The ubiquitin E3 ligase CHIP promotes proteasomal degradation of the serine/threonine protein kinase PINK1 during staurosporine-induced cell death

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Lang Yoo and Kwang Chul Chung

From the Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul, 03722, Korea

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Mutations in the gene for the serine/threonine protein kinase PTEN-induced putative kinase 1 (PINK1) are the second most frequent cause of autosomal recessive Parkinson’s disease (PD). Via its kinase activity, PINK1 regulates neuronal cell survival and mitochondrial quality control. Numerous reports have revealed that PINK1 has diverse and physiologically significant functions, and therefore its activity should be tightly regulated. However, the molecular mechanisms regulating PINK1 stability and the modulator(s) involved have not been elucidated. In this study, we demonstrate that the ubiquitin E3 ligase carboxyl terminus of Hsp70-interacting protein (CHIP) promotes PINK1 ubiquitination and decreases its steady-state levels. Moreover, PINK1 levels were strongly reduced in HEK293 and SH-SYSY cells exposed to the apoptosis-inducer staurosporine. Of note, we found that this reduction resulted from CHIP-mediated PINK1 ubiquitination. Accordingly, siRNA-mediated CHIP knockdown reduced susceptibility to staurosporine-induced cell death. Taken together, these findings suggest that CHIP plays a role in negative regulation of PINK1 stability and may suppress PINK1’s cytoprotective effect during staurosporine-induced mammalian cell death. We propose that this PINK1 regulatory pathway might contribute to Parkinson’s disease pathogenesis.

Parkinson’s disease (PD) is a progressive neurodegenerative disorder (NDD) characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. Although most cases of PD are sporadic, 5–10% have occurred in familial cases of PD.2 Further, a recent article on Future-leading Research Initiative of 2015 Grant 2015-22-0055 (to K. C. C.).

The abbreviations used are: PD, Parkinson’s disease; CHIP, carboxyl terminus of Hsp70-interacting protein. Of note, it is highly likely that Hsp70/90-dependent CHIP affects the stability of PINK1 (10–12). For the first time, we identified CHIP as a novel ubiquitin E3 ligase that targets...
Figure 1. CHIP binds to PINK1 in mammalian cells. A, HEK293 cells were transfected for 24 h with plasmid encoding Myc-PINK1 and/or Xpress-CHIP, and treated for an additional 6 h with 10 μM MG132. Cell lysates were immunoprecipitated with anti-Myc antibody, followed by immunoblotting with the indicated antibodies. Actin served as a loading control. B and C, SH-SYSY cell lysates (B) and mouse brain lysates (C) were immunoprecipitated with anti-PINK1 IgG, followed by immunoblotting with the indicated antibodies. As a negative control, cell lysates were immunoprecipitated with pre-immune IgG (IgG). D, to assay PINK1-CHIP interaction in vitro, bacterial recombinant GST-PINK1-KD (amino acids 112–496) and His-tagged CHIP were purified as indicated in “Experimental Procedures.” The purified proteins were immunoprecipitated with IgG or anti-PINK1 antibody, followed by immunoblotting with the indicated antibodies. Purity of the proteins assessed by Coomassie Brilliant Blue staining. E, representative confocal images of immunostaining of a SH-SYSY cell expressing both Myc-PINK1 (red) and HA-CHIP (blue). Mitochondria were stained with TOM20 (green). F, SH-SYSY cells were treated for 6 h with 10 μM MG132 before fixation. Representative confocal images of immunostaining using endogenous PINK1 (red) and endogenous CHIP (green). Nuclei were counterstained with DAPI (blue). Scale bars, 10 μm.

Results

CHIP binds to PINK1 in mammalian cells

Based on the previous finding that the upstream regulators of PINK1 stability are in some way associated with the chaperone machinery and proteasomal degradation (11), we examined biochemical and functional interactions of PINK1 with chaperone-dependent ubiquitin E3 ligase CHIP. To first determine whether PINK1 and CHIP physically interact in mammalian cells, we performed co-immunoprecipitation (co-IP) analysis of lysates of cells transfected with plasmid encoding Myc-tagged PINK1 alone or together with plasmid encoding Xpress-tagged CHIP. These experiments revealed that exogenous CHIP binds exogenous PINK1 in HEK293 cells (Fig. 1A). The interaction between endogenous CHIP and endogenous PINK1 was further confirmed in SH-SYSY cells, as well as in mouse brain lysates (Fig. 1, B and C). We then examined whether CHIP binds directly to PINK1 using pulldown assays and bacterial GST-fused or His-tagged recombinant proteins. As shown in Fig. 1D, highly purified CHIP was found to interact with GST-tagged PINK1 (consisting of amino acids 112–496), indicating that CHIP binds directly to PINK1 under in vitro conditions. Immunostaining revealed that not only overexpressed Xpress-CHIP and PINK1-Myc but also endogenous PINK1 and CHIP are co-localized, primarily in the cytoplasm (Fig. 1, E and F).

