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Cytosolic cleaved PINK1 represses Parkin translocation to mitochondria and mitophagy

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Abstract

PINK1 is a mitochondrial kinase proposed to have a role in the pathogenesis of Parkinson's disease through the regulation of mitophagy. Here, we show that the PINK1 main cleavage product, $PINK1_{52}$, after being generated inside mitochondria, can exit these organelles and localize to the cytosol, where it is not only destined for degradation by the proteasome but binds to Parkin. The interaction of cytosolic PINK1 with Parkin represses Parkin translocation to the mitochondria and subsequent mitophagy. Our work therefore highlights the existence of two cellular pools of PINK1 that have different effects on Parkin translocation and mitophagy.

Keywords mitochondria; mitophagy; Parkin; Parkinson's disease; PINK1
Subject Categories Membrane & Intracellular Transport; Autophagy & Cell Death
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Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder [1]. The discovery of mutations in the genes encoding the PTEN-induced putative kinase-1 (PINK1) and Parkin, which are linked to rare familial forms of PD, has led to the hypothesis that a defect in mitochondrial quality control may contribute to PD [2]. Upon reduction of the mitochondrial membrane potential ($\Delta\Psi_m$) by chemicals such as Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or valinomycin, cytosolic Parkin translocates to the mitochondria [3] in a PINK1-dependent manner [4–9]. Once the mitochondria are decorated with Parkin, they cluster and migrate toward the perinuclear area of the cell where they co-localize with autophagy/lysosomal markers [4,5]. Eventually, these mitochondria disappear, leaving cells, such as HeLa, alive but devoid of mitochondria [3]. Yet, if instead of expressing wild-type Parkin and PINK1, cells express PD pathogenic forms of Parkin and/or PINK1, Parkin translocation to the mitochondria and ensuing mitophagy are no longer observed, even if mitochondria have a low $\Delta\Psi_m$ [4–9]. These findings support the notions that both Parkin and PINK1 contribute to the normal turnover of mitochondria [10,11] and that PD mutations, by affecting this quality control mechanism, ultimately cause neurodegeneration [2].

Although PINK1 appears necessary for the recruitment of Parkin, its subcellular distribution and turnover remain debated. Together with our previous findings [12] and those of others [13–16], we show here that, following the processing of full length PINK1 ($PINK1_{63}$) inside the mitochondria, cleaved PINK1 ($PINK1_{52}$) accumulates at the mitochondrial outer membrane (MOM) to ultimately end up in the cytosol. We also show that once in the cytosol $PINK1_{52}$ represses the translocation of Parkin to the mitochondria and the ensuing mitophagy by physically binding to cytosolic Parkin. Ultimately, cleaved PINK1 is degraded by the proteasome. We believe that our data provide further details about the life cycle of PINK1, which should be taken into consideration if PINK1 becomes a therapeutic target [17]. In addition, this study further supports the notion that PINK1 may be acting as a non-canonical mitochondrial protein, like fumarase and aconitase [18], and thus has a dual subcellular localization and a dual function.

Results and Discussion

Protease resistance of cleaved PINK1 is not due to its sheltering inside the mitochondria

We sought to revisit the question of the sub-mitochondrial localization of the PINK1 main cleavage product, $PINK1_{52}$, since debates about where $PINK1_{52}$ resides within the mitochondria have re-emerged [13,14,19–22]. Adding to the debate surrounding $PINK1_{52}$ topology is the uncertainty about whether this cleaved frag-

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ment is the mature form of PINK1 endowed with functional roles or is merely a byproduct destined to be degraded [16,21–23].

