PINK1 Content in Mitochondria is Regulated by ER-Associated Degradation

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Maintaining a pool of functional mitochondria requires degradation of damaged ones within the cell. PINK1 is critical in this quality-control process: loss of mitochondrial membrane potential causes PINK1 to accumulate on the mitochondrial surface, triggering mitophagy. However, little is known about how PINK1 is regulated. Recently, we showed that PINK1 content is kept low in healthy mitochondria by continuous ubiquitination and proteasomal degradation of its mature form via a mechanism inconsistent with the proposed N-end rule process. Herein, we now demonstrate that once generated within the mitochondria, 52-kDa PINK1 adopts a mitochondrial topology most consistent with it being at the mitochondrial-endoplasmic reticulum (ER) interface. From this particular submitochondrial location, PINK1 interacts with components of the ER-associated degradation pathway, such as the E3 ligases gp78 and HRD1, which cooperate to catalyze PINK1 ubiquitination. The valosin-containing protein and its cofactor, UFD1, then target ubiquitinated PINK1 for proteasomal degradation. Our data show that PINK1 in healthy mitochondria is negatively regulated via an interplay between mitochondria and ER, and shed light on how this mitochondrial protein gains access to the proteasome.

Parkinson's disease (PD) is an adult-onset neurodegenerative disorder that is characterized pathologically by the loss of ventral midbrain dopaminergic neurons and by the presence of intracytoplasmic proteinaceous inclusions, or Lewy bodies, in the few neurons that are spared (Dauer & Przedborski, 2003). Although PD presents primarily as a sporadic condition, mutations in *PINK1*, which encodes a mitochondrial kinase, have been associated with an early-onset form of the disease, inherited as an autosomal-recessive trait (Valente, bou-Sleiman et al., 2004).

Most of our current knowledge about the function of PINK1 and how *PINK1* mutations can cause neuronal death are derived from studies in invertebrate and cell-culture models (Clark, Dodson et al., 2006, Narendra, Jin et al., 2010, Park, Lee et al., 2006, Thomas, Andrews et al., 2014, Valente et al., 2004, Vives-Bauza, Zhou et al., 2010). One conclusion from these studies is that PINK1 cooperates with the E3 ubiquitin ligase Parkin to promote the turnover of mitochondria by autophagy (also called mitophagy). Upon a profound loss of mitochondrial membrane potential ($\Delta\Psi_m$), PINK1 accumulates at the surface of mitochondria, where it phosphorylates ubiquitin (Kane, Lazarou et al., 2014, Kazlauskaite, Kondapalli et al., 2014, Koyano, Okatsu et al., 2014) thereby promoting the recruitment of cytosolic Parkin to the organelle (Geisler, Holmstrom et al., 2010, Matsuda, Sato et al., 2010, Narendra et al., 2010, Vives-Bauza et al., 2010). Thereafter, Parkin-decorated mitochondria migrate toward the perinuclear area of the cell, where they are eliminated by the autophagy/lysosomal pathway (Geisler et al., 2010, Matsuda et al., 2010, Narendra et al., 2010, Vives-Bauza et al., 2010). This process of mitophagy involves the autophagy receptors optineurin and NDP52 (Lazarou, Sliter et al., 2015) and the autophagy adaptor TBK1 (Heo, Ordureau et al., 2015).

In mammalian cells deficient in PINK1, the lowering of $\Delta\Psi_m$ fails to trigger Parkin-mediated mitochondrial recruitment and mitophagy (Geisler et al., 2010, Matsuda et al., 2010, Narendra et al., 2010, Vives-Bauza et al., 2010). Conversely, Parkin translocation and mitophagy are observed in cells overexpressing wild-type PINK1, but not its kinase-dead

mutant counterpart, even when $\Delta\Psi_m$ is preserved (Vives-Bauza et al., 2010). These findings indicate that the fine-tuning of the mitochondrial content of PINK1 is not only essential to drive Parkin-dependent mitophagy, but also to allow the autophagic machinery to discriminate between damaged (low $\Delta\Psi_m$) and healthy (normal $\Delta\Psi_m$) mitochondria, a process that is fundamental to preserving a pool of functional mitochondria within cells. The PARL-processed ("mature") 52-kDa form of PINK1, located in part on the mitochondrial outer membrane (MOM), retains most of the known functional properties of its full-length 63-kDa counterpart. We and others have shown that PINK1 is expressed constitutively, its level is kept low in healthy mitochondria by the preferential polyubiquitination and ensuing proteasomal degradation of its 52-kDa form (Jin, Lazarou et al., 2010, Lin & Kang, 2008, Liu, Guardia-Laguarta et al., 2017, Takatori, Ito et al., 2008, Zhou, Huang et al., 2008). However, our recent analyses of the PINK1 degradation process indicate that the bulk of ubiquitinated (Ub)-PINK1 is mitochondrially anchored and not cytosolic and that the N-terminal phenylalanine (F104) of PINK1, which forms an N-degron motif, is located inside the MOM (Liu et al., 2017). These data provide compelling evidence that the low PINK1 steady-state level in normal mitochondrial is primarily regulated by a conventional polyubiquitin/proteasome process and not by an N-end rule mechanism, as previously proposed (Yamano & Youle, 2013). However, how mitochondrial PINK1 becomes ubiquitinated and then is turned over to the cytosolic proteasome remains poorly understood.

Here we show that, in mammalian cells, degradation of 52-kDa PINK1 by the proteasome relies on the AAA+ ATPase segregase valosin-containing protein (VCP) and on components of the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway, including the E3 ligases gp78 and HRD1. The ERAD-assisted degradation of 52-kDa PINK1 correlates with the accumulation of mature PINK1 in the vicinity of the ER. Our study emphasizes the key role of 52-kDa PINK1 at all points of this protein metabolic pathway, including ubiquitination, involvement of the ER, and proteasomal degradation. Moreover, by

studying PINK1 processing and turnover, we have uncovered a new functional link between mitochondria and ER, as well as a role for ERAD in regulating mitochondrial protein content.

