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Loss of HIPK2 protects neurons from mitochondrial toxins by regulating Parkin protein turnover

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ABSTRACT

Mitochondria are important sources of energy, but they are also the target of cellular stress, toxin exposure, and aging-related injury. Persistent accumulation of damaged mitochondria has been implicated in many neurodegenerative diseases. One highly conserved mechanism to clear damaged mitochondria involves the E3 ubiquitin ligase Parkin and PTEN-induced kinase 1 (PINK1), which cooperatively initiate the process called mitophagy that identifies and eliminates damaged mitochondria through the autophagosome and lysosome pathways. Parkin is a mostly cytosolic protein, but is rapidly recruited to damaged mitochondria and target them for mitophagy. Moreover, Parkin interactomes also involve signaling pathways and transcriptional machinery critical for survival and cell death. However, the mechanism that regulates Parkin protein level remains poorly understood. Here, we show that loss of homeodomain interacting protein kinase 2 (HIPK2) in neurons and mouse embryonic fibroblasts (MEFs) has broad protective effect from cell death induced by mitochondrial toxins. The mechanism by which *Hipk2*$^{-/-}$ neurons and MEFs are more resistant to mitochondrial toxins is in part due to the role of HIPK2 and its kinase activity in promoting Parkin degradation via the proteasome-mediated mechanism. Loss of HIPK2 leads to higher cytosolic Parkin protein level at basal conditions and upon exposure to mitochondrial toxins, which protects mitochondria from toxin-induced damage. In addition, *Hipk2*$^{-/-}$ neurons and MEFs show increased expression of PGC-1α, a Parkin downstream target that can provide additional benefits via transcriptional activation of mitochondrial genes. Together, these results reveal a previously unrecognized avenue to target HIPK2 in neuroprotection via Parkin-mediated pathway.

SIGNIFICANCE STATEMENT

In this study, we provide evidence that HIPK2 and its kinase activity promote Parkin degradation via the proteasome-mediated pathway. Loss of HIPK2 increases cytosolic and mitochondrial Parkin protein levels under basal condition and upon exposure to mitochondrial toxins, which protect mitochondria from toxin-induced damage. In addition, *Hipk2*$^{-/-}$ neurons and mouse embryonic fibroblasts also show increased expression of PGC-1α, a Parkin downstream target that can provide additional benefits via transcriptional activation of mitochondrial genes. These results indicate that targeting HIPK2 and its kinase activity can have neuroprotective effects by elevating Parkin protein levels.

INTRODUCTION

Mitochondria provide important sources of cellular energy via the execution of a wide range of functions, including respiratory complex assembly, ATP production, Ca^{2+} homeostasis and radical oxygen species (ROS) production. In addition, mitochondria interact with many intracellular organelles, such as endoplasmic reticulum (ER), autophagosomes, lysosomes and microtubules, to maintain cellular homeostasis (Sheng and Cai, 2012; Youle and van der Bliek, 2012). Dysfunctions in mitochondria have been implicated in neurodevelopmental disorders and neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis. In addition to genetic mutations that directly disrupt mitochondrial quality control, there is compelling evidence that misfolded proteins associated with

neurodegenerative diseases, including α-synuclein, amyloid-β, tau, TDP-43 and mutant SOD1 proteins, can also disrupt normal mitochondrial functions (Eckert et al., 2010; Israelson et al., 2010; Nakamura et al., 2011; Wang et al., 2016).

First identified in patients with familial Parkinson's disease, mutations in *Parkin* (*PARK2*) and *PINK1* (*PARK6*) interfere with the normal functions of Parkin and PINK1 in the ubiquitination of the outer membrane of mitochondria to promote mitophagy, an important and evolutionarily conserved mitochondrial quality control process that identifies impaired mitochondria and removes them from the mitochondrial network (Klein and Westenberger, 2012; Narendra and Youle, 2011). Parkin is an E3 ubiquitin ligase that is predominantly cytosolic under basal conditions. However, when cells are treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP), which uncouples mitochondrial membrane potentials by increasing the inner membrane permeability to protons, Parkin is rapidly recruited to the outer membrane of mitochondria (Narendra et al., 2008). Several lines of evidence indicate that PINK1 promotes Parkin recruitment to the mitochondria, and that both Parkin and PINK1 are required to promote the degradation of mitochondria via the autophagosome pathway (Matsuda et al., 2010; Vives-Bauza et al., 2010). In addition to chemical uncouplers, chronic oxidative stress and mitochondrial dysfunction can also promote Parkin recruitment to mitochondria.

Besides its role in mitophagy, Parkin can affect mitochondrial function via transcriptional mechanism. For instance, yeast two-hybrid screens identified human PARIS (ZNF746) as a Parkin-interacting substrate that contains a Kruppel-associated box (KRAB) in its N-terminus and a C2HC/C2H2 zinc finger domain at its C-terminus (Shin et al., 2011). Down-regulation or inactivation of Parkin leads to increased PARIS,

112 which suppresses the transcription of PGC-1α and PGC-1α downstream target genes
113 that are critical for mitochondrial functions. Consistent with these results, co-expression
114 of Parkin and PGC-1α increases the number of mitochondria, enhances mitochondrial
115 respiration, and promote functional recovery of mitochondria after membrane
116 uncoupling (Zheng et al., 2017). Despite the robust evidence supporting the role of
117 Parkin in mitophagy, the exact mechanism that regulates the distribution of Parkin in the
118 cytosol and its recruitment to mitochondria upon membrane uncoupling remains poorly
119 understood. It is also unclear what controls Parkin protein levels and whether increasing
120 cytosolic Parkin protein level has any beneficial effect in protecting neurons from
121 mitochondrial toxicity.

