AIMP2 TARGETS PINK1 FOR PROTEOSOMAL DEGRADATION: INSIGHT INTO MITOCHONDRIAL MAINTENANCE AND PARKINSON’S DISEASE

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ABSTRACT

A mitochondrial serine/threonine kinase, PINK1, is a common genetic risk factor that has been implicated in the pathology of autosomal recessive Parkinson’s disease (PD). PINK1 identifies damaged mitochondria and recruits another PD-related E3 ubiquitin ligase parkin to trigger the autophagic clearance of the impaired mitochondria: mitophagy. The tight regulation of PINK1 protein expression in mitochondria as well as in cytoplasm is important for this process because loss of function of PINK1 leads to failure in mitophagy. However, the exact mechanisms by which the steady-state level of PINK1 is maintained are poorly understood. Here, we show that AIMP2, previously found as a pathogenic substrate of parkin, functions as a novel regulator for ubiquitin-proteasome-dependent degradation of PINK1. AIMP2 interacts with both PINK1 and 26S proteasome regulatory subunits, RPN1 and RPN3 subunits to promote PINK1 degradation through the ubiquitin-proteosome system. Overexpression of AIMP2 decreases PINK1 levels, whereas depletion of AIMP2 increases PINK1 levels both in vitro and in vivo. Moreover, AIMP2-mediated regulation of PINK1 leads to differential kinetics of parkin recruitment onto CCCP-induced damaged mitochondria. These results suggest that AIMP2 serves as a key regulator of PINK1 expression and PINK1-induced parkin recruitment for mitophagy.

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Introduction

The number of mitochondria and their quality are regulated through biogenesis and removal of mitochondria by autophagosomes and lysosomes (a.k.a mitophagy) to meet the metabolic needs of different cell types. Disruption of the fine control of mitochondrial quality is implicated in the etiopathogenesis of various neurodegenerative disorders including Parkinson’s disease (PD).\footnote{1} Starting with the observation that mitochondrial toxins can cause parkinsonism in humans and animal models, scientists have accrued a large body of evidence confirming that mitochondrial alterations can promote the degeneration of dopamine-producing neurons typically shown in the brain of PD patients. A number of genes that have been identified and characterized to have links with inherited forms of PD encode mitochondrial proteins or proteins implicated in mitochondrial dysfunction, supporting the fundamental involvement of mitochondria in PD. A serine-threonine kinase PINK1 (PTEN-induced kinase-1) is one of the autosomal-recessive genes whose loss-of-function mutations cause mitochondrial dysfunction in PD.\footnote{2, 3} 

Localized in cytoplasm and mitochondria,\footnote{2, 3} PINK1 plays a vital role in the elimination of damaged mitochondria. Imported from cytoplasm to the outer membrane of mitochondria, under normal conditions, PINK1 quickly undergoes the voltage-dependent proteolysis by a mitochondrial intramembrane cleaving protease, Presenilin Associated Rhomboid-Like (PARL).\footnote{4} The cleaved forms of PINK1 have not yet been characterized, but they are rapidly degraded by proteosome.\footnote{5} This proteolytic mechanism maintains full-length PINK1 at much lower levels on healthy, polarized mitochondria to prevent unnecessary mitophagy. As mitochondria become dysfunctional, however, the mitochondria lose their electrochemical gradient. This change in the membrane potential
(depolarization) leads to not only up-regulation of PINK1 expression in cytoplasm but also inactivation of the PARL1-mediated proteolytic cleavage of PINK1 to stabilize its protein expression at the outer membrane of the damaged mitochondria. The resulting PINK1 accumulation then recruits another recessive PD-linked protein, E3 ligase parkin, to trigger a selective clearance of the non-functional mitochondria.\textsuperscript{6, 7} Thus, PINK1 serves as a scout to search for damaged mitochondria, and it is important for cells to tightly regulate PINK1 levels in order to produce a quick response to mitochondrial damage and maintain mitochondrial homeostasis. However, it is still unclear how PINK1 is targeted for proteasomal degradation. Understanding the mechanism of PINK1 regulation is significant because it can provide clinically relevant insights into improving mitochondrial dysfunction observed in PD.