Results

Ub-PINK1 interacts with valosin-containing protein. The degradation of the mature form of PINK1 by the ubiquitin/proteasome pathway has recently been clarified (Liu et al., 2017). However, the mechanism by which Ub-PINK1 gains access to the cytosolically-located proteasome remains uncertain. To identify PINK1-interacting partners that might provide clues as to how it is extracted from mitochondria and then targeted to the proteasome, we immunoprecipitated (IP) PINK1 from HeLa cells, separated the resulting material by SDS-PAGE gels, excised coomassie brilliant blue-stained bands, and subjected them to liquid chromatography-mass spectrometry LC-MS/MS (Figure 1A). We identified ~480 candidate interaction partners (Supplemental Table 1), including VCP (Figure 1A, band #2), as well as several VCP cofactors, including ubiquitin conjugation factor E4B (UBE4B or UFD2A) (Figure 1A, band #1) and nuclear protein localization protein 4 homologue (NPL4) (Figure 1A, band #3). We were particularly interested in these candidates, as VCP, which is an AAA+ ATPase, is known to facilitate the degradation of ubiquitinated proteins anchored in intracellular membranes (Meyer & Weihl, 2014) and was found, upon mutation, to phenocopy the mitochondrial defect caused by PINK1 mutations in flies (Kim, Tresse et al., 2013). To validate these candidates, we performed a series of co-IP experiments in HEK293T cells stably transfected with wild-type PINK1 and treated with the proteasome inhibitor MG132 to enrich the cell content in Ub-PINK1. As shown in Figure 1B, VCP was successfully pulled-down upon IP PINK1 with a previously validated antibody (Zhou et al., 2008); more importantly, the reciprocal IP showed that 52-kDa PINK1 was the PINK1 species that bound preferentially to VCP. Despite low levels of endogenous PINK1 in cell with healthy mitochondria (Liu et al., 2017, Zhou et al., 2008), some

endogenous 52-kDa PINK1 was pulled-down upon IP VCP in absence of MG132 and showed a slight increase in the ratio of PINK1 over VCP in the presence of MG132 (Figure 1C). Collectively, these experiments support a *bona fide* physical interaction between these two proteins. Furthermore, consistent with VCP's affinity for ubiquitinated proteins, co-IP experiments using HEK293T cells expressing the ubiquitination-defective mutant PINK1^{K137R} (Liu et al., 2017) failed to interact with VCP (Figure 1D). We also validated the interaction between PINK1 and UFD2A (Figure 1E).

To confirm the functional significance of the interactions of Ub-PINK1 with UFD2A and with VCP, we silenced each of these two factors in HeLa cells stably expressing PINK1, using shRNA lentiviral vectors. Consistent with a role of VCP in PINK1 degradation, we found that upon knockdown of VCP using shRNA with a ≥85% efficiency), we observed an increase in the amount of the 52-kDa form, as well as that of Ub-PINK1 species, in cells both treated and untreated with MG132, compared to control cells (Figure 1F). As for UFD2A, its near complete silencing in HeLa cells was associated with a moderate increase in both 52-kDa PINK1 and Ub-PINK1 (Figure 1G).

UFD1 encodes a key adaptor protein that contributes to the VCP-specific activity in the removal of ER proteins (Ye, Meyer et al., 2001) and, interestingly, of damaged mitochondria as well (Kim et al., 2013). Thus, to further support the role of VCP in PINK1 degradation process, we targeted UFD1. Strikingly, we found that silencing *UFD1* phenocopied the effects of VCP knockdown, in that it markedly increased 52-kDa PINK1 and Ub-PINK1 (Figure 1H).

Thus, our data indicate that conditions in which the functions of VCP, UFD1 and, to a lesser extent, UFD2A are reduced, promote 52-kDa PINK1 stability, suggesting that all of these three factors operate in the molecular pathway between Ub-PINK1 and its proteasomal degradation.

52-kDa PINK1 interacts with the ERAD machinery.

The VCP machinery collaborates with the ER-associated degradation (ERAD) pathway to remove and degrade selected integral membrane proteins (Brodsky, 2012). Thus, in light of the above, we then asked whether PINK1 interacts with ER-resident components of the ERAD pathway, namely, the factors ERLIN-1 and -2, Sec61β (that is also associated with the VCP retrotranslocation complex), and the E3 ubiquitin ligase gp78 (gene *AMFR*) (Christianson & Ye, 2014, Habeck, Ebner et al., 2015, Scott & Schekman, 2008, Spiro, 2004, Wang, Heath-Engel et al., 2008). We found no evidence of interactions between PINK1 and ERLIN-1 and -2 (data not shown). In contrast, we found that PINK1 interacts with Sec61β in both MG132-treated and non-treated HEK293T cells stably expressing PINK1 (Figure 2A); this interaction was also detected in cells expressing endogenous PINK1 (Figure 2B). Moreover, in a similar experiment, we found that gp78 was successfully pulled down by overexpressed PINK1 IP in both MG132-treated and non-treated HEK293T cells; the reciprocal IP also indicated that 52-kDa PINK1 binds to gp78 (Figure 2C). Our results indicate that PINK1 interacts direct or indirectly with specific members of the ERAD machinery, raising the possibility that PINK1 degradation involves the ERAD pathway.

PINK1 polyubiquitination and degradation rely on ERAD machinery and E3 ligases. To determine the contribution of Sec61β to PINK1 stability, cells stably overexpressing PINK1 were infected with lentiviral shRNAs to knock down Sec61β. Two independent Sec61β shRNAs, whose efficiencies were >75% (Figure 2D), caused a consistent increase in 52-kDa PINK1 and Ub-PINK1 in whole-cell extracts (Figures 2D and E). Thus, Sec61β interacts with and contributes to PINK1 stability.