To determine which domain(s) of PINK1 and CHIP are important for binding, several deletion mutants were generated lacking the conserved functional and/or structural domain(s) of each protein. PINK1 contains the N-terminal mitochondrial localization signal, a transmembrane domain, N-terminal regulatory domain, and a kinase domain. The kinase domain of PINK1 consists of N lobe (amino acids 156–320) and C lobe (amino acids 320–509) (17). On the other hand, CHIP has a TPR domain responsible for chaperone binding, a charged coiled-coil domain, and a U-box domain that is essential for ubiquitin ligase activity. The coiled coil domain of CHIP is essential for CHIP dimerization, but rarely binds its substrates (13). These mutants of PINK1 and CHIP were expressed in HEK293 cells, and their interaction assessed by co-IP analysis (Fig. 2A). Co-IP analyses of cell lysates revealed that the full-length PINK1 and its mutant PINK1-Δ1, consisting of residues 111–581, bind well to CHIP. However, mutants PINK1-Δ2, consisting of 156–584, and PINK1-Δ3, consisting of residues 300–581, exhibited greatly diminished binding to CHIP (Fig. 2B). These results suggest that the region of PINK1 containing amino acids 111–299 is necessary for CHIP binding. Additional co-IP analyses using CHIP deletion mutants CHIP-Δ1, consisting of residues 1–215, and CHIP-Δ2, consisting of residues 141–300 revealed that they interacted with PINK1 to an extent similar to that seen with full-length CHIP. However, CHIP-Δ3, consisting of amino acid residues 216–303, exhibited greatly reduced interaction with PINK1, suggesting that the coiled-coil domain (amino acids 141–215) of CHIP is necessary for PINK1 binding (Fig. 2C).
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Taken together, the overall results indicate that PINK1-CHIP binding takes place in mammalian cells via the coiled-coil domain of CHIP and the centrally located region of PINK1 consisting of amino acid residues 111–299, which includes the N lobe region of the kinase domain.

CHIP decreases the protein stability of PINK1

Next, we investigated whether ubiquitin E3 ligase CHIP affects the steady-state level of PINK1. The level of exogenous PINK1 was greatly reduced by exogenous CHIP in a dose-dependent manner (Fig. 3A). To investigate whether the catalytic activity of CHIP plays a role in the down-regulation of PINK1, PINK1-Myc was co-expressed with wildtype Xpress-CHIP, the indicated Myc-tagged PINK1 deletion mutant (B), or the indicated Xpress-tagged CHIP deletion mutants (C) alone or in combination. Cells were treated for an additional 6 h with 10 μM MG132. Cell lysates were immunoprecipitated with anti-Myc antibody, followed by immunoblotting with the indicated antibodies. Tubulin served as a loading control.

Moreover, the CHIP-mediated degradation of PINK1 was prevented by pretreatment of cells with the proteasome inhibitor MG132, but not with the lysosomal inhibitor NH₄Cl, suggesting that PINK1 degradation occurs through the proteasome-dependent pathway (Fig. 3C). In contrast, siRNA-mediated CHIP knockdown in HEK293 cells resulted in a significant increase in endogenous PINK1 level (Fig. 3D). Furthermore, the same pattern of increase in endogenous PINK1 level was observed in CHIP⁻/⁻ mouse embryonic fibroblasts (MEFs) derived from CHIP-KO mice (Fig. 3E) relative to the level in CHIP⁺/+ MEFs. We next assessed whether the effect of CHIP on PINK1 stability occurs through proteolysis by measuring PINK1 half-life. Over-expression of wildtype PINK1 having a C-terminal Myc-tag in HEK293 cells showed that its half-life was ~25–30 min. Co-expression of wildtype CHIP and PINK1-Myc reduced the half-life of PINK1-Myc to about 15 min (Fig. 3F). In contrast, CHIP knockdown caused an increase of PINK1-Myc half-life up to ~55 min (Fig. 3G).