122 Homeodomain interacting protein kinase 2 (HIPK2) is a serine/threonine kinase that
123 regulates tumorigenesis, neural development and tissue fibrosis (Blaquiere and
124 Verheyen, 2017; Fan et al., 2014; Hofmann et al., 2013). In our recent studies, we have
125 shown that loss of HIPK2 promotes neuronal survival when exposed to endoplasmic
126 reticulum (ER) stress induced by misfolded $SOD1^{G93A}$ proteins or by mitochondrial toxin
127 1-Methyl-4-(2′-methylphenyl)-1,2,3,6-tetrahydropyridine hydrochloride (MPTP)(Lee et al.,
128 2016; Shang et al., 2018). Here, we show that *Hipk2*$^{-/-}$ neurons have broader resistance
129 to mitochondrial toxins and that such neuroprotective effect is due in part to a persistent
130 increase of Parkin protein levels in the cytosol and mitochondria under normal growth
131 conditions and upon mitochondrial membrane uncoupling. We further show that HIPK2
132 promotes Parkin degradation via the proteasome-mediated mechanism and this
133 process requires HIPK2 kinase activity. Consistent with the elevated Parkin protein level,
134 *Hipk2*$^{-/-}$ neurons show increased expression of PGC-1α, which can activate the

transcription of target genes that promote mitochondrial functions. Taken together, these results support the idea that targeting HIPK2 and its kinase activity may promote neuronal survival under stress conditions by regulating Parkin protein level.

MATERIALS AND METHODS

Animals. *Hipk2*$^{-/-}$ mice (*Hipk2*^{tm1Ejh}/*Hipk2*^{tm1Ejh}, RRID:MGI:5008273 and RRID:MGI:3510466) have been described previously (Wiggins et al., 2004; Zhang et al., 2007). *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mice in the mixed C67BL/6 and 129 background were used at 2 months old. *TH-IRES-Cre* mice (*Th*$^{tm1(cre)Te}$, MGI Cat# 3056580) were previously described (Lindeberg et al., 2004; Tang et al., 2009). *R26R*^{HIPK2} mice were described previously (Shang et al., 2018) and maintained in mixed C57BL/6 and 129 background. Mice of both genders were selected and assigned to each age or treatment group randomly. Animal care was approved by the Institutional of Animal Care and Use Committee (IACUC) at the University of California San Francisco and followed the NIH guidelines.

MPTP treatment in *Hipk2*$^{+/+}$, *Hipk2*$^{-/-}$ and *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$ mice. Treatment of *Hipk2*$^{+/+}$, *Hipk2*$^{-/-}$ and *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$ mice with 1-Methyl-4-(2′-methylphenyl)-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) was conducted according to previously published methods (Shang et al., 2018). Briefly, 4 mg/kg of MPTP (Sigma-Aldrich Cat# M103) or equivalent volume of PBS was injected intraperitoneally (IP) into two-month-old male and female mice (littermates) by one injection per day for ten consecutive days. The mice were monitored according to the

158 approved IACUC protocol and their health was scored prior to each injection. Seven
159 days after the last MPTP treatment mice were euthanized and perfused with 4% PFA
160 prior to brain extraction. The brains were post-fixed in 4% PFA overnight, followed by
161 serial cryoprotection in 15% and 30% sucrose for 24 hours each. The brains were
162 embedded in OCT for cryosectioning and cut into 40 μm coronal sections.
163
164 **Electron microscopy and quantification of mitochondria morphology.** Mice were
165 deeply anesthetized with avertin (150 mg/kg) and were perfused transcardially with 2%
166 paraformaldehyde (PFA)/0.2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 with
167 gravity. Brains were quickly removed, fixed overnight in 2% PFA at 4°C, and cut into 60
168 μm-thick frontal sections with vibratome. Finally, the sections were post-fixed in 0.5%
169 osmium tetroxide for 10 min, dehydrated, and embedded in resin (Durcupan ACM). To
170 identify dopaminergic (DA) neurons to electron microscopic analysis, we prepared
171 100μm brain sections at the level of Bregma -2.75 from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mice. The
172 substantia nigra was then dissected using a Nikon Stereomicroscope. Serial ultrathin
173 sections of *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ substantia nigra were cut with a Reichert Ultracut S,
174 contrasted with lead citrate. Ultra-thin sections were cut at 1-μm thick with a Reichert
175 Ultracut S, contrasted with lead citrate, and imaged in a Phillips Tecnai10 transmission
176 electron microscope using FEI software. Mitochondrial morphology in was characterized
177 by measuring the ferret diameter and surface area of mitochondria in DA neurons in the
178 substantia nigra of control and MPTP-treated *Hipk2*$^{+/+}$, *Hipk2*$^{-/-}$ and *TH-IRES-*
179 *Cre;R26R*$^{HIPK2/HIPK2}$ mice. The measurements were performed in three mice per
180 genotype and mitochondria in 10 DA neurons were selected for quantification.

