).

Because mitochondrial translocation of PARKIN has also been observed recently in primary neurons (12, 23), we investigated whether BAG2 also affects mitochondrial translocation of PARKIN in primary neurons after mitochondrial depolarization. We found that BAG2 expression significantly increased mitochondrial translocation of PARKIN induced by CCCP. The BAG2-mediated increase in PARKIN translocation was

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completely dependent on PINK1 because PARKIN translocation did not occur in PINK1-deficient neurons (Fig. 5). As an alternative control, we also examined whether BAG2 might affect levels of PARKIN as an alternative explanation of the observed increase in PARKIN translocation. However, the expression level of BAG2 appeared to have no effect on PARKIN levels (Figs. 3D and 4D, *top panel*). Taken together, this suggests a model in which BAG2 contributes to increased PARKIN levels on mitochondria through a PINK1-mediated pathway that is independent of any global effect on PARKIN levels.

BAG2 Protects Neuronal Death through a PINK1/PARKIN Pathway—Both PINK1 and PARKIN have also been reported to promote the survival of neurons in response to multiple stresses. Given that overexpression of PINK1 protects cells against reactive oxygen species-induced death (5, 39), and BAG2 promotes PARKIN translocation through PINK1, we investigated whether BAG2 protects neurons against oxidative stress induced by MPP⁺ through the PINK1/PARKIN signaling cascade. First, we evaluated whether BAG2 itself protects cultured primary cortical neurons in an MPP⁺-induced cell model of PD. As shown in Fig. 6A, *top panel*, overexpression of BAG2 protected neurons against MPP⁺-induced neuronal death. We have reported previously that acute down-regulation of PINK1 sensitizes neurons to MPP⁺ (39). We recapitulate this observation here and also confirm the same with Parkin down-regulation. Interestingly, siRNA-mediated down-regulation of BAG2 led to the same degree of sensitization to death observed with down-regulation of either PARKIN or PINK1. Furthermore, combined down-regulation of BAG2 and PINK1 or PARKIN did not further increase death when compared with either alone. This was also true of co-treatment with siRNA to both *parkin* and *pink1* (Fig. 6A, *bottom panel*). We also assessed whether the survival effects of BAG2 expression were dependent on PINK1 or PARKIN. We utilized *pink1*- or *parkin*-deficient neurons obtained from germ line KO animals. In this case, sensitization of death induced by loss of *pink1* or *parkin* alone was not observed, as shown with acute knockdown. This may be due to compensation resulting from long-term *pink1/parkin* loss. Indeed, *pink1/parkin* KO animals do not show dopaminergic loss (40, 41). Importantly, however, the protection observed in WT cultures with BAG2 expression is abolished with either *parkin* or *pink1* deficiency (Fig. 6B). Taken together, these data suggests that BAG2 can increase neuronal viability through a PINK1/PARKIN pathway.

Discussion

Previous work has shown that PINK1 is degraded by the proteasomal pathway (42, 43). After being processed by a number

of proteases, PINK1 is degraded by the proteasome (13, 44–46), resulting in a low abundance of PINK1 in healthy cells. Upon loss of mitochondrial membrane potential, accumulated full-length PINK1 (FL-PINK1) on the outer mitochondrial membrane recruits PARKIN to mitochondria, leading to mitochondrial clearance via autophagy (16–19). Some reports have also suggested that cleaved PINK1 can perform the same function (46, 47). These observations indicate that regulation of PINK1 processing and turnover is critical to its biological function. Here we identify BAG2 as one mechanism by which PINK1 levels are regulated and link its effect on PINK1 with its downstream biological effects.

BAG2 is one member of the BAG family. Its members are reported to have a diverse range of effects, particularly on stimulating or interfering with ubiquitin E3 ligase activity (36, 48). For example, BAG1 stimulates chaperone-associated E3 ubiquitin ligase activity, facilitating proteasomal activity for protein degradation. BAG2 inhibits chaperone-associated E3 ubiquitin ligase activity, interrupting protein degradation (36, 48, 49). Beyond regulation of chaperone-associated E3 ubiquitin ligase activity, BAG5 also inhibits the E3 ligase activity of PARKIN through its functional interaction with PARKIN (50).