Furthermore, given the physical interaction between PINK1 and gp78 (Figure 2C), we also asked whether this E3 ubiquitin ligase catalyzes PINK1 polyubiquitination. Silencing gp78

in PINK1 and HA-Ub co-transfected HEK293T cells reduced the signal for Ub-PINK1 in association with a moderate increase of 52-KDa PINK1, but in an unusual pattern (Figure 2F): the reduction in the Ub-PINK1 was greatest in the region corresponding to the higher molecular mass species (Figure 2F), suggesting that PINK1 ubiquitination, and in particular that of the lower molecular mass species, may not be mediated solely by gp78. Another ER-related E3 ligase, HRD1, can form a transient interaction and cooperate with gp78 to catalyze ubiquitination of both luminal and membrane ERAD substrates (Bernardi, Williams et al., 2010, Ishikura, Weissman et al., 2010, Zhang, Xu et al., 2015). Accordingly, to examine whether HRD1 is also involved in PINK1 ubiquitination, we performed an *in vivo* ubiquitination assay in HRD1-deficient HEK293T cells (Zhang et al., 2015). Strikingly, the lack of HRD1 did abrogate the Western blot signal for all Ub-PINK1 species (Figure 2G). These results suggest that PINK1 polyubiquitination arises from the dual action of HRD1 and gp78, where the former initiates the formation of the polyubiquitin chain, while the latter elongates it, an interpretation consistent with our previous results that PINK1 polyubiquitination is made of heterotypic chains (Liu et al., 2017).

PINK1 co-localizes with both mitochondria and the ER. In light of the proposed link of PINK1 with ERAD, we revisited PINK1's subcellular distribution. Strikingly, confocal microscopy revealed that PINK1 immunoreactivity in HeLa cells transfected stably with wild-type PINK1 co-localized with the ER marker Sec61β (M1 co-localization coefficients [CCs] = 0.593) (Figure 3A; $P<0.001$) as well as with the mitochondrial markers MitoTracker® Green FM and cytochrome *c* (M1 CCs = 0.531 and 0.685, respectively). In contrast, PINK1 did not co-localize with the Golgi marker GM130 (M1 CC = 0.045) (Figure 3A). Similar results were also obtained with super-resolution structured illumination microscopy (SIM) imaging (Gustafsson, Shao et al., 2008):

COS-7 cells co-transfected with PINK1 and Sec61β-GFP or Mito-DsRed showed that PINK1 co-localized with both Mito-DsRed and Sec61β-GFP (Figure 3B and S1A-B).

Because these microscopy methods do not allow one to conclude whether the observed co-localization is linked to PINK1 immunofluorescence generated by the full-length or cleaved form, or both, two additional methods were employed. First, we used gradient-centrifugation to generate enriched subcellular fractions to detect endogenous 52-kDa PINK1. With this method endogenous mature 52-kDa PINK1 was unambiguously detected in the ER-enriched fractions of HEK293T cells treated with MG132 (Figure 3C). Second, we carried out a subcellular biotinylation assay using an intermembrane space-specific targeted ascorbate peroxidase (IMS-APEX2) (Hung, Zou et al., 2014, Rhee, Zou et al., 2013) to investigate specific cellular compartments. Upon addition of biotin to cells co-transfected with PINK1 and IMS-APEX2, biotin-labeled 52-kDa PINK1 was recovered from the both the ER and mitochondrial fractions following sequential pull-down with anti-PINK1 and streptavidin magnetic beads (Figure 4A-B). The IMS-resident protein SMAC was also biotinylated (Figure 4C). In contrast, none of the ER-resident proteins tested, such as BIP, Sec61β, or calreticulin, were biotinylated by the IMS-APEX2-catalyzed reaction (Figure 4C). These results are consistent with 52-kDa PINK1 residing in or close proximity of both mitochondria and the ER, such as the mitochondrial-ER interface.

Ub-PINK1 is found in both the ER and mitochondrial fractions. The observed partitioning of 52-kDa PINK1 between the mitochondrial and the ER enriched fractions raises the question as to whether PINK1 is differentially ubiquitinated in these two fractions. We therefore performed an *in vivo* ubiquitination assay using purified subcellular fractions isolated from MG132-treated HEK293T cells. We found that Ub-PINK1 was associated with both mitochondrial and ER fractions (Figure 4D) and, using alkaline extraction, that Ub-PINK1 was poorly extractable from both subcellular fractions (Figure 4E-F). To confirm these observations, we carried out an *in*

vivo ubiquitin-mediated fluorescence complementation assay (Fang & Kerppola, 2004) using one plasmid that encoded for ubiquitin fused to the N-terminal half of the fluorescent protein Venus (Vn-Ub), and another that encoded for PINK1 fused to the remaining C-terminal half of Venus (Pink1-Vc). As shown in Figure 4G, Venus fluorescent signal was only observed in HeLa cells successfully transfected with both plasmids (left panel). In those cells, the yellow fluorescence co-localized with both the ER markers Sec61β and KDEL immunofluorescence (M1 CCs = 0.847 and 0.628, respectively) and mitochondrial marker TOM20 (M1 CCs = 0.381), but not with the Golgi matrix protein GM130 (M1 CCs = 0.065, right panel, Figure 4G). Our findings thus provide compelling evidence that Ub-PINK1 is associated with both the ER and the mitochondria-enriched fractions. Furthermore, we showed, by immunoblot and SIM, that the Ub-inefficient PINK1^{K137R} mutant was also found in the mitochondrial (Figure 4H-I) and in the ER fractions, indicating that ubiquitination is not required for 52-kDa PINK1 dual subcellular association.