To address these questions, we exposed crude mitochondrial preparations from HeLa cells transiently transfected with HA-tagged human PINK1 to increasing concentrations of Proteinase K (PK) with and without detergent as described previously [12,20]. In intact mitochondria (no detergent), Western blot analysis revealed that $PINK1_{52}$ was more resistant to proteolysis than $PINK1_{63}$ (supplementary Fig S1A), a finding that is consistent with that of Jin *et al* [21]. After permeabilization of membranes, evidenced by the PK digestion of SMAC/Diablo, the differential susceptibility of $PINK1_{52}$ versus $PINK1_{63}$ persisted (supplementary Fig S1A). Greater resistance of $PINK1_{52}$ over $PINK1_{63}$ was also noted previously in permeabilized mitochondria isolated from HeLa cells overexpressing PINK1 when exposed to trypsin [12], hence excluding the possibility that the greater resistance of $PINK1_{52}$ was restricted to PK only. Yet, the PK assay performed on purified mitochondria from untransfected HeLa cells that were subjected to an *in vitro* import assay [12], showed that $PINK1_{63}$ and $PINK1_{52}$ were equally susceptible to proteolysis (supplementary Fig S1B), irrespective of the membrane permeabilization status. These results indicate that the reported differential sensitivity to PK between $PINK1_{63}$ and $PINK1_{52}$ [21], which is most detectable at high PINK1 expression levels, does not reflect differential submitochondrial localization, but rather an intrinsic lower susceptibility of $PINK1_{52}$ to proteolysis.

Mitochondrial $PINK_{52}$ is loosely attached to the MOM and translocates to the cytosol

Several studies, including our own [4,13,16,20], have shown that proteasome inhibitors, such as MG132 or epoxomicin, lead to the accumulation of $PINK1_{52}$, as evidenced on Western blots of whole cell lysates. Here, we transiently transfected HeLa cells with HA-tagged human PINK1 and treated them with either MG132, valinomycin or both for 8 h. Incubation with MG132 was associated with a marked increase in $PINK1_{52}$, not only in the crude mitochondrial fraction but also in the cytosolic fraction (Fig 1A), confirming that $PINK1_{52}$ is present in both cellular compartments [12–16]. Conversely, incubation with valinomycin was associated with a decrease in $PINK1_{52}$ in both subcellular fractions while there was an increase in $PINK1_{63}$ in the mitochondrial fractions (Fig 1A). Finally, the combination of valinomycin and MG132 decreased the content of $PINK1_{52}$ in both subcellular fractions compared to MG132 alone (Fig 1A). PINK1 signal quantification is provided in supplementary Fig S2A. These results are consistent with the notion that $PINK1_{52}$ is generated within mitochondria and that collapsing $\Delta\Psi_m$ hinders PINK1 cleavage.

As illustrated by immunocytochemistry (Fig 1B), under basal conditions (DMSO control), there was marginal co-localization between PINK1 and the mitochondrial marker TOM20 (co-localization coefficient $c = 0.16$). In contrast, after 2 h exposure to valinomycin, there was a greater co-localization between PINK1 and TOM20 ($c = 0.58$), with PINK1 immunoreactivity displaying a prominent punctate distribution. Following the incubation of cells with MG132, we saw cytosolic $PINK1^+$ aggregates, many of which were $TOM20^-$. Consequently, the mitochondrial co-localization was limited ($c = 0.32$), again suggesting that a significant portion of $PINK1_{52}$ is localized in the cytosol.

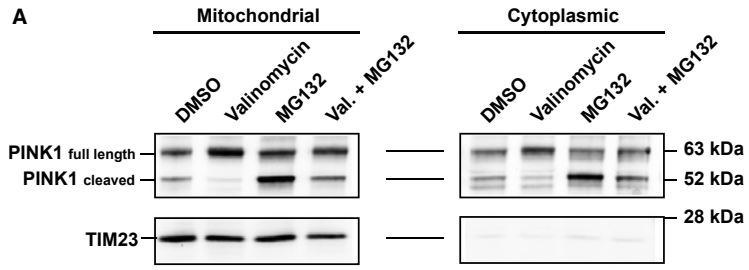
Once $PINK1_{63}$ or at least its *N*-terminal part is imported into mitochondria, it undergoes two sequential proteolytic processing steps which, according to Greene *et al* [24], are mediated first by matrix processing peptidase (MPP) and then by presenilin-associated rhomboid-like protease (PARL) and the m-AAA protease. Previously, we have estimated that the second cleavage should be between amino acids 91 and 104 [20], a prediction confirmed by Deas *et al* [25] who showed that PINK1 is indeed cleaved within the mitochondria by PARL between A103 and F104.