Aminoacyl-tRNA synthetase complex interacting multifunctional protein-2 (AIMP2) has been found as a pathogenic substrate of parkin that consistently accumulates in degenerating conditions of PD.\textsuperscript{8, 9} AIMP2 was originally discovered as a scaffolding protein required for the assembly of the tRNA synthetase complex,\textsuperscript{10} but it has been suggested that AIMP2 can have diverse regulatory functions that are not directly related to protein synthesis\textsuperscript{11}. In addition to its characteristic accumulation in degenerating neurons, recent studies have shown that AIMP2 can destabilize some of its interacting proteins by driving their poly-ubiquitination and proteasomal degradation.\textsuperscript{12, 13} Furthermore, AIMP2 deletion/inactivation is associated with the process of tumorigenesis.\textsuperscript{14, 15} Although the exact mechanism by which AIMP2 regulates protein levels is unknown, the emerging evidence suggests its central involvement in proteosomal degradation. Because the interaction between AIMP2 and PINK1 was
observed in our screening experiment assessed via co-immunoprecipitation, we hypothesized that AIMP2 can regulate the steady-state levels of PINK1 through the ubiquitin-proteosome degradation system.

Here, we report AIMP2 as a novel regulator and adaptor for PINK1 degradation. AIMP2 interacted with both PINK1 and 26S proteosomal subunits, RPN1 and RPN3, and facilitated ubiquitination and proteasomal targeting of PINK1. The AIMP2-induced alteration in basal PINK1 levels led to differential kinetics of parkin recruitment to damaged mitochondria. Taken together, these data demonstrates that AIMP2 is a key regulator of PINK1 expression and its mitochondrial function to orchestrate mitochondrial homeostasis. This discovery may provide a clue to unravel the formerly uncharacterized pathway of PINK1 degradation.

Materials and Methods

Antibodies

The following antibodies were used. Rabbit antibody to GFP (cat# ab290, 1:1,000, Abcam), rabbit antibody to AIMP2 (ProteinTech Group), rabbit antibody to PINK1 (Novus Biologicals), rabbit antibody to PINK1 (Cayman), mouse antibody to FLAG (M2, 1:5,000, Sigma-Aldrich), mouse antibody to MYC (cat# 11 667 149 001, 1:1,000, Roche Diagnostics), mouse antibody to V5 (cat# 46-0705, 1:500, Invitrogen), mouse antibody to GAPDH (GT239, 1:5,000, GeneTex), mouse antibody to Succinate Dehydrogenase (SDHA) (ab 14715, 1:2,000, Abcam), rabbit antibody to Mfn2 (gift from Dr. Richard J. Youle), mouse antibody to parkin (Park8, 1:2,000, Cell Signaling), mouse antibody to Tom20 (sc-17764, 1:500, Santa cruz biotechnology), Horseradish peroxidase (HRP)-conjugated antibody to HA (Roche), HRP-conjugated mouse antibody to β-actin (AC15,
Sigma-Aldrich). For secondary antibodies, we used HRP-conjugated sheep antibody to mouse IgG (cat# RPN4301, 1:5,000, GE Healthcare), HRP-conjugated donkey antibody to rabbit IgG (cat# RPN4101, 1:5,000, GE Healthcare), Cy3–conjugated donkey antibody to mouse (H+L) (cat# 715-165-151, 1:250, Jackson ImmunoResearch).

**Plasmids**

pEGFP-C1-\textit{PINK1}, pLenti6-DEST-\textit{PINK1}-V5, and YFP-\textit{parkin} were purchased from Addgene. pDsRed2-\textit{Mito} was purchased from Clontech. pRES2-HA-\textit{PINK1} was provided by Dr. Serge Przedborski (Columbia University). pEGFP-\textit{AIMP2} was cloned into a pEGFP-C1 vector (Clontech). pRK5-\textit{MYC-\(\alpha\)-\textit{synuclein}} was cloned into a pRK5-myc vector (Stratagene). pcDNA-V5-\textit{VPS35} was kindly provided by Dr. Matthew Farrer (University of British Columbia, Canada). The following constructs used here were described previously:, pcDNA-MYC-\textit{LRRK2},\textsuperscript{22} pCMV-Tag2a-\textit{FLAG-\textit{AIMP2}},\textsuperscript{16} HA-\textit{ubiquitin},\textsuperscript{23} shDsRed, pCDNA-\textit{FLAG-Rpn1, Rpn2, Rpn3, Rpn5, Rpn6, and Rpn9}.\textsuperscript{24} A plasmid containing \(\beta\)-galactosidase, empty vector, or GFP cDNA was used as a control in the cell-culture experiments.