Ubiquitination-defective PD-linked PINK1 mutations accumulate in the ER fraction. We note that K137, which is required for ubiquitination (Liu et al., 2017), is located in a 130-aa "linker" region (aa 104-233) connecting the transmembrane domain and the kinase domain. To confirm the importance of the PINK1 linker region and the ER to PINK1 degradation, we screened a series of nine pathogenic PD mutations (Bonifati, 2005, Song, Jang et al., 2013) that reside in the linker region in the vicinity of K137. Of the nine such PINK1 mutations examined, we found that five were associated with little or no ubiquitination of PINK1, even after treatment with MG132 (Figure 5A). Remarkably, all five of these ubiquitination-defective mutants were disproportionally enriched in the ER-enriched fractions in the absence of MG132, as revealed by the ratio of 52-kDa: 63-kDa PINK1 (Figure 5B). In contrast, we did not detect ubiquitination defects or ER enrichment in two representative kinase-domain mutants, PINK1^{G309D} and PINK1^{G386A} (Okatsu, Oka et al., 2012, Valente et al., 2004) (data not shown). These results

confirm that PINK1 degradation relies on ubiquitination and the ERAD machinery, and suggest that the PINK1 ER-assisted degradation and kinase activity may be two independent determinants of PINK1 pathogenicity in PD.

Discussion

We show here that 52-kDa PINK1 can be found in both the mitochondria and the ER-enriched fractions, that it interacts with the ERAD machinery, and that its degradation by the proteasome is controlled by the ERAD E3 ubiquitin ligases HRD1 and gp78 and by the ERAD-associated proteins VCP, UFD1, and UFD2A. Thus, these data begin to reveal a novel picture of PINK1 metabolic processing that revolves around possible mitochondria-ER interface and involvement of the ERAD machinery.

Our discoveries of PINK1 linkage to heterotypic polyubiquitin chains (Liu et al., 2017) and interaction with the components of the ERAD pathway have enabled us to obtain further insights into PINK1 degradation process. We note that some E3 ligases (e.g. UBE3C (You & Pickart, 2001)) can generate both K29 and K48 linkages, whereas in other cases, the formation of heterotypic K29/K48 polyubiquitin chains requires at least two E3 enzymes (Koegl, Hoppe et al., 1999, Saeki, Tayama et al., 2004). In keeping with the latter scenario, we found that silencing the ERAD-associated E3 ligase gp78 decreased mainly the higher order polyubiquitin species (Figure 2F), whereas silencing the E3 ligase HRD1 abolished essentially all PINK1 ubiquitination, suggesting that HRD1 initiates PINK1 ubiquitination and that gp78 extends it. This is reminiscent of the proteasomal degradation of two other ERAD substrates, the cytokine autocrine motility factor (Wang, Ha et al., 2014) and the cystic fibrosis transmembrane conductance regulator (Morito, Hirao et al., 2008), where, following the initiation of ubiquitination by one E3 ligase (i.e. TRIM25 and RMA1, respectively (Morito et al., 2008), gp78 then

recognizes the ubiquitin chains already conjugated to the substrates and operates as an ubiquitin chain *elongase* (i.e. E4 ubiquitin factor) to catalyze further polyubiquitination.

The role of the ubiquitin-proteasome system in the degradation of mitochondrial proteins, and especially MOM-localized proteins, is increasingly recognized (Karbowski & Youle, 2011). Even though the knowledge of the direct involvement for PINK1 in mitochondrial-ER crosstalk is limited (ref: https://www.ncbi.nlm.nih.gov/pubmed/28815529), a recent study showed that endogenous PINK1 and BECN1 were both found to re-localize at MAM, where they promoted the enhancement of ER-mitochondria contact sites and the formation autophagosome precursors (ref: https://www.ncbi.nlm.nih.gov/pubmed/28368777). However, how integral MOM proteins are removed from the membrane and targeted to the cytosolic proteasome remains uncertain. Mounting evidence indicates that VCP, which is critically important for the extraction of ubiquitinated proteins from the ER and from other organelle membranes, is also instrumental in the removal and ensuing proteasomal degradation of MOM proteins, such as mitofusins, in mammalian cells (Karbowski & Youle, 2011). The specific action and subcellular recruitment of the multifunctional protein VCP is governed via its interaction with a variety of adaptors. For example, it has been shown in yeast that for VCP to be recruited to the mitochondria and to contribute to the ubiquitin-dependent degradation of MOM proteins, specific VCP adaptors, such as Vms1 (Heo, Livnat-Levanon et al., 2010), are necessary. Although our data demonstrate that VCP is involved in PINK1 turnover, the knockdown of ANKZF1 (the human homologue of yeast Vms1) (see Figure S1C) or of mitochondria-associated E3 ligases (e.g. Parkin, Mulan, and March V) did not affect PINK1 ubiquitination/degradation (Liu et al., 2017). Conversely, the physical interaction between PINK1 and ER proteins, including Sec61β, and components of the ERAD pathway, such as HRD1, gp78, UFD1, and UFD2A, suggests that the VCP-dependent degradation of PINK1 is associated with the ER, not with the mitochondria.

Relevant to the proposed PINK1 association with the ER proteastasis machinery, it is also worth mentioning that Weihofen, Ostaszewski et al. (2008) showed that PINK1 can be detected in the ER-rich microsome fractions of cell homogenates. Our work is consistent with this observation.

At this time, there is no evidence for PINK1 co-translation in either mitochondria or ER nor for PINK1 import in the ER. Although protein movement between the mitochondria and the ER have been reported, especially for proteins belonging to the Bcl-2 family (Saita, Shirane et al., 2013), It is more likely that our results reflect the localization of PINK1 at the contact sites, which refers to zones of close contact between mitochondrial and ER membranes (Rowland & Voeltz, 2012). Consistent with this interpretation are the observations made by both Silvestri, Caputo et al. (2005) and ourselves (Zhou et al., 2008) that PINK1 is found in both the soluble and particulate fractions of mitoplast preparations and is poorly extractable by detergents, features often linked to proteins residing at the mitochondrial/ER contact side.