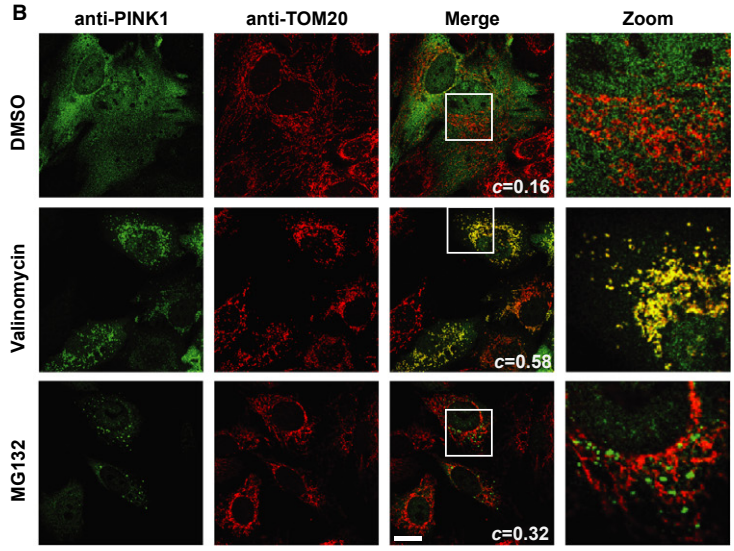
Given that $PINK1_{52}$ is generated by cleavage within the transmembrane (TM) domain, we hypothesized that it may be less firmly integrated into the MOM. We thus subjected mitochondria isolated from HeLa cells which transiently express HA-tagged PINK1 to alkaline extraction and assessed the relative amounts of $PINK1_{63}$ and $PINK1_{52}$ (Fig 1C). We found that approximately 40% of $PINK1_{63}$ remained in the particulate fraction, even at pH 12, whereas < 4% of $PINK1_{52}$ did so. Comparable results were obtained for the alkaline extraction assay on isolated mitochondria after *in vitro* import of radiolabeled [^{35}S]-PINK1 (supplementary Fig S2B). This suggests that $PINK1_{63}$ is more strongly integrated into the MOM than $PINK1_{52}$.

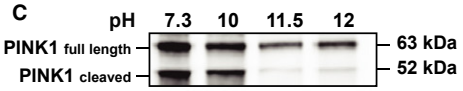
To determine if $PINK1_{52}$ can exit mitochondria, we performed an *in vitro* export experiment with radiolabeled PINK1 and intact mitochondria from HeLa cells. This experiment revealed the accumulation of a signal for $PINK1_{52}$ in the supernatants (Fig 1D) indicating that at least some $PINK1_{52}$ does exit the mitochondria spontaneously.