**shRNA-\textit{AIMP2} construct and AAV2 shRNA \textit{AIMP2} virus.**

PLKO.1 shRNA plasmids encoding small interfering RNAs (siRNAs) targeting \textit{AIMP2} were purchased from the genomics resources in the Hit center (the Johns Hopkins University). TRCN0000099157 vector was effective in knocking down \textit{AIMP2} protein expression. As a control, shRNA-dsRed coexpressing GFP and short hairpin sequence (AGTTCCAGTACGGCTCCAA) was used. Scramble shRNA (AAV-GFP-U6-shRNA) and human \textit{AIMP2} shRNA AAV2 (adeno-associated virus 2) was purchased from the vector biolabs (Cat#shAAV-230409). The AAV2-shRNA \textit{AIMP2} virus successfully
knocked down AIMP2 protein expression in cultured cells and in vivo as shown in Figure 2E.

**Cell culture and transfection**

Human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA) were grown in DMEM containing 10% FBS (vol/vol) and antibiotics in a humidified 5% CO2/95% air atmosphere at 37°C. For transient transfection, cells were transfected with indicated target vectors using Lipofectamine and Plus reagents (Invitrogen) according to manufacturer’s instructions. Unless otherwise indicated, lysates were prepared 48 hours after transfection. HeLa cells stably expressing YFP-parkin (Hela-YFP-parkin) were provided by Dr. Richard J. Youle (NIH). Aimp2+/+ and -/- MEF cells were kindly provided by Dr. Sunghoon Kim (Seoul National University, Korea)

**Co-immunoprecipitation**

For co-immunoprecipitation from SH-SY5Y cells, 48 hours after transfection cells were washed with cold PBS and harvested in immunoprecipitation buffer (1% NP-40, 2 mg per ml aprotinin, and 100 mg per ml PMSF in PBS). The lysate was then rotated at 4°C for 1 h, followed by centrifugation at 14,000 rpm for 20 min. The supernatants were then combined with protein G Sepharose beads (Amersham Biosciences) preincubated with antibodies against FLAG or GFP or AIMP2 or PINK1, followed by rotating at 4°C over night. The protein G Sepharose was pelleted and washed two times using immunoprecipitation buffer and additional two times with 500 mM NaCl buffer. The precipitates were resolved on SDS-PAGE gel and subjected to immunoblot analysis. Immunoblot signals were visualized with chemiluminescence (Pierce, Rockford, IL). The densitometric analyses of the bands were performed using Image J (NIH,
Subcellular fractionation

SH-SY5Y cells or mouse brain tissues were subcellular fractionated into the cytosol, mitochondria, and the nucleus by using Qproteome Mitochondria Isolation Kit (Qiagen) following the instructions in the manual. Cytosolic fraction was further concentrated with acetone precipitation. Purity of each fractions was validated with western blots using antibodies to marker proteins for cytosolic (GAPDH), and mitochondria (SDHA) subcellular fractions.

Cellular ubiquitination assay

For the ubiquitination assay, SH-SY5Y cells were transiently transfected with pMT123-HA-ubiquitin, pEGFP-PINK1 in the presence of FLAG-AIMP2. Total cell lysates were prepared by harvesting the cells after washing with PBS, followed by solubilizing the pellets in 200 ml of 2% SDS, followed by sonication, 48 hours after transfection. The lysates were then rotated at 4°C for 1 h, diluted to 1 ml with PBS, and then boiled and sonicated. The samples were used as input and for immunoprecipitation. Immunoprecipitation was performed using an antibody to GFP. The precipitates were subjected to immunoblotting with HA or GFP antibodies to detect PINK1 ubiquitination.