Primary dopaminergic (DA) neuron cultures and mouse embryonic fibroblasts (MEFs). Primary DA neurons were prepared according to published procedures (Luo et al., 2016; Tang et al., 2010; Zhang et al., 2007). Briefly, E13.5 *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mouse embryos were collected from time-pregnant *Hipk2*$^{+/-}$ females. The ventral mesencephalon was dissected, dissociated after treatment with trypsin and cultured in DMEM-F12 medium (Invitrogen, Cat# 11765-054) supplemented with 10% FBS, 1X penicillin/streptomycin, and 2mM glutamine (Gibco) on cover slides coated with poly-DL-ornithine hydrobromide (Sigma-Aldrich, Cat# P8638) overnight and laminin (Sigma-Aldrich, Cat# L2020) for 2 hours. On DIV1, the medium was replaced with DMEM-F12 medium with 1X N2 supplement (Gibco, Cat# 17-502-048), 20 ng/ml FGF2, 100 ng/ml FGF8, 1X penicillin/streptomycin for two days. To characterize neurotoxic responses, *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ DA neurons were treated with 1-methyl-4-phenylpyridinium (MPP+)(Sigma-Aldrich, Cat# D048, 5 μM), rotenone (Sigma-Aldrich, Cat# R8875, 0.25, 0.5, 1, 2.5 or 5 nM), paraquat dichloride (1,1'-Dimethyl-4,4'-bipyridinium dichloride, Sigma-Aldrich, Cat# 856177, 5, 10, 25 or 50 μM), or carbonyl cyanide-m-chlorophenylhydrazone (CCCP)(Sigma-Aldrich, Cat# C2759, 0.25, 0.5, 1, 2.5 or 5 nM) on DIV3 for 24 hours. As control, each treatment regimen included identical primary neuron cultures where the neurons were incubated with control media without any neurotoxin. After 24 hours incubation, cultured neurons were fixed with 4% PFA for 10 min, and stained with anti-TH antibody (Chemicon, Cat# AB152) and anti-Tuj1 antibody (Covance, Cat# PRB-435P), following the standard straining procedures. Confocal images of the cultured neurons were captured using the Leica confocal microscope

(TCS SP, Leica). Laser intensity (measured as the PMT levels) for each fluorophor was kept within the linear range. TH+ or TuJ1+ Cell number was determined by NIH ImageJ online software. The percentage of surviving neurons was calculated using the total number of TH+ or TuJ1+ neurons in neurotoxin-treated coverslips divided by the total number of TH+ or TuJ1+ neurons in control DMSO-treated coverslips. Data represented results from four independent biological replicates.

Measurements for Parkin recruitment to mitochondria, mitochondrial mass, membrane potentials, and energetics using Seahorse XF Cell Mito Stress Test.

Hipk2$^{+/+}$ and *Hipk2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and HEK293 cells have been reported in our previous studies (Lee et al., 2016; Shang et al., 2018; Wei et al., 2007). To characterize Parkin recruitment to mitochondria, *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were transfected with FLAG-Parkin cDNA using Lipofectamine (ThermoFisher Scientific, Cat# L3000-015). Twenty-four hours after transfection, cells were treated with DMSO or 5 μM CCCP for 2 hours, fixed in 4% PFA for 30 min and processed for immunofluorescent staining with anti-FLAG M2-monoclonal antibody (Sigma, Cat# F3165, 1:1,000 dilution) and anti-Tom20 antibody (Santa Cruz Biotechnology, Cat# sc-11415, 1:1,000 dilution). Images of these cells were captured using Nikon C2 Confocal Microscope. Parkin and Tom20 fluorescent signal intensity was measured using NIS-Elements software.

To measure mitochondrial mass, MitoTracker Green FM Dye (ThermoFisher Scientific, Cat# M7514) and MitoTracker Red FM Dye (ThermoFisher Scientific, Cat# M22425) were added to *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs according to manufacturer's instructions. Afterward, *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were treated with DMSO or 5 μM

CCCP for 6 or 24 hours, and collected for fluorescence-activated cell sorting (FACS) analyses using FACSAria™ III (BD Biosciences, San Jose, CA) at UCSF Parnassus Flow Cytometry Core. Respiratory and glycolytic rates in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were measured using the Seahorse Extracellular Flux (XF) Analyzer 96-well plate reader (Agilent Technologies) as previously described (Mendelsohn et al., 2018). Briefly, *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were seeded at 150,000 cells per well in Seahorse assay medium, supplemented with 10 mM pyruvate in 96-well plate precoated with 22.4 μg/ml Cell-Tak and Tissue Adhesive (Corning, Cat# CB40240). Respiration and glycolysis were simultaneously measured based on oxygen consumption rates (OCRs) and extracellular acidification consumption rate (ECAR), respectively. OCR and ECAR were measured 3 times before injection and 3 times after sequential injection of 2 μg/ml oligomycin or 2 μM carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) (Sigma-Aldrich, Cat# C2920). The measurements at each time point were normalized to the value of the first time point on a well-by-well basis.

Protein lysate preparation, mitochondria isolation and western blot analysis. Mitochondria were isolated from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ brain and MEFs using Mitochondria Isolation Kits for tissues and cells, respectively (ThermoFisher, Cat# 89801 and 89874). Protein lysates were prepared from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs or mitochondria isolated from these cells using NP-40 lysis buffer (1% NP-40, 20 mM Tris, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitor cocktail. To prepare protein lysates from brain tissues, we first micro-dissected substantia nigra or cerebral cortex from 2 months old *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mice and

lysed the tissues using RIPA buffer (0.1% SDS, 1% sodium deoxycholate, 1% NP-40, 20 mM Tris, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitor cocktail. Protein lysates were separated by SDS-PAGE and transferred to the PVDF membrane (Millipore). The membrane was blocked in 4% BSA (for phosphor-antibodies) or 5% non-fat milk for non-phosphorylated antibodies before incubated with primary antibodies overnight at 4°C. The sources and conditions for the primary antibodies were: anti-Parkin antibody (Abcam, Cat# ab15954, 1:2,000 dilution for western blot), anti-FLAG M2 antibody (Sigma, Cat# F3165, 1:1,000 for western blot), anti-NeuN antibody (Millipore, Cat# ABN78, 1:500 dilution for IF microscopy), anti-PGC-1α antibody (Calbiochem, Cat# KP9803, 1:1,000 dilution for western blot and IF microscopy), anti-Tom20 antibody (Santa Cruz Biotechnology, Cat# sc-11415, 1:5,000 for western blot), anti-VDAC antibody (Cell Signaling, Cat# 4661s, 1:1,000 dilution for western blot), anti-cytochrome c antibody (BD Biosciences, Cat# 556432, 1:500 for IF microscopy), and anti-actin antibody (Calbiochem, Cat# CP01, 1:5,000 dilution for western blot). The membranes were washed with 0.1% TBST washing buffer followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Western blots were developed by ECL Chemiluminescence (ThermoFisher Scientific, Cat# 32132).