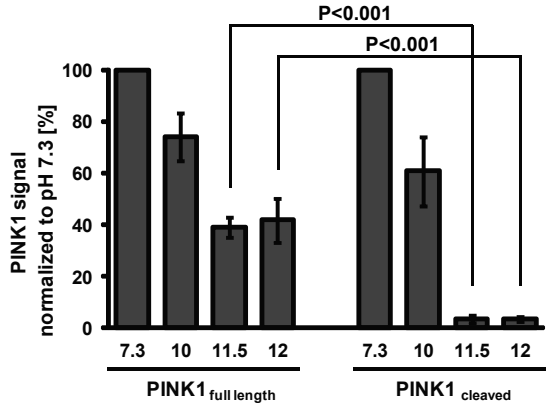
$PINK1_{52}$ binds Parkin

Although cleaved PINK1 is degraded by the proteasome, we doubt that the sole reason for mitochondrial $PINK1_{52}$ to enter the cytosol is to be disposed of. Instead, we predict that cytosolic $PINK1_{52}$ plays a role in the cytosol. Germane to this possibility, we wondered whether cytosolic $PINK1_{52}$ might bind to cytosolic Parkin, and in doing so, whether it might prevent Parkin translocation to mitochondria and ensuing mitophagy. To test this question, we first performed co-immunoprecipitation experiments of whole cell extracts and confirmed that PINK1 physically interacts with Parkin [5,26,27] (Fig 2A). Since specific domains of Parkin are necessary to allow cytosolic Parkin to translocate to mitochondria [27,28], we next sought to determine the PINK1 and Parkin binding domains. Accordingly, immunoprecipitation experiments with several truncated forms of PINK1 carrying a *C*-terminal Flag-tag were performed (Fig 2A). In transfected HEK293T cells, endogenous Parkin was predominantly pulled-down by two PINK1 fragments (Fig 2A, lower panel), both containing the middle section of the kinase domain (aa 156–507 and aa 310–428) and, to a lesser extent, by the truncation encompassing the first part of the kinase domain (aa 156–309). The *N*-terminal part of the protein that encompasses the mitochondrial targeting sequence and the TM (aa 1–155) and the *C*-terminal part of PINK1 (aa 429–581) did not bind to endogenous Parkin. The kinase domain of PINK1 is thus essential for the physical interaction between PINK1 and Parkin. We then used the PINK1 kinase domain fragment (aa 156–507) to determine the PINK1 binding domain of Parkin. Co-immunoprecipiation was performed in HEK293T cells co-transfected with *$PINK1_{156\text{-}507}$-Flag* and various truncated forms of *Parkin* carrying an *N*-terminal Myc-tag (Fig 2B). Although not all our Parkin fragments were expressed and/or successfully pulled-









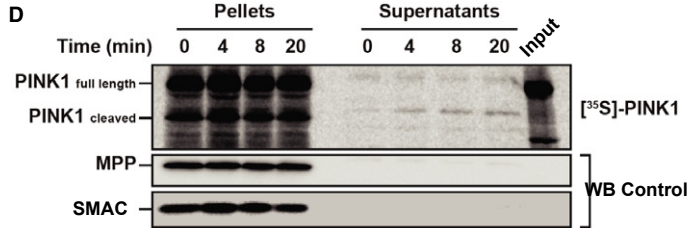


Figure 1. Subcellular localization of full-length and cleaved PINK1.

A PINK1 immunoblot of cytoplasmic and mitochondrial fractions from HeLa cells transfected with PINK1 after 8 h exposure to either DMSO, valinomycin, MG132 or both. TIM23 = mitochondrial marker.

B Representative images of PINK1-transfected HeLa cells after 2 h valinomycin or MG132. TOM20 = mitochondrial marker. *c* = co-localization coefficient. Scale bar = 20 μm.

C Alkaline extraction of PINK1 from isolated mitochondria. Upper panel: Representative immunoblot. Lower panel: Quantification of PINK1 in the particulate fraction (not extractable portion) at varying pH. There is a significant interaction between PINK1 fragments and pH (2W-ANOVA, $F_{3,16} = 253.11$, $P < 0.001$). Newman–Keuls test indicates that PINK1 full length is significantly less extractable than PINK1 cleaved at both pH 11.5 and 12. Values are means ± s.e.m. of three independent experiments.

D Autoradiogram of *in vitro* export assay of [^{35}S]-labeled PINK1 in mitochondria and supernatants (see Materials and Methods). Immunoblots for mitochondrial markers MPP and SMAC.

down, the results revealed that only the products comprising the R1 domain of Parkin did bind to the PINK1 kinase domain (Fig 2B). To confirm that cleaved PINK1 is indeed able to bind Parkin, we transfected HeLa cells stably expressing YFP-Parkin with $PINK_{\Delta 1\text{-}103}$, mimicking $PINK1_{52}$. This co-immunorecipitation experiment demonstrates (Fig 2C) that $PINK_{\Delta 1–103}$ effectively binds YFP-Parkin.

Cytosolic cleaved PINK1 places a break on Parkin translocation and mitophagy

Next, we wondered whether such protein-protein interactions that were to take place in the cytosol, could interfere with Parkin translocation. To test this idea, we first pretreated YFP-Parkin HeLa cells with MG132 for 5 h, to increase endogenous levels of $PINK1_{52}$ in the cytosol. We then induced Parkin translocation by dissipating $\Delta\Psi_m$ with valinomycin for 2 h in the presence of MG132. After fixation, cells were immunostained for PINK1 and for the mitochondrial marker TOM20. As shown in Fig 3A, MG132 treatment significantly impaired Parkin translocation to mitochondria. Using the same pretreatment conditions but extending the time of the exposure to valinomycin/MG132 to 19 h, we also found that the proportions of cells with either reduced or abolished $TOM20^+$ mitochondrial network,