We provide evidence that BAG2 is central to the regulation of PINK1 stability. First, BAG2 expression increases the levels of both exogenously expressed and endogenous PINK1. Second, BAG2 expression reduces the ubiquitination of expressed or endogenous PINK1. These results are consistent with a recent report showing that expression of BAG2 reduces ubiquitinated PINK1, possibly through blocking ubiquitin ligase activity (26). Importantly, we also show that down-regulation of endogenous BAG2 leads to the converse observations, providing much clearer evidence that BAG2 plays a central role in regulating PINK1 levels. It is interesting that BAG2 appears to regulate the stability of full-length as well as processed forms of PINK1. This is in contrast to MG132 treatment, which stabilizes only the more processed forms. The reason for this is unclear, but BAG2 does interact with both forms of PINK1. We can only speculate that the interaction of BAG2 with full-length PINK1 may slow down the conversion of full-length PINK1 to processed PINK1 by proteases such as PARL (45).

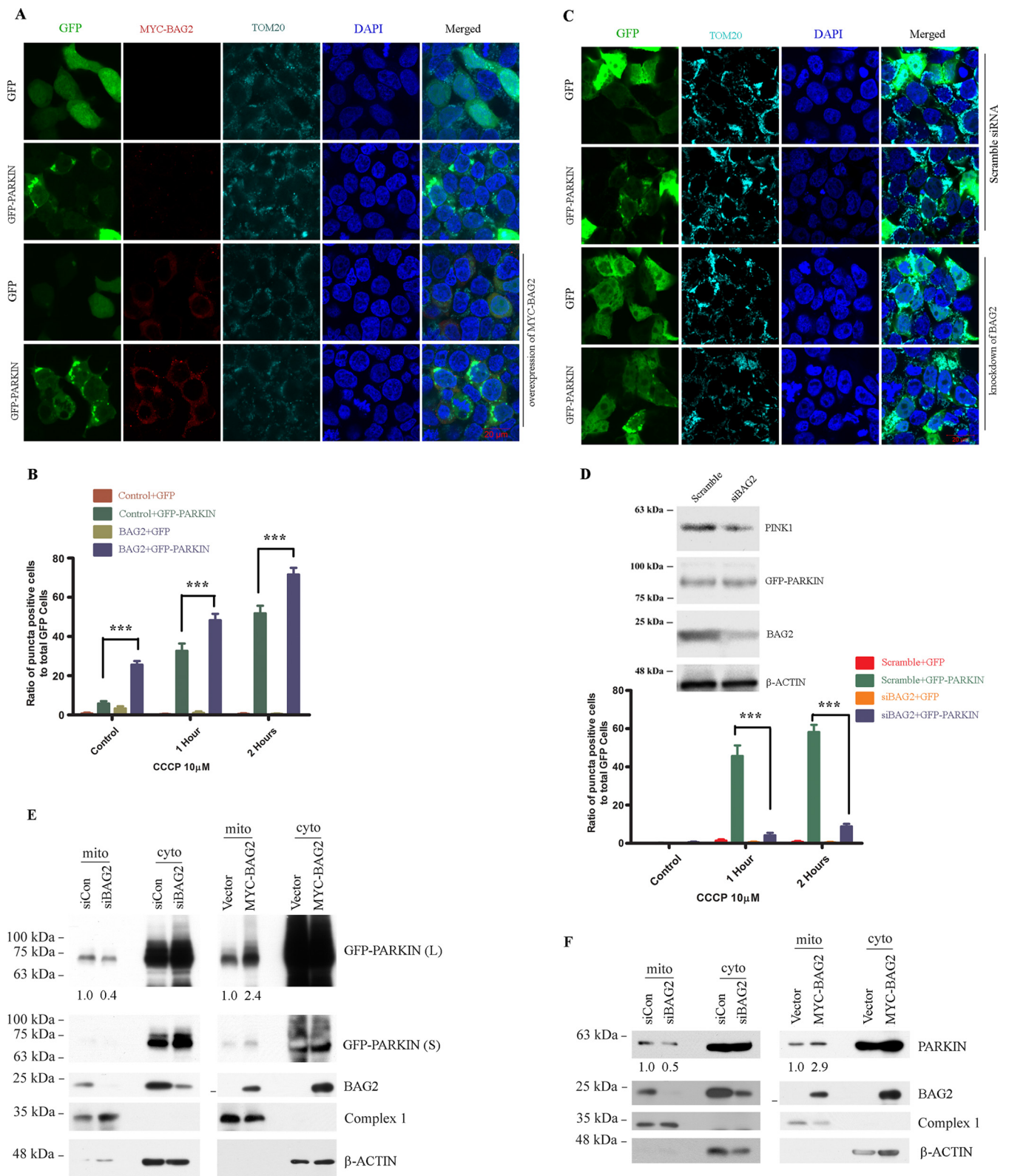
Given the evidence that BAG2 can regulate proteasomal activity through inhibition of CHIP E3 ligase activity, we anticipated that the ubiquitinated level of PINK1 should be up-regulated upon expression of CHIP. However, we observed a decrease in ubiquitinated PINK1 and an increase in PINK1 levels as a result of CHIP expression. Furthermore, we found that HSP90, a chaperone protein important for CHIP-mediated