Quantification of western blot results was performed by normalizing the protein of interest to the loading controls, which included actin for cytosolic proteins and Tom20. Or VDAC1 for mitochondrial proteins. The normalized protein levels were then compared to those in *Hipk2*$^{+/+}$ or basal conditions to obtain the fold change between *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ (Figures 3B and 6C) or different experimental conditions (Figure

3D). The quantitative analysis in Figure 4J was performed by using normalized Parkin level in the cytosol of *Hipk2*$^{+/+}$ MEFs treated with DMSO.

Experimental design and statistical analyses. For both *in vivo* and *in vitro* studies, at least three biological replicates were used in each study. Experiments in which N was greater than 3, the exact number of replicates will be indicated. All data were expressed as mean ± SEM. Data were analyzed using the following statistics with Prism (GraphPad Software, San Diego, CA):

Figure 1B, 2B-2C, 3B, 3D, 4I, 4K, 5C, 5E, 6B, 6D: two-tailed Student's *t* test

Figure 1C-1H: two-way ANOVA

Data was used to compare the differences between the mean values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, whereas $p > 0.05$ is considered non-significant (n.s.).

RESULTS

Loss of HIPK2 protects neurons from mitochondrial toxins

Our previous results showed that *Hipk2*$^{-/-}$ DA neurons are more resistant to mitochondrial toxin MPTP, whereas overexpressing HIPK2 in DA neurons using *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$ mice enhances MPTP-induced cell death (Shang et al., 2018). Given the effects of MPTP as a mitochondrial complex I inhibitor, we asked whether loss of HIPK2 may have broader protective effects on mitochondrial toxins. To test this, we established primary DA neuron cultures from the ventral mesencephalon of *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mice at embryonic day 13.5 (E13.5). Consistent with the in vivo data, TH+;TuJ1+ DA neurons from *Hipk2*$^{-/-}$ mice were more resistant to MPP+-induced

296 toxicity (65% survival in *Hipk2*$^{-/-}$ DA neurons vs 12% in *Hipk2*$^{+/+}$ DA neurons, Figure 1A-
297 B). Interestingly, a direct comparison between TH-;Tuj1+ *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ neurons
298 showed that *Hipk2*$^{-/-}$ non-DA neurons were also more resistant to MPP+ (Figure 1B),
299 suggesting that loss of HIPK2 might have more general neuroprotective effects toward
300 mitochondrial toxicity. To test this, we treated *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ primary neurons with
301 additional mitochondrial toxins, rotenone, paraquat and carbonyl cyanide *m*-
302 chlorophenyl hydrazine (CCCP), which inhibits complex I, generates excess superoxide
303 radicals or uncouples oxidative phosphorylation, respectively (Castello et al., 2007; Li et
304 al., 2003). Consistent with the results from MPP+, both *Hipk2*$^{-/-}$ DA and non-DA neurons
305 showed dose-dependent resistance to these mitochondrial toxins (Figure 1C-H).
306 To further characterize the resistance of *Hipk2*$^{-/-}$ neurons to MPTP, we examined the
307 mitochondrial morphology in the substantia nigra DA neurons of 2 months old *Hipk2*$^{+/+}$,
308 *Hipk2*$^{-/-}$ and *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$ mice. In control PBS-injected mice, the
309 mitochondrial morphology in the substantia nigra DA neurons of *Hipk2*$^{+/+}$, *Hipk2*$^{-/-}$ and
310 *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$ mice showed no significant differences in the maximal
311 diameter or surface area (Figure 2A-C). However, following MPTP treatment, the
312 mitochondrial diameter and surface area in *Hipk2*$^{+/+}$ and *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$
313 neurons were significantly enlarged with dissolution of cristae and disruption of
314 membrane integrity (Figure 2A). In contrast, the mitochondria in the substantia nigra DA
315 neurons in *Hipk2*$^{-/-}$ mice showed no significant enlargement or disruption of morphology.
316 Taken together, these results support the idea that loss of HIPK2 renders neurons more
317 resistant to mitochondrial toxins.
318

Loss of HIPK2 increases Parkin protein level via proteasome-mediated mechanism

The resistance of *Hipk2*$^{-/-}$ neurons to mitochondrial toxins raises the possibility that loss of HIPK2 may alter the protein constituents in the mitochondrial membranes to protect neurons from initiating the cell death process. To test this, we characterized a number of proteins that have been previously shown to regulate mitochondrial membrane potentials and responses to cell death signals, including Bcl-2, Bcl-xL, NRF1, Parkin and PARIS, in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mouse brains (Chipuk and Green, 2008; Riedl and Salvesen, 2007; Youle and Strasser, 2008). Among these candidates, a consistent increase in Parkin protein level in was detected in the lysates from the substantia nigra and cerebral cortex of *Hipk2*$^{-/-}$ mice (Figure 3A). In support of these results, we isolated mitochondria from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mouse brain and showed that Parkin protein level was also significantly elevated in the mitochondria isolated from *Hipk2*$^{-/-}$ mouse brain (Figure 3A-B). No difference was detected in the level of Bcl-xL, NRF1 or PARIS (Figure 3C).