Pulse chase analysis

SH-SY5Y cells were transiently transfected with plasmids and 48 hours after transfection cells were treated with 100 mg/ml cycloheximide and/or 10 mM MG132 and harvested at different time points as indicated in the figure 2D. Cells were then lysed with RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP40, 1% SDS, and 0.5 % sodium deoxycholate plus protease inhibitor mixture). The protein concentration of each
sample was determined using the BCA protein assay kit (Pierce), and subsequently the samples were western blotted. The optical density of bands was measured by the gel analysis function of Image J software (NIH) normalized to β-actin.

**Immunofluorescence**

SH-SY5Y and YFP-parkin-expressing Hela cells were plated onto poly-D-lysine-coated coverslips at 10,000 cells/cm². Cells were fixed with 4% paraformaldehyde in PBS and then blocked in a solution containing 5% normal donkey serum (Jackson ImmunoResearch, BarHarbor, ME), 2% BSA (Sigma) and 0.1% Triton X-100 (Sigma) for 1 h at room temperature. The samples were incubated overnight at 4°C with primary antibodies against V5 or Tom20. After briefly washed with PBS containing 0.1% Triton X-100, the cells were incubated for 1 h at room temperature with Cy3-conjugated secondary antibodies (1:200, Jackson immunoResearch). The coverslips were mounted and fluorescent images were obtained using a confocal microscope (Zeiss Conforal LSM 710).

**Assessment of parkin recruitment to mitochondria**

MEF cells and Hela cells stably expressing YFP-parkin were plated onto poly-D-lysine-coated coverslips at 10,000 cells/cm². Cells were transfected with plasmids as indicated. cells were treated with either vehicle (DMSO) or CCCP. After fixation and Tom20-staining, parkin recruitment to mitochondria was assessed, using a confocal microscope (Zeiss Confocal LSM 710). Colocalization between YFP-parkin and mitochondria was scored for ≥100 cells in three independent experiments.

**Statistics**

Quantitative data are presented as the mean ± s.e.m. Normality of the data was
tested with the Shapiro-Wilk test. The equality of variance was determined with Levene statistics. Statistical significance was assessed either via an unpaired two-tailed Student’s \( t \) test or nonparametric Mann-Whitney \( U \) test for two-group comparison or an ANOVA test with Tukey’s HSD post hoc analysis for comparison of more than three groups. Assessments were considered significant with a \( P < 0.05 \).

**Results**

**AIMP2 interacts with PINK1.**

To identify a potential regulation of PD-related proteins by AIMP2, we performed a small-scale screening experiment, using SH-SY5Y cells transiently transfected with N-terminal FLAG-tagged AIMP2 (FLAG-\textit{AIMP2}) together with several V5- or MYC-tagged PD-associated genes including \textit{PINK1}, Vacuolar Protein Sorting 35 Homolog (\textit{VPS35}), and \( \alpha \)-synuclein. Due to transfection and expression efficiency, MYC-tagged Leucine-rich Repeat Kinase 2 (\textit{LRRK2}) construct and FLAG-\textit{AIMP2} were transfected into HEK 293 cells instead of SH-SY5Y cells. Co-immunoprecipitation with antibodies against FLAG revealed that AIMP2 strongly bound to full-length and processed forms of V5-PINK1 and V5-VPS35, whereas there was no association between AIMP2 and MYC-\( \alpha \)-synuclein or MYC-LRRK2 (Fig. 1A). To further characterize the interaction between AIMP2 and PINK1, SH-SY5Y cells were transiently transfected with FLAG-\textit{AIMP2} and \textit{PINK1}-V5 constructs and subjected to co-immunoprecipitation. Anti-FLAG immunoprecipitation of FLAG-AIMP2 pulled down all forms of V5-PINK1 with a slightly stronger interaction with the 55 kDa processed form of V5-PINK1. Conversely, anti-V5 immunoprecipitation of PINK1-V5 pulled down FLAG-AIMP2 (Fig. 1B).
To determine the subcellular localization of endogenous PINK1 and AIMP2 under basal conditions, SH-SY5Y cells were separated into cytoplasmic and mitochondrial subcellular fractions. From the following western blot, a predominant distribution of PINK1 and AIMP2 was observed in the cytoplasm (Fig. 1C). Consistent with the notion that PINK1 is released from healthy mitochondria to cytoplasm after its cleavage by PARL, the 55 kDa processed form of PINK1 was mainly detected in the cytoplasm of SH-SY5Y cells. Immunoprecipitation of endogenous AIMP2 from the cytoplasmic fraction of SH-SY5Y cells demonstrated its association with endogenous PINK1 (Fig. 1D). Conversely, PINK1 immunoprecipitation pulled down AIMP2 (Fig. 1D). Finally, exogenously expressed AIMP2 and PINK1 showed co-localization in the SH-SY5Y cells as determined by co-labeling of each protein (Fig. 1E). Taken together, these results indicate that AIMP2 interacts with full-length and processed forms of PINK1.