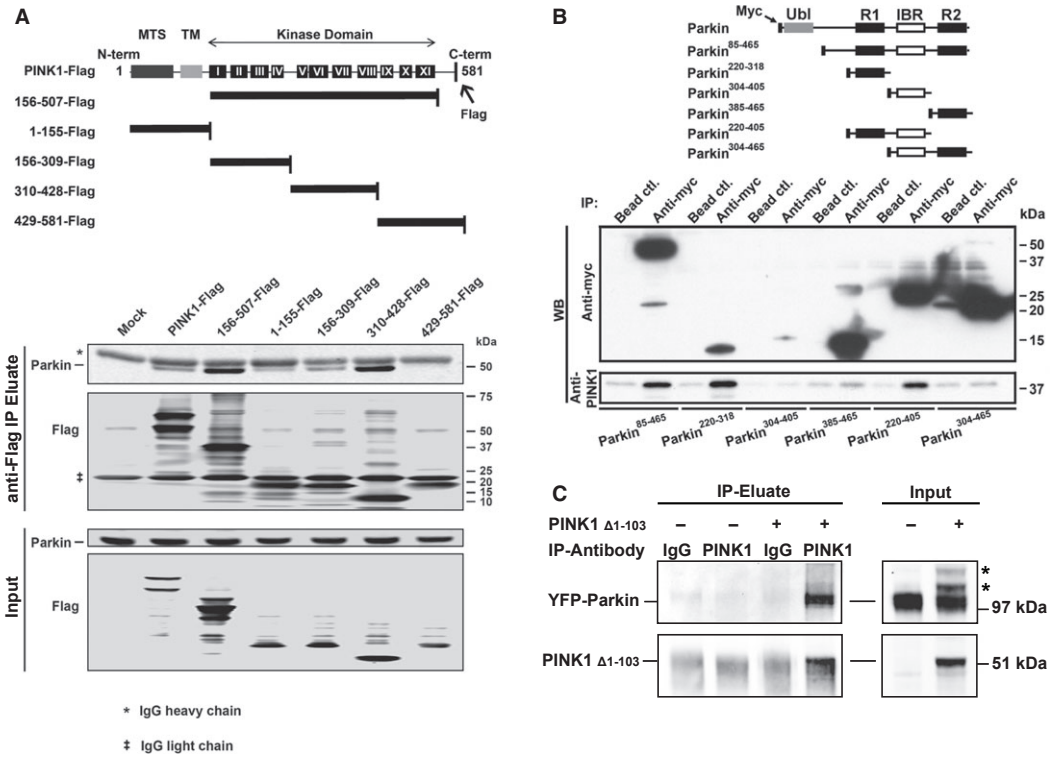


Figure 2. Identification of the PINK1-Parkin binding sites.

A Co-immunoprecipitation assay on lysates of HEK293T cells transfected with *C*-terminal Flag-tagged human PINK1 or PINK1 fragment constructs (schematic) with an anti-Flag antibody, followed by immunoblotting for Flag and Parkin.

B Co-immunoprecipitation assay on lysates of HEK293T cells co-transfected with PINK1 kinase domain ($PINK1_{156–507}$-HA) and selected myc-tagged Parkin domain constructs (schematic).

C $PINK1_{\Delta 1–103}$ (used to mimic cleaved PINK1) binds to YFP-Parkin. Co-immunoprecipitation assay on YFP-Parkin HeLa cells transiently transfected or not with $PINK1_{\Delta 1–103}$. Rabbit IgG = nonspecific binding control; *Parkin high-molecular weight species.