Figure 2. The coiled-coil domain of CHIP binds to the central region of PINK1 containing amino acid residues 111–299. A, diagram of wildtype PINK1 (PINK1-WT) and its deletion mutants (upper panel) and wildtype CHIP (CHIP-WT) and its deletion mutants (lower panel). Results of PINK1-CHIP binding assays are shown on the right. Minus (−), no binding; plus (+), binding (the number represents the extent of binding). B and C, HEK293 cells were transfected for 24 h with plasmid encoding Xpress-CHIP, PINK1-Myc, the indicated Myc-tagged PINK1 deletion mutant (B), or the indicated Xpress-tagged CHIP deletion mutants (C) alone or in combination. Cells were treated for an additional 6 h with 10 μM MG132. Cell lysates were immunoprecipitated with anti-Myc antibody, followed by immunoblotting with the indicated antibodies. Tubulin served as a loading control.
We next investigated the effect of CHIP on two familial PD-linked pathogenic mutants of PINK1, PINK1-G309D, and PINK1-L347P. Previous study reported that, compared with PINK1-WT, the PINK1-G309D and PINK1-L347P mutants exhibit much reduced kinase activity and the protein stability, respectively (18). As shown in the Fig. 3H, CHIP overexpression caused the reduction of these two mutants, and there was not significant difference in the reduction of PINK1-WT, PINK1-G309D, and PINK1-L347P mutants by CHIP. These results indicate that CHIP is capable of degrading both these pathogenic mutants as well as PINK-WT (Fig. 3H).

Taken together, these observations suggest that CHIP promotes the degradation of PINK1 through the proteasomal pathway, possibly through PINK1 ubiquitination.

**CHIP facilitates ubiquitination of PINK1**

As PINK1 protein levels were significantly reduced through CHIP activity (Fig. 3B), it is highly probable that PINK1 is directly polyubiquitinated by CHIP, leading to proteasomal degradation. To explore this, cells were transfected with plasmid encoding PINK1-Myc alone or together with plasmid encoding Xpress-CHIP (F), nonspecific siRNA (NC), or CHIP-siRNA (G). Cells were treated for the indicated times with 20 μg/ml cycloheximide, and cell lysates were immunoblotted with the indicated antibodies (left), and results are quantified (right). The half-lives of each group are marked at the horizontal axis of the graph by an arrowhead. H, HEK293 cells were transfected for 24 h with plasmid encoding Xpress-CHIP-WT, PINK1-Myc, PINK1-L347P-Myc, or PINK1-G309D-Myc alone or in combination. Cell lysates were immunoblotted with the indicated antibodies. Values below top panel indicate the relative intensity of PINK1-Myc band to that of loading control, α-tubulin. Tubulin and actin served as a loading control.
Reduced, comparing with that seen in CHIP+/− MEFs (Fig. 4C).

Moreover, *in vitro* ubiquitination assays of the sample including recombinant CHIP, purified E1/E2 and ubiquitin, and GST-PINK1-KD, demonstrated that CHIP-WT markedly enhances ubiquitination of GST-PINK1-KD (Fig. 4D). Taken together, these results suggest that CHIP directly promotes PINK1 polyubiquitination.

**Hsp70 potentiates CHIP-mediated degradation of PINK1**

As described previously, CHIP acts in dual capacity as ubiquitin E3 ligase and Hsp70 co-chaperone, and Hsp70 is critical for CHIP ubiquitin E3 ligase activity. Therefore, we next determined whether Hsp70 binding to CHIP affects CHIP binding to PINK1 and/or PINK1 ubiquitination. Based on the finding that N-terminal TPR domain of CHIP is required for its interaction with Hsp70 (16), we used the CHIP-K30A mutant, which exhibits its defective binding to Hsp70 via its TPR domain and consequently lacks chaperone-binding activity, as the dominant negative mutant of Hsp70. As CHIP is a U-box–dependent E3 ubiquitin ligase, the CHIP-H260Q mutant, which does not bind to the E2 ubiquitin–conjugating enzyme and so lacks E3 activity, was also used as the catalytic inactive mutant of CHIP (19).