**AIMP2 regulates PINK1 ubiquitination and proteasomal degradation.**

AIMP2 has been reported to facilitate degradation of its interacting proteins, but the mechanisms are unknown. To determine downstream consequences of AIMP2’s interaction with PINK1, PINK1 polyubiquitination was monitored in SH-SY5Y cells transfected with HA-tagged ubiquitin (HA-Ub) with or without FLAG-AIMP2. FLAG-AIMP2 enhanced GFP-PINK1 poly-ubiquitination determined by the western blot of HA and GFP for anti-GFP immunopurified PINK1 (Fig. 2A). Next, we sought to examine a potential adaptor function of AIMP2 to bridge proteasome with lysine-48 ubiquitin conjugated substrates. GFP-AIMP2 co-immunoprecipitated with 26S proteasome non-ATPase regulatory subunit, RPN1 and RPN 3, which comprise part of 19S regulatory cap
subunits of 26S proteasome (Fig. 2B). On the other hand, AIMP2 failed to interact with Rpn2, Rpn5, Rpn6, and Rpn9 (Fig. 2B), demonstrating AIMP2’s specific interaction with Rpn1/3.

To determine whether AIMP2 affects the protein stability of PINK1, different amounts of FLAG-AIMP2 were transfected into SH-SY5Y cells. As AIMP2 dose was increasing, PINK1 steady-state protein levels seemed to decrease proportionally (Fig. 2C). AIMP2-activated PINK1 degradation must be achieved through proteasome since the proteasome inhibitor MG132 blocked PINK1 degradation and resulted in the accumulation of the PINK1 55 kDa form (Fig. 2C). PINK1 levels were monitored over time after cycloheximide treatment to stop further protein translation. AIMP2 expression accelerated degradation of all three forms of PINK1 proteins with more dramatic clearance of 55 kDa PINK1. MG132 treatment completely blocked this effect, suggesting again that AIMP2 enhancement of PINK1 degradation took place through proteasomal degradation (Fig. 2D). To see whether endogenous AIMP2 is involved in the process of PINK1 degradation, we knocked down AIMP2 by transiently transfecting SH-SY5Y cells with shRNA to AIMP2. AIMP2 reduction by shRNA resulted in a significant increase in both 65 kDa and 55 kDa PINK1 levels (Fig. 2E), supporting the role of AIMP2 in the clearance of PINK1. To address a potential non-targeting effect of shRNA to AIMP2, we also used AIMP2 null mice to assess PINK1 stability. Because AIMP2 null background in these mice generally leads to postnatal lethality due to a defective lung differentiation, we used AIMP2-deficient mouse embryonic fibroblasts (MEF) and embryonic brain lysates to monitor PINK1 protein levels. Consistent with the results in
AIMP2 knockdown study in SH-SY5Y cells, AIMP2 ablation led to an accumulation of PINK1, especially 55 kDa forms.

To better understand the relationship between AIMP2 and PINK1 in the in vivo system, we evaluated PINK1 levels in the striatum of conditional transgenic mice expressing human AIMP2 in the central nervous system. In agreement with the notion that AIMP2 regulates PINK1 stability, AIMP2 expression in mouse brains led to a significant reduction of steady-state PINK1 protein levels (Fig. 2H). Taken together, these results suggest that AIMP2 is involved in the regulation of PINK1 proteasomal degradation through its association with proteasomal subunits. Either down-regulation or accumulation of AIMP2 can give rise to abnormal PINK1 steady-state protein levels.