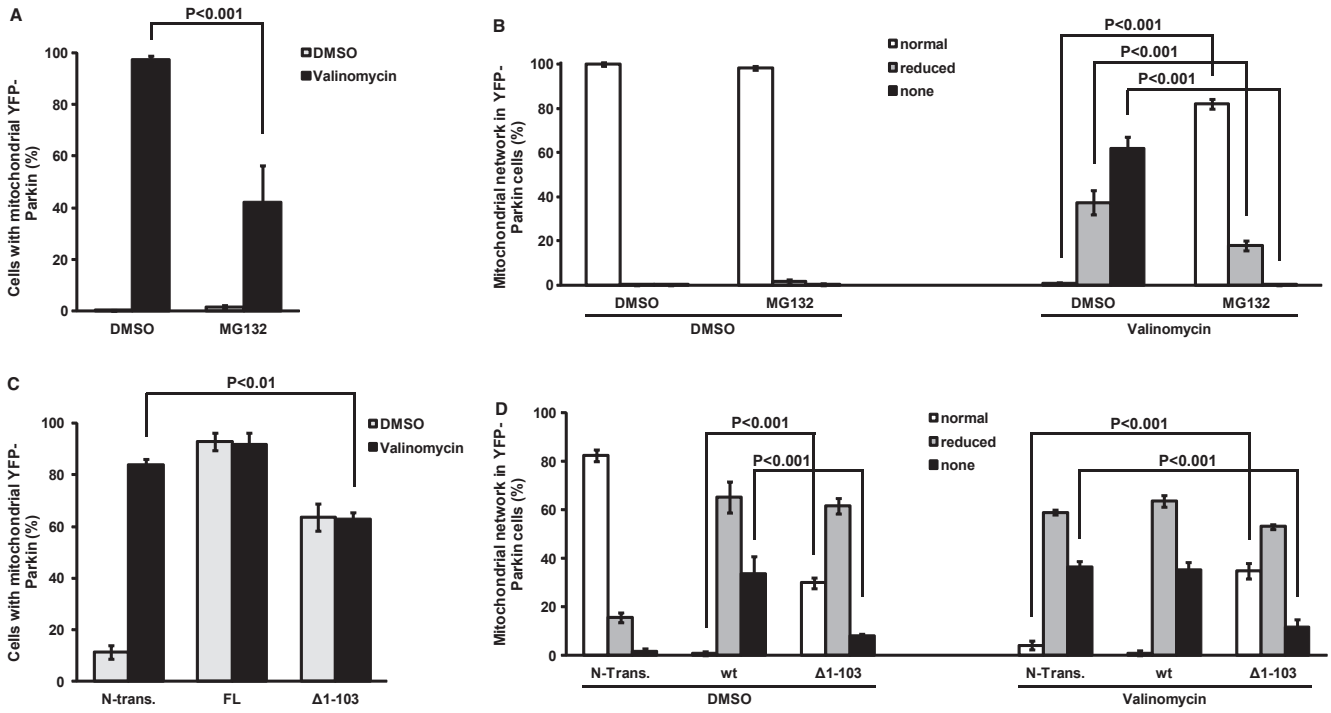


Figure 3. Cleaved PINK1 attenuates Parkin translocation and mitophagy.

A, B YFP-Parkin HeLa cells were pretreated with MG132 (5 h) to increase endogenous cleaved PINK1 and then exposed to valinomycin/MG132 for: (A) Parkin translocation (2 h); (B) mitophagy (19 h). Mitophagy was inferred by the disappearance of the mitochondrial network (see representative images in supplementary Fig S3). In (A), there is a significant interaction between membrane potential and proteasome inhibition (2W-ANOVA, $F_{[1,8]} = 16.12$, $P < 0.001$). Newman–Keuls test indicates that the number of cells with mitochondrial YFP-Parkin is significantly higher in the DMSO/valinomycin than in the MG132/valinomycin group. In (B), there is a significant interaction among membrane potential, proteasome inhibition, and mitochondrial network (3W-ANOVA, $F_{[2,24]} = 239.01$, $P < 0.001$). Newman–Keuls test indicates that the number of cells with normal mitochondrial network is significantly lower in the DMSO/valinomycin than in the MG132/valinomycin group. It also shows that the number of cells with reduced mitochondrial network or no mitochondrial network is significantly higher in the DMSO/valinomycin than in the MG132/valinomycin group.