First, HEK293 cells were transfected with plasmid encoding PINK1-Myc, Xpress-CHIP-WT, Xpress-H260Q, or CHIP-ΔU alone or in combination, and treated for 6 h with 10 μM MG132. Cell extracts were immunoprecipitated with anti-Myc antibody, followed by immunoblotting with the indicated antibodies. B, HEK293 cells were transfected for 48 h with PINK1-Myc, nonspecific control siRNA (NC), or CHIP-siRNA alone or in combination, and treated for additional 6 h with 10 μM MG132. Cell extracts were immunoprecipitated with anti-Myc antibody, followed by immunoblotting with the indicated antibodies. C, CHIP-null (CHIP−/−) and control (CHIP+/+) MEFs were treated for 6 h with 10 μM MG132. Cell lysates were immunoprecipitated with anti-PINK1 antibody, followed by immunoblotting with the indicated antibodies. D, where specified, bacterial recombinant E1 and E2 proteins were incubated for 24 h with highly purified GST-ubiquitin, His$_6$-CHIP, GST, or GST-PINK1-KD alone or in combination. Samples were analyzed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue (CBB) or immunoblotting with the indicated antibodies. Tubulin and actin served as loading control, as indicated. Relative ubiquitination level of PINK1-Myc was quantified (bottom of A, B, and C).
extent of polyubiquitination of both exogenous expressed (Fig. 6C) and endogenous (Fig. 6D) PINK1; however, this stimulatory effect was not seen with CHIP-K30A and CHIP-H260Q. Furthermore, overexpression of Hsp70 enhanced the extent of CHIP-mediated PINK1 degradation (Fig. 6, E and F). Conversely, pretreatment with Hsp70 inhibitor methylene blue partially inhibited CHIP-mediated PINK1 degradation, compared with the control sample treated with vehicle alone (Fig. 6, G and H). Furthermore, the knockdown of Hsp70 recovered the reduced PINK1 level up to much more than the control level (Fig. 6I).

Taken together, these data suggest that Hsp70 and its binding to CHIP are necessary for PINK1 ubiquitination and degradation through promotion of the interaction between CHIP and PINK1.

CHIP exacerba tes STS-induced cell death via negative regulation of PINK1 level

Previous reports revealed that PINK1 exerts a cytoprotective effect against various physical and chemical insults in mammalian cells (7, 20, 21). The current finding that CHIP promotes the degradation of PINK1 raises the possibility that CHIP-induced PINK1 degradation affects cell death in response to various stress inducers. To test this possibility, we first examined the effect of treatment with STS, a potent pan-specific protein kinase inhibitor that is commonly used as an apoptotic cell death—inducing agent, on PINK1 proteolysis and viability in HEK293 cells. When HEK293 cells were exposed to STS, both exogenous expressed and endogenous PINK1 levels were greatly decreased (Fig. 7, A–C). Accompanying cell death, the amount of PINK1 ubiquitination was correspondingly increased with STS treatment (Fig. 7D, lanes 1–3). In contrast, PINK1 ubiquitination was remarkably inhibited by siRNA-mediated knockdown of endogenous CHIP. The effect of CHIP knockdown on STS-induced PINK1 ubiquitination was restored after reconstitution of CHIP-WT, but not CHIP-H260Q. (Fig. 7, D, lane 4 and E). These results suggest that STS treatment causes the CHIP-mediated ubiquitination of PINK1 and its degradation.

We then explored whether STS-induced PINK1 degradation affects cell viability. Dopaminergic neuroblastoma SH-SY5Y cells were mock-transfected or transfected for 24 h with plasmid encoding CHIP and/or PINK1. Cell were then treated with vehicle or 0.5 μM STS for an additional 24 h. Measurement of cell viability, using an LDH cytotoxicity assay, revealed that STS treatment alone induces cell death by ~30% in mock-transfected cells, as expected (Fig. 8A). In addition, cells expressing exogenous PINK1 showed an ~5% lower level of STS-induced cytotoxicity, verifying the previous finding that PINK1 exerts a cytoprotective effect against STS in SH-SY5Y cells. Interestingly, cells expressing exogenous CHIP only showed a further decrease in viability with STS treatment relative to the mock-transfected cells. Furthermore, STS treatment of cells expressing exogenous CHIP and PINK1 caused an ~5% greater level of cytotoxicity than in cells expressing exogenous PINK1 alone. Consistent with these data, siRNA-mediated CHIP knockdown markedly decreased the cytotoxicity of STS, compared with cells transfected with nontargeting control siRNA (Fig. 8B). Lastly, when cells were transfected with both CHIP- and PINK1-siRNA and then treated with STS, a much greater level of cell death was induced than in cells with CHIP knockdown alone.

Overall, these data suggest that CHIP potentiates the cytotoxicity of STS in mammalian cells, and this occurs through PINK1 ubiquitination and blockade of PINK1 cytoprotective effect.