FIGURE 3. BAG2 regulates PINK1 degradation in HEK293T cells. A, ubiquitination of overexpressed PINK1 is inhibited by BAG2. *PINK1*-FLAG or *PINK1*(K219M)-FLAG along with *MYC*-BAG2 was coexpressed in HEK293T cells. Transfection of *PINK1*-FLAG and an empty vector was used as a control. Where indicated, transfected cells were treated with MG132 for 2 h. FLAG-tagged PINK1 or its mutant was immunoprecipitated for ubiquitination analysis by Western blot (*left* and *center panels*). *Left panel*, the ubiquitination analysis was performed from HEK293T cells with expression of *PINK1*-FLAG and overexpression of *MYC*-BAG2. *Center panel*, the ubiquitination analysis was performed for detection of *PINK1*-FLAG or *PINK1*(K219M)-FLAG with expression of *MYC*-BAG2. *Right panel*, the ubiquitination level of *PINK1*-FLAG was examined with knockdown of BAG2. *B*, immunoblot; *Ubi*, ubiquitin. *B*, expression or knockdown of BAG2 affects the ubiquitination of endogenous PINK1. The ubiquitination of PINK1 was detected by IP of PINK1 from HEK293T cells that either expressed *MYC*-BAG2 (*left panel*) or knockdown of BAG2 (*right panel*). *C*, the level of CHIP affects the PINK1 level through regulating the ubiquitination of PINK1. *PINK1*-FLAG was transfected into HEK293T cells along with overexpression of CHIP or knockdown of CHIP. *Top panels*, 24 h after transfection, the total cell extracts were subjected to Western blots for *PINK1*-FLAG. *Bottom panels*, 24 h after transfection, cells were treated with MG132 for 2 h for ubiquitination analyses. *D*, BAG2 has no effect on PARKIN level. *MYC*-BAG2 and *GFP*-PARKIN were cotransfected into HEK293T cells. 24 h after transfection, cell lysates were subjected to Western blot analysis. Asterisks, full-length PINK1; #, processed PINK1.

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proteasomal degradation (51), did not influence the level of PINK1, further indicating that CHIP-mediated protein degradation may not have a critical role in PINK1 degradation. Taken together, these results suggest that an alternative E3 ligase other than CHIP is a target for BAG2.