In *Drosophila*, it seems that PINK1 follows two metabolic routes, one in which a fraction of PINK1 is degraded by the protease Lon inside of the mitochondria and a second that involves the proteasome (Thomas et al., 2014). While the intra-mitochondrial/Lon route has not been found in vertebrate cells (Greene, Grenier et al., 2012, Jin et al., 2010), the role of the cytosolic/proteasome route in PINK1 degradation is well established, and our study and that those already in the literature lead us to propose the following PINK1 metabolic cycle for vertebrate organisms (Figure 6). Upon translation of PINK1, the N-terminal part of the full-length 63-kDa PINK1 is imported into mitochondria where it is cleaved by the combination of MPP and PARL proteases, producing 52-kDa PINK1. This shorter form of PINK1 is subjected to two critical events. First, it adopts a distinct conformation, and engages in interactions with bilayer membranes, distinct from those of the 63-kDa form, thereby, promoting exposure of the lysine residues of the transmembrane-kinase domain linker region, such as K137, which are critical for ubiquitin linkage to PINK1. Second, PINK1 is transferred to the ER, at which point 52-kDa PINK1 interacts with the ERAD machinery, including HRD1, gp78, and VCP, enabling its

ubiquitination and transfer to the proteasome for degradation. While conventional ERAD substrates are degraded when misfolded, PINK1, akin to the non-canonical BCL-2 factor BOK (Llambi, Wang et al., 2016), is degraded continuously in healthy cells by a gp78/VCP-dependent process. Thus, the novel molecular pathway outlined here allows for PINK1 metabolic stability and activity to be tightly regulated in a timely and spatially restricted manner, and ensures that PINK1 serves its function as a mitochondrial quality control factor.

Methods

Cell culture, transfection and plasmids. 293T Hrd1 CRISPR cells were generous gifts from Yihong Ye (National Institute of Health). Transient transfections were performed as previously described (Becker, Richter et al., 2012, Vives-Bauza et al., 2010). Epitope-tagged PINK1 and PINK1 mutants or deletion constructs were generated by PCR-based DNA mutagenesis. Ufd2A-Flag was a gift from David Sidransky (Chatterjee, Upadhyay et al., 2008). The backbone vector used to construct C-terminal Venus-tagged PINK1 (Vc-PINK1) was from Kjungjin Kim (Lee, Lee et al., 2010). The APEX backbone was from Addgene, and the IMS-APEX2 plasmid was a generous gift from Alice Ting (MIT, Cambridge).

Subcellular fractionation, protease K protection assay and alkaline extraction. Crude mitochondria, purified mitochondria and ER from cultured cells were fractioned as described previously (Area-Gomez, 2014, Guardia-Laguarta, Area-Gomez et al., 2014). The alkaline extraction was performed as described (Zhou et al., 2008).

Immunoblotting, immunoprecipitation and in vivo ubiquitination. Immunoblotting and immunoprecipitation assays were carried out as previously described (Vives-Bauza et al., 2010,

Zhou et al., 2008). Co-IP protocols for PINK1-gp78 interaction were adapted from Zhang et al. (2015). In vivo ubiquitination assay was performed in stringent buffer containing 1% SDS before proceeding to immunoprecipitation as described previously (Vives-Bauza et al., 2010). APEX technology was adapted from previously published (Hung et al., 2014, Rhee et al., 2013).

Immunofluorescence and imaging. Immunofluorescence procedures were performed as described (Guardia-Laguarta et al., 2014). The Manders colocalization coefficient M1 (Manders, Verbeek et al., 1993) was calculated using the Fiji distribution (Schindelin, Arganda-Carreras et al., 2012) of ImageJ (Schneider, Rasband et al., 2012). PINK1 and organelle images were manually threshold by a skilled observer. The background-subtracted integrated intensity was calculated for each label and for the overlapping areas. The co-localization coefficient was calculated as the percentage of PINK1 integrated density overlapping with the indicated organelle. For super-resolution Structured Illumination Microscopy (SIM), cells were imaged on a Nikon N-SIM, based on an Eclipse Ti inverted microscope using an SR Apo-TIRF 100x/1.49 oil-immersion objective and an Andor iXon 3 EMCCD camera.

Mass spectrometry analysis. PINK1 purified protein complexes were resolved using SDS-polyacrylamide gel electrophoresis and protein bands were excised, digested with trypsin and purified using Poros 50 R2 (Applied Biosystems) reversed-phased beads, then purified peptide mixtures were subjected to nanoLC-MS/MS analysis as described (Beverly, Lockwood et al., 2012).

Statistical analysis. Data are presented as mean ± SEM from at least three independent experiments. Samples were compared by one- or two-way ANOVA followed by a Student-Newman-Keuls test. The Null hypothesis was rejected at the 0.05 level. All analyses were performed with Sigmaplot for Windows version12.0 (Systech Software Inc., CA).

Supplemental Information

Supplemental information includes Materials and Methods, one figure and one table.

Author Contributions

Y.L., X.J. and S.P. designed the research; Y.L., C.G.-L., J.Y., K.H.L., H.E.-B. and B.M. performed experiments; Y.L., J.Y., C.G.-L., T.C.S., M.J., H.E.-B and S.P. analyzed the data; Y.L., C.G.-L and S.P. wrote the paper.

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Conflict of interest

The authors declare that they have no conflict of interest

References

Area-Gomez E (2014) Assessing the function of mitochondria-associated ER membranes. Methods Enzymol 547: 181-97

Becker D, Richter J, Tocilescu MA, Przedborski S, Voos W (2012) Pink1 kinase and its membrane potential (Deltapsi)-dependent cleavage product both localize to outer mitochondrial membrane by unique targeting mode. J Biol Chem 287: 22969-87

Bernardi KM, Williams JM, Kikkert M, van Voorden S, Wiertz EJ, Ye Y, Tsai B (2010) The E3 ubiquitin ligases Hrd1 and gp78 bind to and promote cholera toxin retro-translocation. Mol Biol Cell 21: 140-51

Beverly LJ, Lockwood WW, Shah PP, Erdjument-Bromage H, Varmus H (2012) Ubiquitination, localization, and stability of an anti-apoptotic BCL2-like protein, BCL2L10/BCLb, are regulated by Ubiquilin1. Proc Natl Acad Sci U S A 109: E119-26

Bonifati V (2005) Genetics of Parkinson's disease. Minerva Med 96: 175-86

Brodsky JL (2012) Cleaning up: ER-associated degradation to the rescue. Cell 151: 1163-7