The elevated Parkin protein level in multiple regions of *Hipk2*$^{-/-}$ mouse brain raised the possibility that HIPK2 may regulate Parkin via transcription or post-translational mechanism. Since our previous transcriptomic analyses showed no difference in *parkin* mRNA level in *Hipk2*$^{-/-}$ mouse brain (Shang et al., 2018), we asked whether HIPK2 may regulate Parkin protein turnover via proteasome-mediated degradation. To this end, we expressed FLAG-tagged Parkin and HIPK2 in HEK293 cells and determined Parkin protein levels in these cells using western blots. Our results showed that wild type HIPK2 reduced Parkin protein level by ~50%, whereas kinase inactive HIPK2^{K221A} did

not consistently affect Parkin protein levels (Figure 3D-E). Interestingly, the effects of wild type HIPK2 to promote the reduction in Parkin was blocked by proteasome inhibitor MG132. Together, these results support the idea that HIPK2 regulates Parkin protein level via proteasome-mediated mechanisms and this activity requires HIPK2 kinase activity.

Elevated Parkin protein level in the mitochondria of *Hipk2*$^{-/-}$ MEF

Given the elevated Parkin protein levels in *Hipk2*$^{-/-}$ mouse brain tissues, we asked how this might affect mitochondrial morphology during CCCP-induced membrane uncoupling. To test this, we expressed FLAG-Parkin in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs and treated these cells with DMSO or 5 μM CCCP. In control DMSO-treated conditions, Parkin fluorescent intensity in the cytoplasm and mitochondria was consistently higher in *Hipk2*$^{-/-}$ MEF than that in *Hipk2*$^{+/+}$ MEF (Figure 4A-B, E-F, I). Upon CCCP treatment, *Hipk2*$^{+/+}$ MEF showed rapid recruitment of Parkin to Tom20-positive mitochondria and a marked reduction in overall cytoplasmic Parkin protein level (Figure 4C-D). However, unlike *Hipk2*$^{+/+}$ MEF, *Hipk2*$^{-/-}$ MEF continued to have a high abundance of Parkin in the cytoplasm similar to the level at basal conditions (Figure 4G-H). Consistent with these results, quantification of Parkin fluorescent intensity indeed confirmed the higher Parkin protein levels in *Hipk2*$^{-/-}$ MEF compared to *Hipk2*$^{+/+}$ MEF (Figure 4I).

To further characterize the subcellular distribution of Parkin, we used western blots to characterize Parkin protein levels in the cytosol and mitochondria from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs that had been treated with DMSO or CCCP. Consistent with the results from immunofluorescent microscopy, western blots using protein lysates from the

cytosol showed that the basal Parkin protein level in the cytosol of *Hipk2*$^{-/-}$ MEF was higher than that in *Hipk2*$^{+/+}$ MEF (Figure 4J-K). Upon CCCP treatment, *Hipk2*$^{+/+}$ MEF showed a modest reduction in Parkin protein level in the cytosol, whereas *Hipk2*$^{-/-}$ MEF showed a significant increase of Parkin in the cytosol. Western blot analyses using mitochondria from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEF showed that under control DMSO treatment condition Parkin protein level was low in *Hipk2*$^{+/+}$ MEF, but CCCP treatment induced Parkin recruitment to the mitochondria in *Hipk2*$^{+/+}$ MEF (Figure 4J-K). In contrast to *Hipk2*$^{+/+}$ MEF, mitochondria in *Hipk2*$^{-/-}$ MEF showed significantly higher basal Parkin protein level in the mitochondria before and after CCCP treatment (Figure 4J-K). Taken together, these results support that loss of HIPK2 results in consistently high Parkin protein levels in the cytosol and mitochondria at basal level and after CCCP-induced mitochondrial membrane uncoupling.

Mitochondria in *Hipk2*$^{-/-}$ MEFs are resistant to chemical-induced membrane uncoupling

To characterize how loss of HIPK2 affects the mitochondrial membrane potentials, we incubated *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs with tetramethylrhodamine methyl ester (TMRM), a vital dye that detected mitochondrial membrane potentials, followed by sequential treatments with 2 μg/ml oligomycin to inhibit ATP synthase in complex V and 2 μM carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) to disrupt the proton gradient in mitochondrial membrane potential. This approach revealed no difference in TMRM signal intensity between *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs following oligomycin or FCCP treatment up to 10 minutes (Figure 5A). Furthermore, Seahorse

388 mitochondrial stress assays showed no difference in oxygen consumption rates (OCR)
389 or extracellular acidification consumption rate (ECAR) between *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$
390 MEFs (data not shown).

391 To further determine how loss of HIPK2 affects mitochondrial membrane potentials
392 and integrity, we incubated *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs with MitoTracker Red and
393 MitoTracker Green, which measured mitochondrial membrane potential and the
394 mitochondrial mass by labeling the inner membrane, respectively. After incubation with
395 these dyes, *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were treated with 5 μM CCCP and collected at 2,
396 6, and 24 hours after treatment for fluorescence-activated cell sorting (FACS) analyses.
397 Our results showed that CCCP did not alter the distribution and relative intensity of
398 mitochondrial membrane potentials in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs at 2 hours after
399 treatment. Interestingly, beginning at 6 hours and more prominently at 24 hours CCCP
400 treatment increased MitoTracker Red and MitoTracker Green staining intensity in 23-
401 30% of *Hipk2*$^{+/+}$ MEF (Figure 5B-C). In contrast, only 12-17% of *Hipk2*$^{-/-}$ MEF showed
402 similar changes at 6 and 24 hours. Consistent with these results, double
403 immunofluorescent confocal microscopy revealed that whereas CCCP treatment
404 induced dissociation of cytochrome C and Tom20 in >50% of *Hipk2*$^{+/+}$ MEF (arrowheads,
405 Figure 5D), only ~15% *Hipk2*$^{-/-}$ MEF showed similar dissociation of cytochrome C from
406 their mitochondria (Figure 5D-E). These results suggest that mitochondria in *Hipk2*$^{-/-}$
407 MEF were more resistant to CCCP-induced changes in mitochondrial membrane
408 potentials and integrity.
409