Importantly, we also assessed the biological consequences of BAG2 expression. We first assessed the effects of BAG2 on the Parkin-mediated mitochondrial control pathway. Given that accumulation of PINK1 recruits PARKIN to the outer membrane of mitochondria (8, 16, 18, 19), the stabilization of PINK1



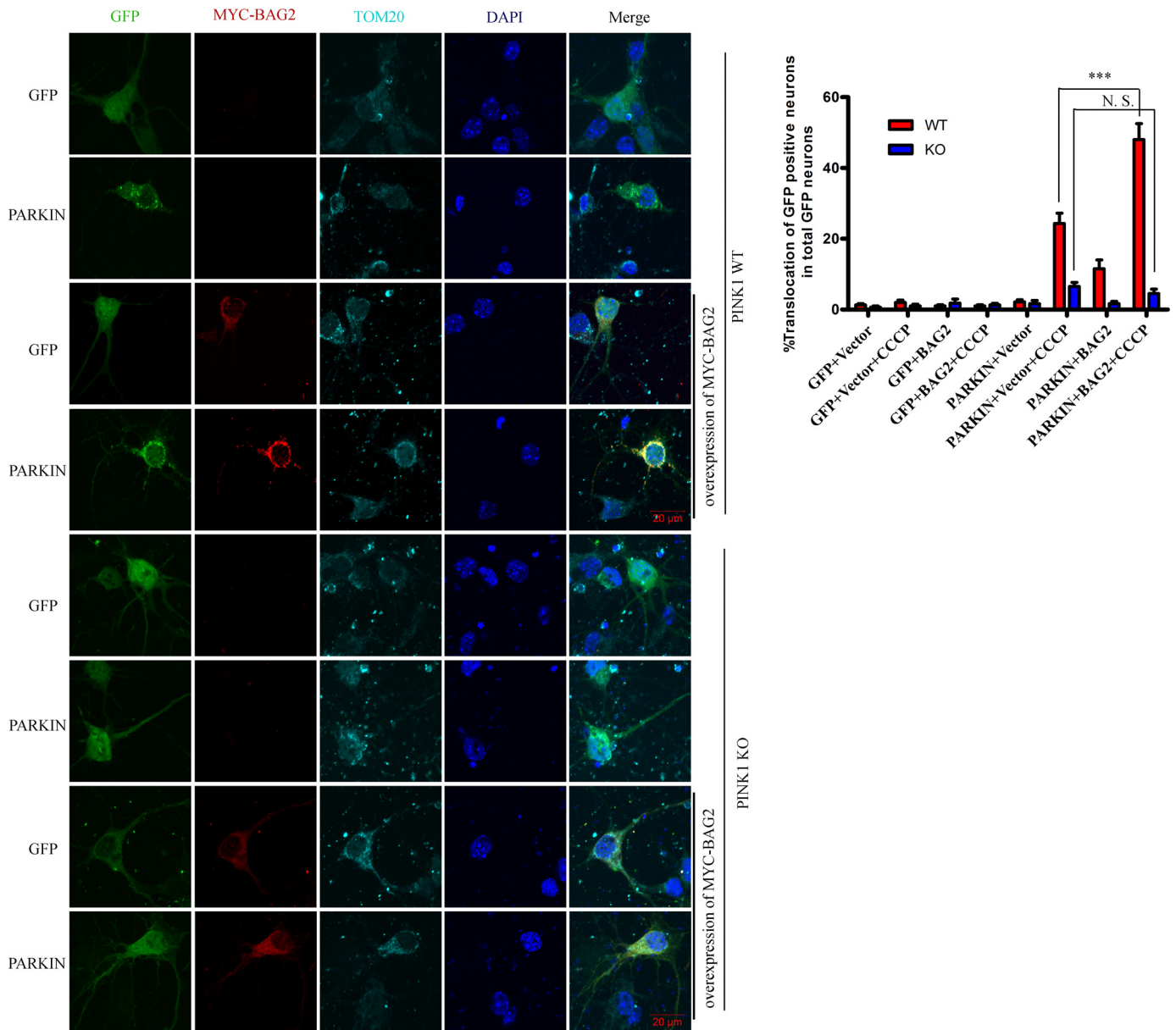


FIGURE 5. **PARKIN translocation is regulated by BAG2 via PINK1 in cortical neurons.** Primary cortical neurons were transfected with plasmids as described in Fig. 4, A and B. A, representative images showing the distribution of GFP or GFP-PARKIN in wild-type or PINK1-deficient neurons with CCCP treatment with and without BAG2 expression, as indicated. Shown are GFP/GFP-PARKIN (green), MYC-BAG2 (red), mitochondrial TOM20 (cyan), and nuclei (blue). B, the percentage of puncta in GFP-positive neurons colocalized with TOM20 was evaluated by comparing to the total number of GFP-positive neurons. Data are presented as mean \pm S.E. of at least three independent experiments. ***, $p < 0.001$; N.S., not significant.

should augment mitochondrial translocation of PARKIN upon depolarization of mitochondria. Indeed, expression of BAG2 significantly increased PARKIN translocation to the mitochondria.

In addition, down-regulation of BAG2 by siRNA led to the converse observation, dramatically blocking PARKIN recruitment to the mitochondria. Importantly, we also assessed