Chatterjee A, Upadhyay S, Chang X, Nagpal JK, Trink B, Sidransky D (2008) U-box-type ubiquitin E4 ligase, UFD2a attenuates cisplatin mediated degradation of DeltaNp63alpha. Cell Cycle 7: 1231-7

Christianson JC, Ye Y (2014) Cleaning up in the endoplasmic reticulum: ubiquitin in charge. Nat Struct Mol Biol 21: 325-35

Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Yoo SJ, Hay BA, Guo M (2006) Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature 441: 1162-1166

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39: 889-909

Fang D, Kerppola TK (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. Proc Natl Acad Sci U S A 101: 14782-7

Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol 12: 119-31

Greene AW, Grenier K, Aguileta MA, Muise S, Farazifard R, Haque ME, McBride HM, Park DS, Fon EA (2012) Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. EMBO Rep 13: 378-85

Guardia-Laguarta C, Area-Gomez E, Rub C, Liu Y, Magrane J, Becker D, Voos W, Schon EA, Przedborski S (2014) alpha-Synuclein is localized to mitochondria-associated ER membranes. J Neurosci 34: 249-59

Gustafsson MG, Shao L, Carlton PM, Wang CJ, Golubovskaya IN, Cande WZ, Agard DA, Sedat JW (2008) Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. Biophys J 94: 4957-70

Habeck G, Ebner FA, Shimada-Kreft H, Kreft SG (2015) The yeast ERAD-C ubiquitin ligase Doa10 recognizes an intramembrane degron. J Cell Biol 209: 261-73

Heo JM, Livnat-Levanon N, Taylor EB, Jones KT, Dephoure N, Ring J, Xie J, Brodsky JL, Madeo F, Gygi SP, Ashrafi K, Glickman MH, Rutter J (2010) A stress-responsive system for mitochondrial protein degradation. Mol Cell 40: 465-80

Heo JM, Ordureau A, Paulo JA, Rinehart J, Harper JW (2015) The PINK1-PARKIN Mitochondrial Ubiquitylation Pathway Drives a Program of OPTN/NDP52 Recruitment and TBK1 Activation to Promote Mitophagy. Mol Cell 60: 7-20

Hung V, Zou P, Rhee HW, Udeshi ND, Cracan V, Svinkina T, Carr SA, Mootha VK, Ting AY (2014) Proteomic mapping of the human mitochondrial intermembrane space in live cells via ratiometric APEX tagging. Mol Cell 55: 332-41

Ishikura S, Weissman AM, Bonifacino JS (2010) Serine residues in the cytosolic tail of the T-cell antigen receptor alpha-chain mediate ubiquitination and endoplasmic reticulum-associated degradation of the unassembled protein. J Biol Chem 285: 23916-24

Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ (2010) Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J Cell Biol 191: 933-942

Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, Banerjee S, Youle RJ (2014) PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. J Cell Biol 205: 143-53

Karbowski M, Youle RJ (2011) Regulating mitochondrial outer membrane proteins by ubiquitination and proteasomal degradation. Curr Opin Cell Biol 23: 476-82

Kazlauskaite A, Kondapalli C, Gourlay R, Campbell DG, Ritorto MS, Hofmann K, Alessi DR, Knebel A, Trost M, Muqit MM (2014) Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65. Biochem J 460: 127-39

Kim NC, Tresse E, Kolaitis RM, Molliex A, Thomas RE, Alami NH, Wang B, Joshi A, Smith RB, Ritson GP, Winborn BJ, Moore J, Lee JY, Yao TP, Pallanck L, Kundu M, Taylor JP (2013) VCP is essential for mitochondrial quality control by PINK1/Parkin and this function is impaired by VCP mutations. Neuron 78: 65-80

Koegl M, Hoppe T, Schlenker S, Ulrich HD, Mayer TU, Jentsch S (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell 96: 635-44

Koyano F, Okatsu K, Kosako H, Tamura Y, Go E, Kimura M, Kimura Y, Tsuchiya H, Yoshihara H, Hirokawa T, Endo T, Fon EA, Trempe JF, Saeki Y, Tanaka K, Matsuda N (2014) Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature 510: 162-6

Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, Sideris DP, Fogel AI, Youle RJ (2015) The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. Nature 524: 309-14

Lee Y, Lee J, Kwon I, Nakajima Y, Ohmiya Y, Son GH, Lee KH, Kim K (2010) Coactivation of the CLOCK-BMAL1 complex by CBP mediates resetting of the circadian clock. J Cell Sci 123: 3547-57

Lin W, Kang UJ (2008) Characterization of PINK1 processing, stability, and subcellular localization. J Neurochem 106: 464-74

Liu Y, Guardia-Laguarta C, Yin J, Erdjument-Bromage H, Martin B, James M, Jiang X, Przedborski S (2017) The Ubiquitination of PINK1 Is Restricted to Its Mature 52-kDa Form. Cell Rep 20: 30-39

Llambi F, Wang YM, Victor B, Yang M, Schneider DM, Gingras S, Parsons MJ, Zheng JH, Brown SA, Pelletier S, Moldoveanu T, Chen T, Green DR (2016) BOK Is a Non-canonical BCL-2 Family Effector of Apoptosis Regulated by ER-Associated Degradation. Cell 165: 421-33

Manders EMM, Verbeek FJ, Aten JA (1993) Measurement of co-localization of objects in dual-colour confocal images. J Microsc 169: 375-382

Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, Sou YS, Saiki S, Kawajiri S, Sato F, Kimura M, Komatsu M, Hattori N, Tanaka K (2010) PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol 189: 211-21

Meyer H, Weihl CC (2014) The VCP/p97 system at a glance: connecting cellular function to disease pathogenesis. J Cell Sci 127: 3877-83

Morito D, Hirao K, Oda Y, Hosokawa N, Tokunaga F, Cyr DM, Tanaka K, Iwai K, Nagata K (2008) Gp78 cooperates with RMA1 in endoplasmic reticulum-associated degradation of CFTRDeltaF508. Mol Biol Cell 19: 1328-36

Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, Youle RJ (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 8: e1000298