AIMP2 regulation of PINK1 and CCCP-induced parkin recruitment to mitochondria.

The role of PINK1 has been best characterized in the mitophagy process in which PINK1 is stabilized selectively on damaged mitochondria, recruiting parkin for subsequent ubiquitination and elimination of the nonfunctional mitochondria. The amount of PINK1 is critically important because PINK1 accumulation is necessary and sufficient for initiating mitophagy. Since AIMP2 is capable of regulating PINK1 stability, we investigated whether AIMP2 can modulate the mitophagy via PINK1 regulation. We monitored PINK1 stability in SH-SY5Y cells treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP), which depolarizes mitochondria by dissipating $H^+$ gradient across the inner cell membrane of mitochondria. The CCCP-induced damage on mitochondria resulted in the gradual accumulation of PINK1, whereas the transient expression of FLAG-AIMP2 delayed this process (Fig. 3A). Parkin
translocation to depolarized mitochondria is dependent on PINK1. Therefore, we examined whether AIMP2-driven degradation of PINK1 affects parkin recruitment in YFP-parkin expressing Hela cells under CCCP treatment. Consistent with the delayed and reduced accumulation of PINK1, the kinetics of parkin recruitment after CCCP treatment were impaired in response to AIMP2 overexpression, when monitored by percentage of cells showing YFP-parkin concentration on mitochondria (Fig. 3B).

Next, we determined the role of endogenous AIMP2 in the process of CCCP-induced mitophagy. In contrast to AIMP2 overexpression, knockdown of AIMP2 by shRNA in SH-SY5Y cells increased the basal and CCCP-responsive accumulation of PINK1 (Fig. 3D). In agreement with the enhancement of PINK1 stabilization in the AIMP2-knockdown background in the SH-SY5Y cells, aimp2−/− MEFs exhibited more efficient recruitment of YFP-parkin to CCCP-damaged mitochondria than the wild type MEFs, and this trend was reversed by AIMP2 supplementation (Fig. 3E). Thus, AIMP2 plays an important role in the regulation of PINK1 stability in both basal conditions and those of mitochondrial damage as well as in the parkin recruitment onto the damaged mitochondria.

Discussion

The major finding of this study was the discovery of a novel role of AIMP2 in PINK1 degradation. The accumulation of AIMP2 is implicated in the degeneration of dopaminergic neurons in vitro and in vivo. Several lines of evidence indicate that AIMP2, originally found as a component of aminoacyl-tRNA synthetase for protein synthesis, has a non-canonical function to modulate ubiquitination and proteasomal degradation of its interacting partners. Here, we extended this role of AIMP2 to the
regulation of PINK1 protein levels both under basal conditions and under CCCP-induced mitochondrial damage. Although the exact mechanism by which AIMP2 mediates ubiquitination and degradation of PINK1 is not known, some E3 ligases linked to PINK1 ubiquitination, such as UBR1, 2, and 3,\textsuperscript{18} may be part of this pathway to direct PINK1 polyubiquitination. Notably, the fact that AIMP2 interacts with several subunits of proteasome suggests that AIMP2 can directly deliver PINK1 to the proteosome for degradation. AIMP2 makes not only the processed form of PINK1 (55 kDa) but also the full-length PINK1 (65kDa) subject to proteasomal degradation because AIMP2 can bind to all of these forms of PINK1 and drive their degradation (Fig. 1B and Fig. 2D). Since the PARL-cleaved 55 kDa PINK1 displays a stronger binding affinity to AIMP2 as well as a more rapid degradation in response to AIMP2 expression, it is likely that AIMP2 functions to efficiently remove the cytosolic 55 kDa PINK1, which is released from healthy mitochondria by PARL-mediated cleavage.