FIGURE 4. **BAG2 regulates PARKIN translocation via PINK1.** A and B, BAG2 expression regulates PARKIN translocation in HEK293T cells. HEK293T cells were cotransfected with empty vector and GFP, empty vector and GFP-PARKIN, MYC-BAG2 and GFP, or MYC-BAG2 and GFP-PARKIN as indicated. A, representative images from transfected cells treated with CCCP for 1 h. Shown are GFP/GFP-PARKIN (green), MYC-BAG2 (red), mitochondrial TOM20 (cyan), and nuclei (blue). B, cells were left untreated or treated with CCCP as indicated. GFP colocalization with the mitochondrial marker TOM20 was analyzed under fluorescence microscopy. The percentage of GFP/mitochondria colocalization was ascertained as a ratio relative to total GFP-positive cells. ***, $p < 0.001$. C and D, GFP-PARKIN or GFP was expressed in HEK293T cell with control siRNA or BAG2 siRNA. C, representative images from transfected cells, as indicated, treated with CCCP for 1 h. D, top panel, siRNA for BAG2 significantly knocked down BAG2, resulting in a decrease of PINK1 but no effect on GFP-PARKIN. Bottom panel, quantification of mitochondrial PARKIN colocalized with TOM20 with and without CCCP treatment as indicated. Data are presented as mean \pm S.E. of at least three independent experiments. ***, $p < 0.001$. E and F, the mitochondrial translocation of PARKIN was analyzed by Western blots after cellular fractionation. E, GFP-PARKIN was expressed in HEK293T cells along with overexpression of MYC-BAG2 or knockdown of BAG2 in the presence of CCCP. F, HEK293T cells were transfected with MYC-BAG2 or siRNA for BAG2. After CCCP treatment and cellular fractionation, the level of PARKIN was analyzed by Western blots. GFP-PARKIN or endogenous PARKIN was normalized to mitochondrial marker complex 1 for relative levels of PARKIN and then compared with control siRNA (siCon) or vector controls. mito, mitochondrial; cyto, cytosolic.