Okatsu K, Oka T, Iguchi M, Imamura K, Kosako H, Tani N, Kimura M, Go E, Koyano F, Funayama M, Shiba-Fukushima K, Sato S, Shimizu H, Fukunaga Y, Taniguchi H, Komatsu M, Hattori N, Mihara K, Tanaka K, Matsuda N (2012) PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. Nat Commun 3: 1016

Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, Bae E, Kim J, Shong M, Kim JM, Chung J (2006) Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature 441: 1157-1161

Rhee HW, Zou P, Udeshi ND, Martell JD, Mootha VK, Carr SA, Ting AY (2013) Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. Science 339: 1328-31

Rowland AA, Voeltz GK (2012) Endoplasmic reticulum-mitochondria contacts: function of the junction. Nat Rev Mol Cell Biol 13: 607-25

Saeki Y, Tayama Y, Toh-e A, Yokosawa H (2004) Definitive evidence for Ufd2-catalyzed elongation of the ubiquitin chain through Lys48 linkage. Biochem Biophys Res Commun 320: 840-5

Saita S, Shirane M, Nakayama KI (2013) Selective escape of proteins from the mitochondria during mitophagy. Nat Commun 4: 1410

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9: 676-82

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671-5

Scott DC, Schekman R (2008) Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. J Cell Biol 181: 1095-105

Silvestri L, Caputo V, Bellacchio E, Atorino L, Dallapiccola B, Valente EM, Casari G (2005) Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. HumMol Genet 14: 3477-3492

Song S, Jang S, Park J, Bang S, Choi S, Kwon KY, Zhuang X, Kim E, Chung J (2013) Characterization of PINK1 (PTEN-induced putative kinase 1) mutations associated with Parkinson disease in mammalian cells and Drosophila. J Biol Chem 288: 5660-72

Spiro RG (2004) Role of N-linked polymannose oligosaccharides in targeting glycoproteins for endoplasmic reticulum-associated degradation. Cell Mol Life Sci 61: 1025-41

Takatori S, Ito G, Iwatsubo T (2008) Cytoplasmic localization and proteasomal degradation of N-terminally cleaved form of PINK1. Neurosci Lett 430: 13-7

Thomas RE, Andrews LA, Burman JL, Lin WY, Pallanck LJ (2014) PINK1-Parkin pathway activity is regulated by degradation of PINK1 in the mitochondrial matrix. PLoS Genet 10: e1004279

Valente EM, bou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del TD, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B et al. (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304: 1158-1160

Vives-Bauza C, Zhou C, Huang Y, Cui M, de Vries RL, Kim J, May J, Tocilescu MA, Liu W, Ko HS, Magrane J, Moore DJ, Dawson VL, Grailhe R, Dawson TM, Li C, Tieu K, Przedborski S (2010) PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. Proc Natl Acad Sci U S A 107: 378-83

Wang B, Heath-Engel H, Zhang D, Nguyen N, Thomas DY, Hanrahan JW, Shore GC (2008) BAP31 interacts with Sec61 translocons and promotes retrotranslocation of CFTRDeltaF508 via the derlin-1 complex. Cell 133: 1080-92

Wang Y, Ha SW, Zhang T, Kho DH, Raz A, Xie Y (2014) Polyubiquitylation of AMF requires cooperation between the gp78 and TRIM25 ubiquitin ligases. Oncotarget 5: 2044-51

Weihofen A, Ostaszewski B, Minami Y, Selkoe DJ (2008) Pink1 Parkinson mutations, the Cdc37/Hsp90 chaperones and Parkin all influence the maturation or subcellular distribution of Pink1. Hum Mol Genet 17: 602-16

Yamano K, Youle RJ (2013) PINK1 is degraded through the N-end rule pathway. Autophagy 9: 1758-69

Ye Y, Meyer HH, Rapoport TA (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 414: 652-6

You J, Pickart CM (2001) A HECT domain E3 enzyme assembles novel polyubiquitin chains. J Biol Chem 276: 19871-8

Zhang T, Xu Y, Liu Y, Ye Y (2015) gp78 functions downstream of Hrd1 to promote degradation of misfolded proteins of the endoplasmic reticulum. Mol Biol Cell 26: 4438-50

Zhou C, Huang Y, Shao Y, May J, Prou D, Perier C, Dauer W, Schon EA, Przedborski S (2008) The kinase domain of mitochondrial PINK1 faces the cytoplasm. Proc Natl Acad Sci U S A 105: 12022-7

Figure Legends

Figure 1. Ub-PINK1 interacts with valosin-containing protein. (**A**) Endogenous PINK1 interacting proteins revealed by Commassie blue-stained SDS-PAGE gel. Endogenous PINK1 and its associated complexes were enriched by IP with anti-PINK1 antibody in HeLa cells. HA antibody and normal IgG were used as controls. Bands labeled as 1, 2 and 3 were identified as Ufd2A, VCP and Npl4, respectively. Data are presented in MEF as the mean ± SE (n≥3). (**B**) PINK1 and VCP interaction in HEK293T cells co-transfected with PINK1 and VCP evidenced by co-IP using either an anti-PINK1 or anti-VCP antibody. * IgG heavy chain. (**C**) Endogenous PINK1 and VCP interaction in SH-SY5Y cells evidenced by co-IP using anti-VCP-antibody. (**D**) As in Fig. 1B, except that cells were transfected either with WT PINK1 or mutant PINK1^{K137R}. * IgG heavy chain. (**E**) PINK1 and Ufd2A interaction in HEK293T cells co-transfected with PINK1 and Ufd2A-flag evidenced by co-IP using an anti-M2 Flag antibody. (**F**) Ub-PINK1 IB in PINK1 stably-transfected HEK293T cells. Endogenous VCP was efficiently knocked down by VCP shRNAs validated by IB. (**G**) Ub-PINK1 IB in PINK1 stably-transfected HEK293T cells. Endogenous Ufd2A was efficiently knocked down by Ufd2A shRNAs validated by IB. (**H**) Ub-PINK1 IB in PINK1 stably-transfected HEK293T cells. Endogenous Ufd1 was efficiently knocked down by Ufd1 shRNAs validated by IB.