Discussion

PINK1 is localized mainly in the mitochondria via a mitochondrial targeting signal; therefore it affects mitochondrial home-
ostasis and dynamics, including mitophagy, bioenergetics, fission, and fusion (2). PINK1 is also detected in the cytosol, where it protects cells against various cytotoxic agents and regulates dendritic morphogenesis in neurons (5, 22). Despite its critical roles in diverse physiological processes, the upstream pathway regulating PINK1 stability is poorly understood. PINK1 is intrinsically unstable in healthy cells and subject to fast turnover. The previous findings that pretreatment of cells with proteasome inhibitor blocks PINK1 degradation but that lysosomal inhibitor has little effect (23, 24), strongly suggest that intracellular proteolysis of PINK1 is primarily mediated by UPS pathway. Several PINK1 binding partners that affect its cellular location and/or stability have been reported. For example, whereas DJ-1 and BAG family members act to negatively regulate PINK1 ubiquitination, the identity of the specific E3 ligase targeting PINK1 is not yet clear (10, 12, 24).

Interestingly, based on the previous finding that the steady-state level of PINK1 is affected by Hsp70 binding and the binding of other chaperone proteins, BAG2 and BAG5, we speculated that the chaperone-dependent ubiquitin ligase CHIP may affect PINK1 degradation. Accordingly, we demonstrated for the first time that CHIP acts as a novel E3 ligase of PINK1, promoting its ubiquitination and subsequent proteasomal degradation, and ultimately resulting in cytotoxicity by reducing the PINK1 cytoprotective effect. In addition, the current study demonstrated that STS-induced ubiquitination and degradation...
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Section of PINK1 is mediated by CHIP, and CHIP knockdown correspondingly suppresses STS-induced cell death in SH-SY5Y cells.

With regard to the subcellular localization and processing of PINK1, nuclear-encoded PINK1 is initially synthesized on cytosolic ribosomes and, under normal unstressed conditions, is imported into mitochondria where it is subject to proteolytic degradation (25). PINK1 is targeted to the mitochondria through its N-terminal mitochondrial targeting sequence, and there it is imported via TOM and TIM complexes (26). During import, PINK1 undergoes a series of proteolytic cleavage steps through the actions of peptidase MPP and rhomboid protease PARL, sequentially cleaving the 64-kDa full-length form into 60- and 52-kDa fragments (27). The 52-kDa fragment is exported back into the cytosol, where it is degraded by the proteasome through an unknown enzyme and/or the N end–rule pathway (9). The present study reveals the involvement of chaperone-bound CHIP in mediating PINK1 ubiquitination and subsequent proteolysis. Despite overall compelling support for this model, many of these localization studies rely on overexpression of exogenous PINK1, because endogenous PINK1 is poorly detected by the available antibodies. It thus appears that the biogenesis and processing of PINK1 is more complex than anticipated and much more study is required for complete understanding.

CHIP E3 ligase activity catalyzes Hsp70-induced ubiquitinilation of client proteins (28). In the CHIP quality control pathways, Hsp70 and Hsp90 play opposite role in determining the fate of the client. Target protein bound to Hsp70 predominantly interacts with CHIP, and then undergoes ubiquitin/proteasome-dependent degradation. In contrast, the formation of a complex between Hsp90 and client protein binds to a co-chaperone other than CHIP, such as p23, and then the client protein undergoes refolding (29). Inhibition of Hsp90 by geldanamycin or 17-AAG increases the concentration of the Hsp70-client complex in the cell, resulting in the rapid degradation of the...
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Figure 8. CHIP promotes STS-induced cell death via PINK1 proteolysis. A, SH-SY5Y cells were mock-transfected or transfected for 24 h with plasmid encoding Myc-tagged PINK1 and/or Xpress-tagged CHIP, and treated for an additional 24 h with DMSO (vehicle) or STS (0.5 μM). Cell toxicity was measured using LDH assay kit. B, SH-SY5Y cells were transfected for 48 h with nonspecific control siRNA (NC), CHIP-siRNA, or PINK1-siRNA, and treated for an additional 24 h with DMSO or STS (0.5 μM). Cell toxicity was measured using LDH assay kit. Data are represented as the mean ± S.D. of three independent experiments (n = 9; *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant).

Regarding its effect on cell viability, CHIP has been considered a double-edged sword because it is able to differentially target proteins in quite different cell-signaling pathways. For example, CHIP exerts a cytotoxic effect through degradation of cell proliferation- and survival-related proteins, including ErBb2, HIF1-α, and c-Myc. In contrast, CHIP may exert a cytoprotective effect on cancer cells through the down-regulation of many tumor suppressor proteins, such as FoxO1, p53, and PTEN (32).