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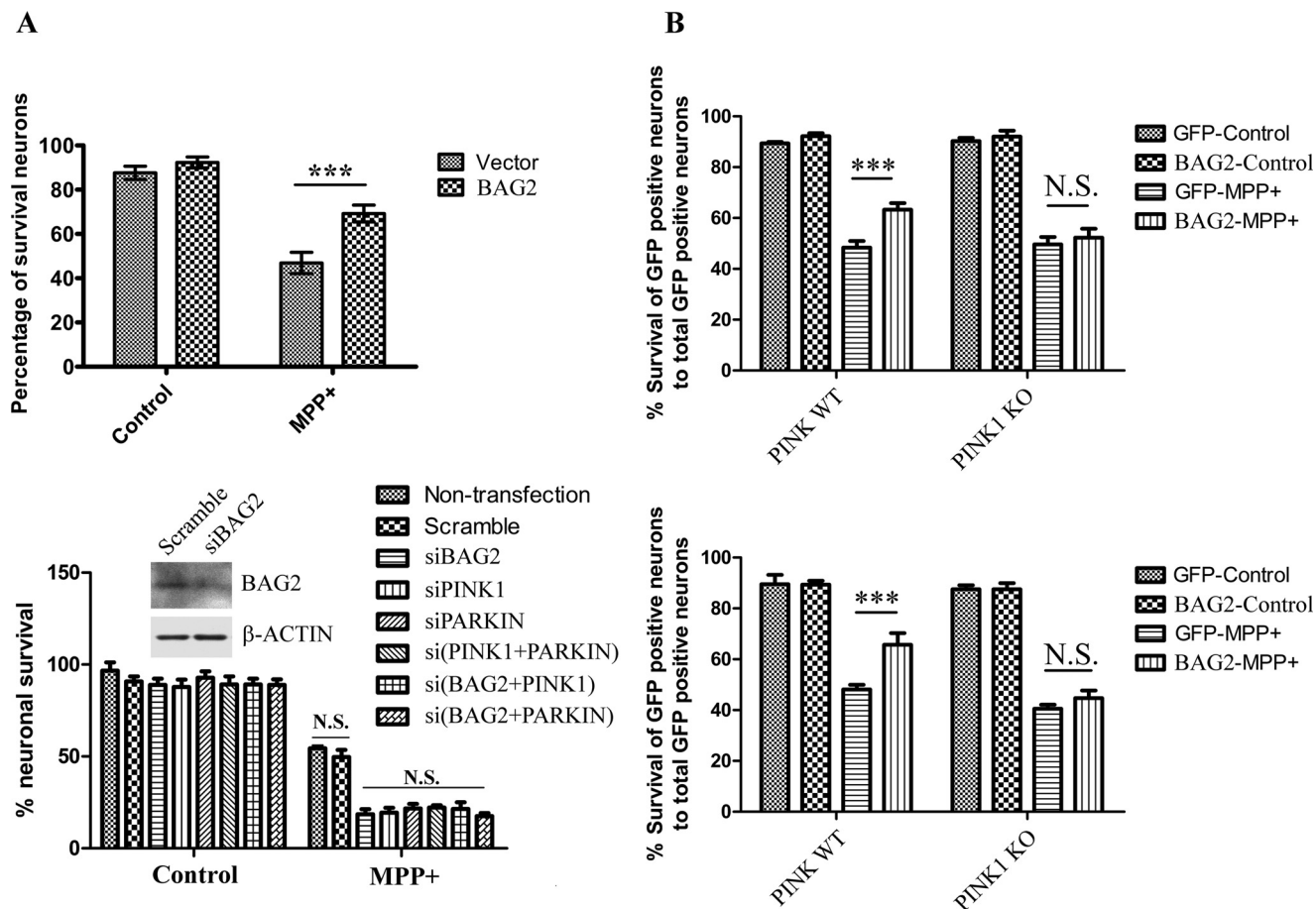


FIGURE 6. BAG2 protects neurons through PINK1 and PARKIN against MPP⁺ challenge. *A*, expression or knockdown of BAG2 in cortical neurons modulates neuronal viability. A plasmid expressing GFP or MYC-BAG2 was transfected into primary cortical neurons. *Top panel*, the number of GFP-positive viable neurons with intact nuclei was normalized to the total number of GFP-positive neurons after MPP⁺ treatment. *Bottom panel*, the neurons were transfected with control scrambled siRNA, bag2 siRNA, pink1 siRNA, parkin siRNA, siRNAs for pink1 and parkin, siRNAs for bag2 and pink1, or siRNAs for bag2 and parkin. Viability was evaluated by MTT assay. The knockdown of BAG2 was confirmed by Western blot analyses. ***, $p < 0.001$; N.S., not significant. *B*, BAG2 plays a protective role through the PINK1/PARKIN signaling pathway. WT, pink1^{-/-}, or parkin^{-/-} neurons were transfected with empty vector or the MYC-BAG2 plasmid. Neuronal viability was evaluated by normalizing GFP-positive neurons with intact nuclei to total GFP-positive neurons. Data are presented as mean \pm S.E. of at least three independent experiments. ***, $p < 0.001$.