C, D YFP-Parkin HeLa cells were either transfected with full-length (FL), cleaved mimicking (Δ1–103) PINK1 or not transfected (N-Trans.) and then exposed to valinomycin (C: Parkin translocation [1.5 h]; D: mitophagy [16 h]). In (C), there is a significant interaction between membrane potential and PINK1 fragment (2W-ANOVA, $F_{[2,12]} = 68.4$, $P < 0.001$). Newman–Keuls test indicates that the number of cells with mitochondrial YFP-Parkin is significantly higher in N-trans cells than cells expressing PINK1 $_{\Delta 1–103}$ after valinomycin treatment. In (D), there is a significant interaction among membrane potential, PINK1 fragment, and mitochondrial network (3W-ANOVA, $F_{[4,36]} = 96.01$, $P < 0.001$). Newman–Keuls test indicates that, after valinomycin treatment, the number of cells without mitochondrial network is significantly higher in N-Trans. cells than in cells expressing PINK1 $_{\Delta 1–103}$.

Data information: All values are means ± s.e.m. of 3 independent experiments.

used as a surrogate of mitophagy, were significantly less than control (Fig 3B).

Since MG132 alters the cellular content of a variety of proteins, we next sought to confirm the relationship between cytosolic PINK1$_{52}$ and Parkin translocation and the ensuing mitophagy by overexpressing PINK$_{\Delta 1–103}$. Consistent with the valinomycin/MG132 data (Fig 3A and B), we found that the proportions of YFP-Parkin HeLa cells overexpressing PINK$_{\Delta 1–103}$ that displayed Parkin translocation or an abolished TOM20^{+} mitochondrial network after valinomycin exposure were significantly less than controls (Fig 3C and D). Since all cells express endogenous levels of PINK1, the attenuation of Parkin translocation and of mitochondrial disappearance in PINK1$_{\Delta 1–103}$-expressing cells (Fig 3C and D) suggests that cytosolic PINK1$_{52}$ exerts a dominant negative effect on Parkin recruitment and subsequent mitophagy. However, overexpression of PINK1$_{\Delta 1–103}$, even in absence of valinomycin exposure, promotes some Parkin translocation and mitophagy (Fig 3C and D). This apparent paradoxical finding perhaps stems from the fact that a fraction of cleaved PINK1 still associates with the mitochondria, as we have previously shown [20]. We believe that in doing so, mitochondrial PINK1$_{\Delta 1–103}$, which retains its kinase function, might be able to stimulate Parkin translocation and mitophagy. However, the rest of overexpressed PINK1$_{\Delta 1–103}$ is cytosolic and thus could interfere with most, but not all, Parkin molecules. Consequently, we hypothesize that, even under the stimulating effect of either high mitochondrial PINK1 levels or valinomycin, only some Parkin molecules can translocate and induce mitophagy. Although more work is required to fully examine this idea, it is consistent with the type of response we observed (Fig 3C and D).

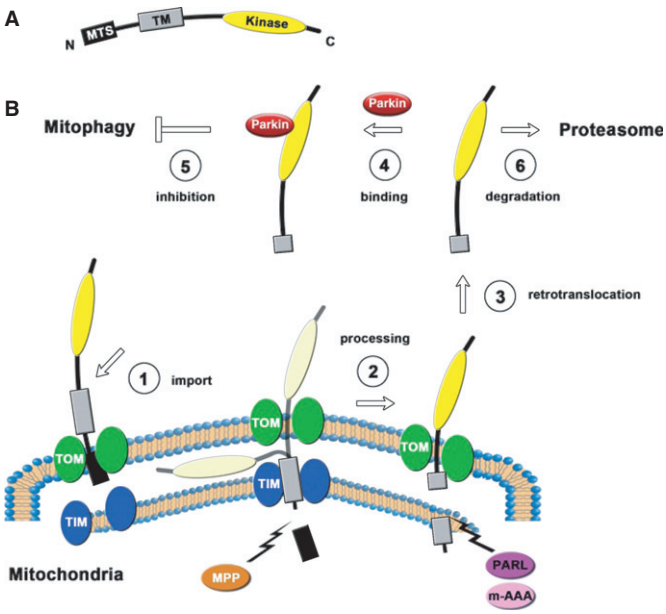


Figure 4. Model of the PINK1 cycle.