Figure 2. ER 52-kDa PINK1 interacts with the ERAD machinery. (**A**) As in Fig. 1B, co-IP was done with anti-PINK1 or anti-Sec61β antibody. (**B**) As in Fig. 1C, co-IP was done with an anti-Sec61β antibody. (**C**) PINK1 and endogenous gp78 interaction in stably PINK1 expressing HEK293T cells evidenced by co-IP using either an anti-PINK1 or anti-gp78 antibody. (**D**) PINK1 IB of PINK1 stably-transfected HeLa cells. Endogenous Sec61β was knocked down by two independent Sec61β shRNAs (#1 and #2) validated by IB. (**E**) Ub-PINK1 IB in PINK1 stably-transfected HEK293T cells. Endogenous Sec61β was efficiently knocked down by Sec61β

shRNAs validated by IB. (**F**) Ub-PINK1 IB in PINK1 stably-transfected HEK293T cells. Endogenous gp78 was efficiently knocked down by gp78 shRNA validated by IB. (**G**) Ub-PINK1 IB in Hrd1 CRISPR HEK293T cells that were transfected with PINK1 WT plasmid.

Figure 3. Ub-PINK1 is localized in both the mitochondria and the ER. (**A**) Co-localization between PINK1 and organelle markers. Cells were transiently transfected with PINK1 (red) and co-stained with cytochrome c (cyt c), Mitotracker, Sec61β-GFP, and GM130 (green). Representative images were shown (left panel) and the quantification (right panel) was done by Image J software. *Scale bar* = 10μm. Data are presented as the mean ± SE (n≥3). (**B**) COS-7 Cells (ATCC) were transfected with PINK1 expression vectors. Images were acquired in 3D-SIM mode using excitation at 488 nm and 561 nm and standard filter sets for green and red emission. Image z-stacks were collected with a z interval of 200 nm. SIM image reconstruction, channel alignment and 3D reconstruction were performed using NIS-Elements AR and Fiji (Schindelin et al., 2012). SIM reconstructed images were threshold, using consistent thresholds for each channel. A binarized mask was created for each channel. Representative images of ER or mitochondria (left panel) PINK1 (center panel), and a composition of the PINK1 mask (depicted in yellow) on top of the ER/mitochondria mask (depicted in red) is shown. Unmasked overlays are shown in Supplemental Figure 1A-B. *Scale bar* = 2 μm. (**C**) PINK1 IB of purified subcellular fractions from HeLa cells. Endogenous PINK1 was enriched by MG132 treatment for 6h, and then proceeded for IP using anti-PINK1 antibody. Note that 52-kDa PINK1 migrates slightly above the heavy chain of IgG (*). See also Figure S1.

Figure 4. Endoplasmic reticulum 52-kDa PINK1 originates from mitochondria. (**A**) HeLa cells were co-transfected with PINK1 and IMS-APEX2, purified mitochondrial (mito) and ER fractions were used in the sequential pull-down. (**B**) Mito-DsRed and Sec61β were used as

mitochondrial and ER outer membrane markers, respectively. IMS-APEX2 specifically co-localized with mitochondrial marker Mito-DsRed in COS-7 cells. *Scale bar* = 10μm. (**C**) 293T cells were co-transfected with PINK1 and IMS-APEX2, labeled with biotin-phenol and processed for IP. Biotin-labeled protein was detected in elutes that was pulled-down by streptavidin magnetic beads using the total cell extract (Input). BIP, Sec61β and Calreticulin were used as ER markers; ATP5A, Smac, and Tom20 were used as mitochondrial markers; PKC and GAPDH were used as cytosolic markers. (**D**) Ub-PINK1 IP followed by IB analysis of purified subcellular fractionation isolated from co-transfected PINK1 and HA-Ub HeLa cell exposed to MG132. (**E-F**) Alkaline extraction (0.1M Na_2CO_3, pH 11) of purified mitochondrial (**E**) or ER fractions (**F**) from the same cells as in Fig. 3D followed by IP and IB analysis. P: particulate; S: supernatant. Tom70, Smac and calnexin are markers of mitochondrial outer membrane, mitochondrial interspace and ER membrane integral proteins, respectively. (**G**) Left panel: The Venus signal (yellow) was only observed in HeLa cells co-transfected with Vn-Ub and PINK1-Vc constructs. Middle panel: Ub-PINK1 signal (yellow) in HeLa cells co-expressing Vn-Ub and PINK1-Vc and immunostained with antibody against Tom20, Sec61β, KDEL or GM130 (red). *Scale bar* = 10μm. Right panel: Quantification of Ub-PINK1 and organelle marker colocalization index. Data are presented as the mean ± SE (n=3) and * ($P<0.05$) and ** ($P<0.01$) indicate difference from GM130 controls (one-way ANOVA followed by a Student-Newman-Keuls test). (**H**) Upper panel: PINK1 IB of purified subcellular fractions of HeLa cells expressing $PINK1^{K137R}$. Lower panel: PINK1 52-kDa:63-kDa ratio relative to WT PINK1 expressing cells. Data are presented as the mean ± SE (n≥3). (**I**) SIM imaging analysis was performed similar to that in Fig. 2B, except that cells were transfected with $PINK1^{K137R}$. *Scale bar* = 100μm.

Figure 5. Ubiquitination level and subcellular fraction in PD pathogenic PINK1 mutants.

(**A**) Ub-PINK1 IB of HEK293T cells co-transfected with HA-Ub and PINK1 pathogenic mutants

exposed to MG132 for 6h. (**B**) Upper panel: PINK1 IB of purified subcellular fractions of cells that express PINK1 pathogenic mutants. Lower panel: PINK1 52-kDa:63-kDa ratio relative to WT PINK1 expressing cells in each subcellular fraction. Data are presented as the mean ± SE (n≥3).

Figure 6. A model for PINK1 metabolic cycle via ER.