whether BAG2 might affect PARKIN levels, thereby explaining the increased PARKIN translocation observed at the mitochondria. However, expression of BAG2 had no effect on PARKIN levels, suggesting that BAG2 did not affect the mitochondrial control pathway through direct regulation of PARKIN levels. Finally, we also determined whether BAG2 might affect PARKIN translocation through a pathway unrelated to PINK1. In this regard, we assessed whether BAG2 might increase PARKIN translocation in the absence of PINK1. However, BAG2 did not increase PARKIN translocation in PINK1 KO neurons. Taken together, our results indicate that BAG2 increases PINK1 levels, which leads to increased PARKIN translocation, an important reported step in mitochondrial quality control.

We also assessed whether and how BAG2 might affect neuronal survival in the presence of mitochondrial stress. We demonstrate that BAG2 expression is protective in this model, in which down-regulation of BAG2 sensitizes neurons to death. Importantly, we showed that the protective effect is completely dependent on the presence of either PARKIN or PINK1. This latter observation is consistent with the notion that both PINK1 and PARKIN have a protective effect in neurons against mito-

chondria and oxidative stress (11, 39, 52). Although our data indicate that the protective effects of BAG2 rely on PINK1/PARKIN, it is important to note that we have not established that mitochondrial quality control is the mechanism by which BAG2 regulates survival. Indeed, some data have indicated that PARKIN-regulated mitophagy does not occur in the presence of mitochondrial damage induced by loss of TFAM (transcription factor A, mitochondrial) (53). However, PARKIN/PINK1-mediated pathways of mitochondrial quality control have been noted in neurons upon oxidative/mitochondrial stress (12, 23). Regardless, our data indicate that BAG2 regulates a protective response through a PINK1/PARKIN-mediated pathway. We propose a model in which this occurs through an increase in PINK1 levels.

Finally, previous works have indicated that import of FL-Pink1 into mitochondria is required for processing of FL-PINK1 to cytosolic PINK1 by different proteases (13, 44–46, 54). The constitutive turnover of PINK1 is presumably a critical regulatory machinery to inhibit the PINK1/PARKIN pathway in healthy cells. On the contrary, FL-PINK1 is accumulated on the outer surface of depolarized mitochondria to

trigger the activation of the PINK1/PARKIN pathway (reviewed in Ref. 55). Our findings demonstrate that BAG2 expression results in the accumulation of different forms of PINK1, especially FL-PINK1, suggesting that BAG2 may block the import of FL-PINK1 into mitochondria, resulting in the accumulation of FL-PINK1. Consistent with this model, we observed more mitochondrial translocation of PARKIN in cells with expression of BAG2 even under conditions lacking mitochondrial stress. We propose two possible mechanisms by which BAG2 manipulates the PINK1 level. First, BAG2 may prevent PINK1 degradation by inhibiting the proteasomal pathway. Second, BAG2 interrupts the import of PINK1 to mitochondria, which would result in inhibition of PINK1 processing and increased FL-PINK1. It will be important to distinguish between these possibilities in future studies.

Author Contributions—D. Q., R. S. S., and D. S. P. designed the study. D. Q. and D. S. P. wrote the paper. D. Q., A. H., and K. D. C. performed the majority of the experiments. E. H., A. J., P. C. M., M. W. C. R., D. S. I., F. S., S. J. H., T. H., and S. C. provided technical assistance. D. D. D. and D. F. designed and performed the proteomic screen. All authors reviewed the results and approved the final version of the manuscript.

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