PINK1 should be rapidly removed from healthy mitochondria to prevent any unwanted recruitment of parkin and subsequent mitophagy. In this respect, additional factors might be necessary to accelerate this PINK1 degradation. Several proteins have been discovered in the regulation of PINK1 processing or stabilization,\textsuperscript{18-21} whereas no protein in the PINK1 degradation has been identified. AIMP2 can be a major regulatory protein in the PINK1 degradation pathway because it serves as an adaptor protein that not only increases PINK1 ubiquitination but also transfers PINK1 to proteasome to ensure a rapid PINK1 clearance from healthy mitochondria.

AIMP2 accumulation in the nigrostriatal pathway along the central nervous system has a selective toxicity to dopaminergic neurons.\textsuperscript{9,16} Our finding that AIMP2
overexpression in the mouse brains causes a significant reduction in PINK1 protein levels suggests the dysfunction of mitochondrial downstream pathway of parkin and its substrate, AIMP2. Regarding that these transgenic mice developed PD phenotypes, AIMP2’s regulation of PINK1 may represent a feed forward mechanism in the pathogenesis of PD where the high expression of AIMP2 leads to the malfunction of mitophagy by decreasing basal PINK1 levels substantially. It will be important to further investigate the stability of PINK1 in PD patients with parkin mutations or parkin knockout mice where AIMP2 accumulate.

Endogenous AIMP2 is required to regulate the steady-state levels of PINK1 protein. AIMP2 depletion up-regulates PINK1 levels and thus enhances parkin recruitments on mitochondria upon CCCP treatment. This result suggests AIMP2 is involved in the process of coordinating PINK1 expression levels with the demands of different cell types or cells under different stress conditions. It is also noteworthy to mention that there is some level of AIMP2 degradation during mitophagy. A slight reduction of AIMP2 in this process (Fig. 3A, C) may contribute to a stable accumulation of PINK1 on damaged mitochondria that have lost their electrochemical gradient. Overall, our results introduce a striking role of AIMP2 in the regulation of basal PINK1 levels by acting as an additional checkpoint of PINK1 degradation machinery.

The dysfunction of the mitochondrial quality control system may not be the only cause of Parkinson’s disease, but there is no denying that it is a primary cause of the disease. Current treatments such as the replacement of dopamine are limited in that they do not completely prevent the loss of neurons or further progression of the disease. Researchers and clinicians are paying attention to mitochondrial function as a
fundamental way to ameliorate the disease. Indeed, it has been reported that interventions focused on improving mitochondrial function are showing positive progress in PD.\textsuperscript{25, 26}

We believe our findings fit into this framework and may help develop a new treatment for PD.

\textbf{References}


Figure 1

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Figure 1. PINK1 interacts with AIMP2

(A) Co-immunoprecipitation of AIMP with PD-related genes, PINK1 (65kDa, 55kDa and 42kDa), VPS35, LRRK2, and α-synuclein. SH-SY5Y (left) or HEK293 (right) cells were cotransfected with the indicated constructs. Whole-cell extracts were used for immunoprecipitation with an anti-FLAG antibody. Similar results were reproduced in multiple independent experiments (n=4).

(B) Immunoprecipitation of FLAG-AIMP2 and PINK1-V5 from SH-SY5Y cells with anti-FLAG and anti-V5 antibodies, respectively.

(C) Subcellular localization of PINK1 and AIMP2 in SH-SY5Y cells. SDHA and GAPDH were used as mitochondrial and cytosolic markers, respectively. Cyto, cytoplasm; Mito, mitochondria.

(D) Immunoprecipitation of endogenous AIMP2 and PINK1 in SH-SY5Y cells. IgG was used as a negative control.

(E) Immunofluorescence images of GFP-AIMP2 (green) and V5-PINK1 (red) in SH-SY5Y cells with anti-V5 antibody.
Figure 2. AIMP2 promotes polyubiquitination of PINK1 and its degradation via proteasomes.

(A) Cell ubiquitination assay demonstrating GFP-PINK1 polyubiquitination determined by western blot using HA or GFP antibodies for immunopurified GFP-PINK1 from SH-SY5Y cells.

(B) Co-immunoprecipitation of Rpn subtypes with GFP-AIMP2 in SH-SY5Y cells.

(C) Western blot of endogenous PINK1 in response to different doses of FLAG-AIMP2 expression in SH-SY5Y cells. MG132 (10 mM) was used as a proteasome inhibitor.