410 **Up-regulation of Parkin target PGC-1α in *Hipk2*$^{-/-}$ brain and MEF**

411 Previous studies have revealed several target genes downstream of Parkin,
412 including PPARγ coactivator-1α (PGC-1α), which is a transcriptional coactivator that
413 regulates a number of genes that have important roles in mitochondrial functions (Lin et
414 al., 2005; Shin et al., 2011; St-Pierre et al., 2006). Given the increase in Parkin protein
415 levels in *Hipk2*$^{-/-}$ neurons and MEF, we asked whether PGC-1α is up-regulated in these
416 cells. In support of this idea, immunofluorescent confocal microscopy showed most DA
417 neurons in the substantia nigra and neurons in the motor cortex in *Hipk2*$^{-/-}$ mice had
418 stronger signal intensity for PGC-1α (Figure 6A-B). These results were further validated
419 using western blots that showed higher abundance of PGC-1α proteins in the substantia
420 nigra and cortex of *Hipk2*$^{-/-}$ mice and in *Hipk2*$^{-/-}$ MEF (Figure 6C-D). Given the role of
421 PGC-1α as a transcriptional coactivator of genes that regulate mitochondrial biogenesis,
422 these results suggested that the elevated PGC-1α in *Hipk2*$^{-/-}$ neurons and MEF most
423 likely provide additional protection from mitochondrial toxicity.
424
425 **DISCUSSION**
426 Results from this study show that HIPK2 can affect the proteasome-mediated
427 degradation of Parkin. Loss of HIPK2 in neurons and MEFs results in higher and
428 saturated Parkin protein levels in the cytosol and mitochondria at basal growth
429 conditions and after uncoupling of mitochondrial membrane. These conditions protect
430 neurons and MEFs from toxicity caused by mitochondrial toxins perhaps through
431 protecting mitochondria from injury, rapid recovery of damaged mitochondria, or both.
432 While the exact mechanism for how HIPK2 regulates Parkin protein turnover remains
433 unclear, several previous studies have implicated HIPK2 in posttranslational

434 modification of proteins via acetylation- and SUMO-mediated degradation pathways.
435 For instance, HIPK2 can phosphorylate E3 ubiquitin ligases SIAH1 and SIAH2, which
436 are the mammalian homologs of the *Drosophila* gene *seven in absentia* that regulate
437 the activity of tumor suppressor p53 (Grishina et al., 2012). Furthermore, elevated
438 reactive oxygen species (ROS) can enhance HIPK2 acetylation and promote cell death
439 (de la Vega et al., 2012). These results support the idea that HIPK2 most likely
440 functions in a highly interconnected regulatory loop where HIPK2 and its interacting
441 partners regulate the turnover of proteins critical for survival and cell death.
442 Of the three members in the HIPK family, HIPK1 is localized mostly in the cytoplasm,
443 HIPK3 is mostly in the nucleus, whereas HIPK2 can be detected in both compartments
444 (Ritter and Schmitz, 2019). These results further support the expanding role of HIPK2 in
445 regulating survival and cell death mechanisms via both transcriptional machinery in the
446 nucleus and proteasome pathways in the cytoplasm. In light of these results, it is
447 Interesting to note that exposure to chemical stressor sodium arsenite leads to a rapid
448 and complete translocation of HIPK2 to the nucleus. It is conceivable that such dynamic
449 regulation of HIPK2 in different subcellular compartments may be coupled with
450 stabilization of potential HIPK2 interacting partners, such as Parkin, that can impact on
451 survival and cell death.
452 Mutations in Parkin are associated with familial Parkinson's disease. Indeed, most
453 functional characterizations of Parkin have provided strong evidence supporting the role
454 of Parkin in repairing damage mitochondria via mitophagy, a form of selective
455 autophagy process (Narendra and Youle, 2011). Interestingly, Parkin deficient mice do
456 not show any detectable degeneration in DA neurons (Goldberg et al., 2003; Von Coelln

et al., 2004). By contrast, overexpression of wild type Parkin in neurons using lentivirus or transgenic approach protects DA neurons from toxicity caused by mutant α-synuclein or MPTP, respectively (Bian et al., 2012; Lo Bianco et al., 2004). Consistent with these results, the robust upregulation of Parkin protein levels in *Hipk2*$^{-/-}$ brain tissues and MEFs provide supporting evidence that *Hipk2*$^{-/-}$ neurons and MEFs are indeed much more resistant to mitochondrial toxins, such as MPTP, rotenone and paraquat.

While most attention on Parkin has focused on its role in mitophagy, there is evidence that Parkin is involved in several non-mitophagy mechanisms. For instance, Parkin ubiquitinates misfolded Pael receptor in the endoplasmic reticulum (ER) and promotes the degradation of Pael receptors (Imai et al., 2001). This presumably alleviates ER stress induced by the accumulation of misfolded Pael receptor. In addition, Parkin can also interact with transcriptional repressor PARIS, which suppresses the expression of transcriptional coactivator PGC-1α and its downstream target genes that can promote mitochondrial biogenesis (Shin et al., 2011). Finally, using a small interfering RNA (siRNA) screen combined with high-content microscopy, Hasson and colleagues identified several candidates that involve in diverse cellular processes that could influence the Parkin signaling pathway, including mitochondrial protein TOMM7 and heat shock protein HSPA1L1 (Hasson et al., 2013).