In addition, CHIP has been linked to various NDDs, which are commonly characterized by the occurrence of uncontrolled protein misfolding and formation of toxic protein aggregates (33). For example, CHIP promotes degradation of several key proteins in these disorders, such as tau and amyloid precursor protein in Alzheimer’s disease, α-synuclein and LRRK2 in PD, and SOD1 in amyotrophic lateral sclerosis (34–38). These reports imply that CHIP plays a neuroprotective role by preventing toxic protein aggregation associated with the pathologies of those NDDs.

In contrast, there is some evidence that CHIP transmits a toxic signal in brain disorders. For example, CHIP was found to be a component of Lewy bodies found in the brains of PD patients, where it co-localizes with α-synuclein and Hsp70 (37). In a cell culture model, endogenous CHIP also co-localizes with α-synuclein and Hsp70 in intracellular inclusions (37). In the protein-aggregation NDDs, the inclusions that form also contain CHIP, ubiquitin, Hsp70, E1 and E2 ubiquitinating enzymes, and even proteasomes (33). These data suggest that CHIP and its inclusion formation might cause neurotoxic effects similar to other Lewy body components, such as α-synuclein, during the pathogenesis of NDD. On the other hand, we cannot exclude the possibility that formation of CHIP-containing protein inclusions may simply reflect blockade of its neuroprotective function. Otherwise, if the UPS is functional in these inclusions, even at a reduced rate, then some reversal of preexisting toxicity may occur over time as the generation of oligomers and aggregates is blocked (33). However, there is no report supporting the latter hypothesis. Interestingly, the current study provides additional evidence of CHIP’s neurotoxic action. The CHIP cytotoxicity may be produced in part via PINK1 degradation. Moreover, the cell death of dopaminergic neuroblastoma SH-SY5Y cells may be induced by CHIP in response to STS.

Similar to CHIP, PINK1 has been purported to have a dual role in cancer biology, with context-dependent pro- and anti-tumorigenic properties (39). PINK1 regulates these activities via phosphatidylinositol 3-kinase/Akt and mitochondrial signaling. PINK1 is also a potential therapeutic target in certain cancers. This is supported by meta-analysis of PINK1 mRNA expression in cancer datasets (i.e. ONCOMINE and COSMIC databases), where PINK1 expression is significantly decreased in several malignancies including ovarian, liver, and renal carcinomas, while being significantly overexpressed in a subset of renal, endometrial, hematopoietic, and parathyroid cancers.

To conclude, the present work proposes a new PINK1 regulatory pathway and demonstrates that CHIP-mediated down-regulation of PINK1 action may contribute to the pathogenesis of PD through the cytotoxic effect of CHIP.

Experimental procedures

Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and Lipofectamine PLUS were purchased from Invitrogen. Protein A Sepharose and glutathione Sepharose 4B beads were obtained from GE Healthcare Life Sciences. Enhanced chemiluminescence (ECL) reagent was purchased from Abclon (Seoul, Korea) and Advansta (Menlo Park, CA). MG132 was purchased from AG Scientific (San Diego, CA). All other chemicals used in the study were analytical-grade commercial products and purchased from Sigma-Aldrich.
Identification of CHIP as PINK1-targeting E3 ubiquitin ligase

For Western blotting, mouse anti-Xpress (1:5000) (46–0528, Invitrogen), anti-Myc (1:1000) (sc-40, Santa Cruz Biotechnology), anti-HA (1:1000) (sc-7392, Santa Cruz Biotechnology), anti-CHIP (1:1000) (sc-133083, Santa Cruz Biotechnology), anti-ubiquitin (1:1000) (sc-8017, Santa Cruz Biotechnology), anti-tubulin (1:1000) (sc-8035, Santa Cruz Biotechnology), and anti-actin (1:1000) (sc-4777, Santa Cruz Biotechnology) antibodies were used. HRP-conjugated anti-rabbit (AP132P) and antimouse (AP124P) secondary antibodies were purchased from EMD Millipore (Billerica, MA). For immunofluorescence, mouse anti-TOM20 (1:100) (sc-17764, Santa Cruz Biotechnology), rabbit anti-CHIP (1:1000) (sc-133083, Santa Cruz Biotechnology), anti-HA (1:1000) (sc-7392, Santa Cruz Biotechnology), anti-Myc (1:1000) (sc-40, Santa Cruz Biotechnology), and anti-ubiquitin (1:1000) (sc-8017, Santa Cruz Biotechnology) antibodies were used. Alexa Fluor 488 conjugated anti-mouse (A-11029) and Alexa Fluor 594 conjugated anti-rabbit (A-11012) secondary antibodies were purchased from Invitrogen.