(D) Western blot of steady-state PINK1 at the indicated hours after cycloheximide treatment of SH-SY5Y cells that were transfected with PINK1 in the presence or absence of AIMP2. MG132 was treated simultaneously with cycloheximide to inhibit proteasomal degradation.

(E) Western blot of PINK1 and AIMP2 in SH-SY5Y cells transfected with PINK1 and shRNA to AIMP2 or DsRed as a control. Quantification of relative protein levels of AIMP2, PINK1 65 kDa, and 55 kDa in AIMP2 knockdown and control SH-SY5Y cells normalized to β-actin (n = 3 per group).

(F) Western blot of PINK1 and AIMP2 in the cytosolic subcellular fraction prepared from aimp2/− and wild-type mouse embryonic brains (left panel). Quantification of PINK1 protein levels in the brains of aimp2/− and wild type mouse embryo (right panel, n = 3 mice per group).

(G) Western blot of PINK1 and SDHA in aimp2/− and wild type MEF cells (left panel). Quantification of PINK1 protein levels in aimp2/− and wild type MEF cells normalized to β-actin (right panel, n = 3 samples per group)
(H) Western blot of PINK1 and AIMP2 in the cytosolic fraction from the striatum of AIMP2 transgenic mice and littermate controls (left panel). Quantification of PINK1 protein levels in the striatum of AIMP2 transgenic mice and littermate controls normalized to β-actin (right panel, n = 3 mice per group).
Quantified data are expressed as mean ± s.e.m. *P < 0.05, unpaired two-tailed Student’s t test. β-actin was used as an internal loading control. GAPDH was used as an internal loading control of cytosolic fraction.
Figure 3

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C

Cells with YFP-Parkin on mitochondria (%)

DMSO

Mock

AIMP2

F

Cells with YFP-Parkin on mitochondria (%)

DMSO

CCCP

AIMP2+/+

AIMP2+/-

AIMP2-/-
Figure 3. AIMP2-dependent regulation of PINK1 and parkin recruitment to damaged mitochondria.

(A) Western blot of PINK1 and FLAG-AIMP2 in SH-SY5Y cells at the indicated time points after CCCP treatment. SH-SY5Y cells were transfected with either FLAG-AIMP2 or mock as control. β-actin was used as an internal loading control.

(B) Representative confocal images of YFP-parkin and Tom20 in Hela cells stably expressing YFP-parkin treated with 10 mM CCCP or DMSO as a control for 1 hour.

(C) Quantification of the percentage of Hela cells having YFP-parkin accumulation on mitochondria after CCCP treatment. Quantified data are expressed as mean ± s.e.m. * P < 0.05, and ***P < 0.001, unpaired two-tailed Student’s t test, or analysis of variance (ANOVA) test followed by Tukey post hoc analysis.

(D) Western blot of PINK1 and AIMP2 in SH-SY5Y cells at the indicated time points after CCCP treatment. SH-SY5Y cells were transfected with either shRNA to AIMP2 or DsRed as control. β-actin was used as an internal loading control.

(E) Representative confocal images of YFP-parkin and mitochondria labeled with MitoRed in wild-type, aimp2^{-/-} MEF cells. Wild-type and aimp2^{-/-} MEF cells were co-transfected with MitoRed, YFP-parkin and FLAG-AIMP2 or mock as control. The transiently transfected MEF cells were treated with 20 mM CCCP or DMSO as vehicle control for 2 hours.

(F) Quantification of the percentage of MEF cells having YFP-parkin concentration on mitochondria after CCCP treatment (right panel). Quantified data are expressed as mean ± s.e.m. * P < 0.05, and ***P < 0.001, unpaired two-tailed Student’s t test, or analysis of variance (ANOVA) test followed by Tukey post hoc analysis.
Biography

Sang Ho. Kwon is currently a M.S candidate in the Biology department, Johns Hopkins University, and is supervised by Dr. Ko, Hanseok, assistant professor of Neurology at Johns Hopkins University. He earned his Bachelor’s degree from Johns Hopkins University with a double major in Molecular and Cellular Biology and Neuroscience and minor in Psychology in 2013.