The implication of HIPK2 in regulating proteasome-mediated Parkin protein degradation raises the question as to whether HIPK2 could be a part of signaling pathway(s) that function upstream of Parkin. In support of this idea, several other kinases have been implicated in Parkin-mediated resistance to neurodegeneration. For example, exposure to neurotoxin MPTP activates non-receptor tyrosine kinase c-Abl,

480 which phosphorylates Parkin on tyrosine residue at position 143. This inactivates the
481 ubiquitin E3 ligase activity in Parkin and mitigates its neuroprotective function (Ko et al.,
482 2010). Perhaps most relevant to our current study is the documented role of JNK in
483 Parkin-mediated signaling pathway. Overexpression of Parkin in neuroblastoma cells
484 significantly attenuates the activation of JNK and caspase 3, and protects cells from
485 toxicity caused by dopamine, 6-OHDA or microtubule-depolarizing toxins (Jiang et al.,
486 2004; Ren et al., 2009). In another study, DA neurons in Parkin loss-of-function
487 *Drosophila* mutants exhibit prominent activation of JNK, which directly contribute to
488 neurodegenerative features and behavioral phenotypes in these mutants (Cha et al.,
489 2005). Given the role of HIPK2 and JNK in neurodegeneration induced by ER stress
490 and in mouse models of Alzheimer's disease and amyotrophic lateral sclerosis (Le
491 Pichon et al., 2017; Lee et al., 2016), it is conceivable that HIPK2 and Parkin may have
492 mutual regulation in response to neurodegenerative conditions. Consistent with this idea,
493 previous studies have shown that Parkin and PINK1 can respond to unfolded protein
494 response in the mitochondria to mitigate proteostasis-induced mitochondrial damage
495 (Burman et al., 2017). Finally, the role of HIPK2 in Parkin-mediated protection of
496 mitochondrial integrity and neuronal survival may depend on other non-proteasome-
497 dependent mechanism(s). Future experiments should aim to uncover whether and how
498 HIPK2 may directly or indirectly interact with other partners that could regulate its
499 proteasome-mediated degradation of Parkin.

500 In summary, the results from this study reveal previously unappreciated role of
501 HIPK2 in regulating Parkin protein level via the proteasome-mediated pathway. The

elevated Parkin protein levels in *Hipk2*$^{-/-}$ neurons and MEFs contribute to the resistance of these cells to toxicity caused by mitochondrial toxins.

FIGURE LEGENDS

Figure 1. *Hipk2*$^{-/-}$ neurons are more resistant to mitochondrial toxins. (A) Immunofluorescent confocal microscopic images of primary neuron cultures using ventral mesencephalon of E13.5 *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ embryos. The primary neurons, immunostained with anti-TH (green) and anti-TuJ1 (red) antibodies, were treated with DMSO (control) or MPP+ (5 μM) for 24 hours before they were fixed and processed for image analyses. (B) Quantification of TH+;TuJ1+ or TH-;TuJ1+ neurons from the ventral mesencephalon of E13.5 *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ embryos showed that *Hipk2*$^{-/-}$ TH+;TuJ1+ and TH-;TuJ1+ neurons were more resistant to MPP+ induced toxicity. For each treatment paradigm, we set up one additional neuron culture without any neurotoxin treatment, which was considered as 0 concentration and % survival in these cultures was counted as 100%. Data represented mean ± s.e.m.. Statistics used Student's *t* test, ns, not significant, * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.001$. (C-H) Dose-response curves for *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ TH+;Tuj1+ and TH-;TuJ1+ neurons treated with rotenone (0.25, 0.5, 1, 2.5 or 5 nM), paraquat (5, 10, 25 or 50 μM), or CCCP (0.25, 0.5, 1, 2.5 or 5 nM). Data represented mean ± s.e.m.. Statistics used Two-way ANOVA test, * $P < 0.05$.

Figure 2. Effects of MPTP on the mitochondrial morphology of DA neurons in *Hipk2*$^{+/+}$, *Hipk2*$^{-/-}$ and *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$ mice. (A) Transmission electron

microscopic (TEM) images of mitochondria in DA neurons in 2 months old *Hipk2*$^{+/+}$, *Hipk2*$^{-/-}$ and *TH-IRES-Cre;R26R*$^{HIPK2/HIPK2}$ mice injected with PBS or MPTP (see Materials and Methods for detailed experimental procedures). (B-C) Quantification of mitochondrial morphology by measuring their diameter and area. Four mice per genotype and 10 neurons in the substantia nigra per mouse were used for this quantification. Data represented mean ± s.e.m.. Statistics used Student's *t* test, ns, not significant, * P < 0.05, ** P < 0.01 and *** P < 0.005.