DNA constructs and RNA interference

Mammalian construct encoding Myc-tagged human wild-type PINK1 (pBOS-3X-Myc-hPINK1-WT; NCBI accession number NM_005861.3) was kindly provided by J. Chung (Seoul National University, Seoul, Korea). The plasmid encoding GST-tagged hPINK1 kinase domain (amino acids 112–496) was provided by M.R. Cookson (National Institutes of Health). Plasmids encoding His-tagged human wildtype CHIP (pHis<sub>6</sub>-Xpress-CHIP-WT; NCBI accession number NM_005861.3) and its deletion mutant lacking the U box (pHis<sub>6</sub>-Xpress-CHIP<sub>ΔU</sub>) were kindly provided from D. H. Lee (Seoul Women’s University, Seoul, Korea). To make constructs encoding various deletion and point mutants of PINK1 and CHIP, site-directed mutagenesis was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), and pBOS-3X-Myc-hPINK1-WT or pHis<sub>6</sub>-Xpress-CHIP-WT as a template, respectively. All cDNA sequences were verified by sequencing (COSMO Genetech, Seoul, Korea). siRNAs targeting human CHIP (siRNA no. 1146289), human PINK1 (siRNA no. 1116919), human Hsp70 (siRNA no. 1071339), and nonspecific control scrambled siRNA (catalogue no. SN-1013) were purchased from Bioneer (Daejeon, Korea).

Cell culture and DNA transfection

Mouse embryonic fibroblasts derived from CHIP-null (CHIP<sup>−/−</sup>) and control (CHIP<sup>+/+</sup>) mice were provided by C. H. Chung (Seoul National University). HEK293 cells, human neuroblastoma SH-SY5Y cells, and CHIP<sup>+/+</sup> and CHIP<sup>−/−</sup> MEF cells were maintained in DMEM containing 10% FBS and 100 units/milliliter penicillin-streptomycin. Cells were grown at 37 °C in 5% CO₂. All DNA transfections were performed using Lipofectamine PLUS, according to the manufacturer’s protocol.

Co-immunoprecipitation and immunoblot analysis

Cell lysates were prepared by rinsing cells twice with ice-cold PBS followed by solubilization in lysis buffer containing 50 mM Tris, pH 7.4, 1.0% Nonidet P-40, 150 mM NaCl, 10% glycerol, and protease inhibitor mixture including 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, and 0.2 mM PMSF. Cell lysates containing ~500 μg of protein were incubated with 0.5 μg of appropriate antibody overnight at 4 °C. Samples were then incubated with Protein A Sepharose beads for 2 h at 4 °C and washed five times with lysis buffer. The immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Whatman, GE Healthcare Life Sciences). Membranes were blocked in Tris-buffered saline with Tween 20 (TBST) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and 5% nonfat dry milk for 1 h at room temperature, and incubated overnight at 4 °C in 3% nonfat dry milk containing the appropriate primary antibodies. The membranes were washed several times in TBST and incubated with HRP-coupled secondary IgG in 3% nonfat dry milk for 1 h. The bands were visualized using ECL reagents (Abclon), following the manufacturer’s instructions.

Preparation of mouse whole brain lysate

The whole brain from C57BL/6 male mouse at the age of 6 weeks (Orient, Seongnam City, Gyeonggi Province, Korea) was homogenized in lysis buffer containing 50 mM Tris, pH 7.4, 1.0% Nonidet P-40, 150 mM NaCl, 10% glycerol, and protease inhibitor mixture, and centrifuged at 13,000 × g for 15 min at 4 °C.