Figure 3. Loss of HIPK2 leads to elevated Parkin protein levels in *Hipk2*$^{-/-}$ brain tissues, whereas HIPK2 overexpression promotes Parkin degradation via the proteasome pathway. (A) Western blot analyses detected Parkin protein levels in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ substantia nigra and cerebral cortex. To characterize Parkin protein levels in mitochondria, fresh brain tissues from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mice were homogenized and mitochondria were isolated using Mitochondria Isolation Kit (ThermoFisher Scientific). (B) Quantification of the relative abundance of Parkin protein levels in the substantia nigra, cerebral cortex and brain mitochondria from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mice. Data represented mean ± s.e.m.. Statistics used Student's *t* test, * P < 0.05 and ** P < 0.01. (C) Western blot results showed no difference in the relative abundance of NRF1, PARIS or Bcl-xL in protein lysates in the substantia nigra or cortex of *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mouse brain. (D) Western blot analysis showed HIPK2 overexpression reduced Parkin protein levels in HEK293 cells, and this effect was not detected in kinase-inactive HIPK2, HIPK2^{K221A}. Treatment with proteasome inhibitor MG132 (10 μM, 4 hours) blocked the effect of HIPK2 to promote Parkin degradation. (E)

548 Quantification of relative Parkin protein levels in HEK293 cells transfected with FLAG-
549 Parkin + wild type HIPK2, FLAG-Parkin + HIPK2^{K221A} or FLAG-Parkin + wild type HIPK2
550 + MG132. Data represented mean ± s.e.m.. Statistics used Student's *t* test, ns, not
551 significant, * P < 0.05 and ** P < 0.01.
552
553 **Figure 4. *Hipk2*$^{-/-}$ MEFs exhibit higher Parkin protein levels in the cytosol and**
554 **mitochondria.** (A-H) Immunofluorescent confocal microscopic images highlight the
555 relatively higher Parkin protein levels in control DMSO-treated and CCCP-treated *Hipk2*$^{-}$
556 $^{/-}$ MEFs. Both *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were transfected with constructs expressing
557 FLAG-Parkin, treated with DMSO or CCCP (5 μM) for 2 hours, fixed in 4% PFA for
558 immunostaining using anti-FLAG and anti-Tom20 antibodies, and processed for image
559 analyses using the Nikon C2 confocal microscope. Insets in panels A, C, E and G
560 represent higher magnification images of the highlighted areas. The scale bar in A, C, E
561 and G is 10 μm and 2.5 μm in insets. FLAG-Parkin and Tom20 immunofluorescent
562 intensities were measured using Nikon NIS-Elements software by drawing a line across
563 the cytoplasm of *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs (white lines in the "Merge" panels of A, C, E
564 and G). The signal intensity of Parkin and Tom20 and the extent of their colocalization
565 were presented in the corresponding panels in B, D, F and H, where arrowheads
566 indicate the colocalization of Parkin and Tom20. (I) Quantification of overall Parkin
567 immunofluorescent signal intensity in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs (panels A, C, E and G).
568 Data represented mean ± s.e.m. from 28 to 32 Parkin-expressing *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$
569 MEFs from 4 independent biological replicates. Statistics used Student's *t* test, *** P <
570 0.005 and **** P < 0.001. (J) Western blot analysis of Parkin protein levels, detected by

anti-FLAG antibody, in the cytosol and mitochondria of *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs before and after CCCP treatment. Antibodies for actin and Tom20 were used as loading controls for cytosolic and mitochondrial fractions, respectively. (K) Quantification of Parkin protein levels in the cytosol and mitochondria of *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs before and after CCCP treatment. Data represented mean ± s.e.m. from 3 independent biological replicates. Statistics used Student's *t* test, * $P < 0.05$ and ** $P < 0.01$.

Figure 5. CCCP-treated *Hipk2*$^{-/-}$ MEFs show more intact mitochondria, but no change in mitochondrial membrane potentials. (A) Mitochondrial membrane potentials in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were measured by tetramethylrhodamine methyl ester (TMRM) dye. *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were treated sequentially with 2 μg/ml oligomycin and 2 μM FCCP to inhibit ATP synthase in complex V and proton gradient, respectively. (B-C) Fluorescent cell-activated sorting (FACS) data from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs, which were pre-loaded with Mitotracker-Red and Mitotracker-Green to measure mitochondrial membrane potentials and mitochondrial mass, respectively. *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were then treated with DMSO (control) or 10 μM CCCP for 2, 6 or 24 hours and collected for FACS analysis. Cells with higher Mitotracker-Green signals were gated for further quantification and the results were presented in panel B. Data represented mean ± s.e.m. from 3 independent biological replicates. Statistics used Student's *t* test. (D-E) *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs were used in immunofluorescent microscopy using anti-cytochrome C and anti-Tom20 antibodies to determine the integrity of mitochondria under control condition or after membrane uncoupling by CCCP treatment. Cells with mitochondria that showed positive

cytochrome C and Tom20 signals were scored as having intact mitochondrial membrane, whereas those with positive cytochrome C but no Tom20 signal were scored as having no intact mitochondria (highlighted by arrowheads). Insets in panel D represent higher magnification of the highlighted areas. Scale bars in inset are 2.5μm. The percentage of *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs with intact mitochondrial membrane was shown in panel E. Data represented mean ± s.e.m. from 30 *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ MEFs from 3 independent biological replicates. Statistics used Student's *t* test, ns, not significant and * $P < 0.05$.

Figure 6. Upregulation of PGC-1α in *Hipk2*$^{-/-}$ neurons and MEFs. (A) Immunofluorescent confocal microscopic images of PGC-1α expression in substantia nigra DA neurons (labeled by anti-TH antibody) and cortical neurons (labeled by anti-NeuN antibody) in *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ mice. (B) PGC-1α protein immunofluorescence intensity in DA neurons and cortical neurons was quantified using NIH ImageJ. Data represented mean ± s.e.m. from 40 *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ DA neurons and cortical neurons from 4 independent biological replicates. Statistics used Student's *t* test, ** $P < 0.01$. (C-D) Western blot analysis of PGC-1α protein levels in lysates from *Hipk2*$^{+/+}$ and *Hipk2*$^{-/-}$ substantia nigra, cerebral cortex and MEFs**.** Antibody for actin was used as loading control. Data represented mean ± s.e.m. from 4 independent biological replicates. Statistics used Student's *t* test, * $P < 0.05$.

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