Purification of bacterial recombinant protein and in vitro binding assay

The plasmids encoding GST-tagged human PINK1 kinase domain (GST-PINK1-KD) containing amino acids 112–496 (pGEX-5X-1-hPINK1-KD) and His<sub>6</sub>-tagged wildtype CHIP (pET28a(+)-CHIP-WT) were expressed in Escherichia coli BL21 cells. Cells were cultured at 37 °C until the A<sub>600</sub> reached 0.7–0.8, and target protein expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 24 h. Cells were harvested and lysed by sonication on ice in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1%, Triton X-100, and protease inhibitor mixture. After centrifugation for 20 min at 12,000 × g, the supernatant was incubated overnight at 4 °C with glutathione Sepharose 4B beads (GE Healthcare Life Sciences) for GST-PINK1-KD or Ni<sup>2+</sup>-NTA agarose beads (Invitrogen) for His<sub>6</sub>-CHIP-WT. Beads were then washed extensively with lysis buffer, and the recombinant proteins were eluted in elution buffer (50 mM Tris-HCl, pH 7.4, and either 10 mM reduced glutathione for GST-PINK1-KD or 250 mM imidazole for His<sub>6</sub>-CHIP-WT). Each 1 μg of purified PINK1 or CHIP was incubated overnight at 4 °C with anti-PINK1 or anti-CHIP antiserum. The mixtures were then incubated with 30 μl of an equal volume of Protein A Sepharose bead suspension for 2 h at 4 °C, and followed by immunoblotting analyses.

In vitro ubiquitination assay

In vitro ubiquitination assays were performed in reaction buffer consisting of 20 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM DTT, 5 mM MgCl<sub>2</sub>, 5 mM ATP, and 1 mM CaCl<sub>2</sub>. The final reaction mixture contained 25 μM ubiquitin, 0.1 μM E1, 2.4 mM UbcH5a as E2 ubiquitin-conjugating enzyme, 1 μM recombinant His<sub>6</sub>-CHIP, and 1 μM recombinant GST-PINK1-KD in...
reaction buffer and was incubated for 2 h at 30 °C. The ubiquitination reaction was terminated by the addition of 5× sample buffer and boiled for 5 min at 95 °C. Protein samples were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes, and immunoblotting analysis were performed with anti-PINK1 antibody.

**Immunocytochemistry analysis**

SH-SY5Y neuroblastoma cells were seeded onto poly-L-lysine–coated cover glasses. After DNA transfection for 24 h, cells were washed twice with PBS and immediately fixed in 3.7% formaldehyde for 10 min at room temperature. After fixation, cells were permeabilized with 0.1% Triton X-100 for 15 min and blocked with 1% BSA in TBST for 30 min at room temperature, followed by immunostaining with rabbit polyclonal anti-c-Myc and/or mouse monoclonal anti-TOM20 antibodies, respectively. Cells were washed and incubated with Alexa Fluor 488 or Alexa Fluor 594 conjugated anti-IgG antibodies. DyLight™ 405 conjugated anti-HA antibody (Rockland Immunchemicals) was used to detect HA-tagged CHIP. Images were captured using a confocal LSM 700 (Carl Zeiss, Oberkochen, Germany), and data were processed using a Zeiss LSM Image Browser (Carl Zeiss).

**Lactate dehydrogenase cytotoxicity assay**

Cytotoxicity was determined using the LDH cytotoxicity assay kit (Takara, Kyoto, Japan), which quantifies LDH release from damaged cells into culture medium. After DNA transfection for 24 h, SH-SY5Y cells were treated with vehicle (DMSO) or 0.5 μM STS for an additional 24 h. Cell-free culture medium was collected and used in the LDH assay, as instructed by the manufacturer. Maximum LDH release (referred as “high control”) was determined by solubilizing cells in 1% Triton X-100, and spontaneous LDH release (“low control”) was determined by incubating cells in medium alone. Reduction of tetrazolium salt into red formazan salt was used as an indicator of LDH release from damaged cells into culture medium. After DNA transfection for 24 h, cells were washed twice with PBS and immediately fixed in 3.7% formaldehyde for 10 min at room temperature. After fixation, cells were permeabilized with 0.1% Triton X-100 for 15 min and blocked with 1% BSA in TBST for 30 min at room temperature, followed by immunostaining with rabbit polyclonal anti-c-Myc and/or mouse monoclonal anti-TOM20 antibodies, respectively. Cells were washed and incubated with Alexa Fluor 488 or Alexa Fluor 594 conjugated anti-IgG antibodies. DyLight™ 405 conjugated anti-HA antibody (Rockland Immunchemicals) was used to detect HA-tagged CHIP. Images were captured using a confocal LSM 700 (Carl Zeiss, Oberkochen, Germany), and data were processed using a Zeiss LSM Image Browser (Carl Zeiss).

**Statistical analysis**

Group means were compared using Student’s t-tests. p < 0.05 was considered statistically significant. Values are reported as the mean ± S.D.

**Author contributions**—L. Y. and K. C. C. conceived and coordinated the study and wrote the paper. L. Y. and K. C. C. designed the experiments and analyzed data. L. Y. performed whole experiments. All authors reviewed the results, edited the manuscript, and approved the final version for submission.

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