A Schematic representation of PINK1. MTS: mitochondrial targeting sequence; TM: transmembrane domain; Kinase: kinase domain.

B Proposed life cycle of PINK1.

Conclusions

The findings presented here, together with those available in the current literature, suggest the following model of PINK1 function, topology and turnover (Fig 4). According to this model, $PINK1_{63}$ or at least its *N*-terminal part is imported into mitochondria by a TOM20-dependent mechanism. PINK1 is then processed sequentially by MPP and PARL/m-AAA. $PINK1_{52}$ then gains access to the cytosol and once there, it attenuates cytosolic Parkin translocation and ensuing mitophagy by binding to Parkin. Yet, overexpressed Parkin, does not overcome the inhibition by endogenous $PINK1_{52}$ through a mass effect, and does not cause mitophagy (Fig 3). This is thus consistent with the finding that in addition to mitochondrial translocation, Parkin has to be activated [29] to induce mitophagy. Eventually, $PINK1_{52}$ is rapidly degraded by the proteasome to ensure a fast turnover and a fast response of the system.

If mitochondria are damaged and exhibit a loss of $\Delta\Psi_m$, no import of PINK1 occurs and thus all of the $PINK1_{63}$ remains at the surface of the mitochondria. As $PINK1_{52}$ is degraded and no-longer replaced, its cytosolic content decreases, hence reducing the repressive action of PINK1 on cytosolic Parkin, and eventually allowing Parkin to translocate to the mitochondria and to contribute to mitophagy. Since the loss of PINK1 repression on Parkin translocation will rely on PINK1 proteasomal degradation, a time-lag between the loss of $\Delta\Psi_m$ and the translocation of Parkin is expected, which is what is experimentally observed. Upon exposure to CCCP the loss of $\Delta\Psi_m$, evidenced with fluorescent probes such as tetramethyl rhodamine methyl ester, is almost instantaneous while overt Parkin translocation is only noticeable after approximately 30 min.

Finally, it has been reported that there is an increased expression of $PINK1_{52}$ protein in PD brains [29]. If our model is correct, this suggests that in PD there may be a deficit in mitophagy by virtue of the fact that cleaved PINK1 may prevent Parkin translocation, hence hampering the elimination of damaged mitochondria.

Materials and Methods

Export assay

Isolation of mitochondria and *in vitro* import of radiolabeled PINK1 was described before [12]. Following 25 min of import at 37°C mitochondria were re-isolated by centrifugation (12 min, 12 000 × *g*, 4°C), re-suspended in fresh mitochondria isolation buffer and incubated at 37°C. After the indicated times, an aliquot was taken and separated into mitochondrial pellet and supernatant by centrifugation (12 min, 12 000 × *g*, 4°C). Samples were analyzed by SDS-PAGE and digital autoradiography.

Alkaline extraction

Crude mitochondria isolated from HeLa cells transfected with HA-tagged wild-type PINK1 were re-suspended in 1 mL of 0.1 mM Na_2CO_3 at indicated pH. After 30 min incubation on ice, samples were centrifuged (1 h, 100 000 × *g*, 4°C). The not extractable portion of PINK1 in the pellet was analysed by SDS-PAGE and Western blotting.

Co-Immunoprecipitation

Flag-IP: HEK293T cells were transfected with PINK1-Flag or Flag-tagged PINK1 fragments. After 24 h, cells were harvested and resuspended in lysis buffer (50 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) supplemented with 2 × protease inhibitor (Roche Diagnostics Corporation, Indianapolis, IN, USA) and agitated (4°C, 1 h). Homogenates were centrifuged (10 min, 11 000 × *g*) and lysate supernatants were collected. Lysates were then incubated with prewashed anti-FLAG M2 antibody affinity gel (A2220; Sigma, St. Louis, MO, USA) overnight (4°C) with constant agitation, followed by washes with lysis buffer. The resins that captured PINK1 were eluted in 2 × SDS sample buffer. Myc-IP: Cells were lysed in buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM Tris (pH 7.5), 0.5 mM PMSF, 0.5 mM EDTA, and 0.5% protease inhibitor cocktail (Sigma). After centrifugation at 14 000 × *g* (10 min), the supernatants were incubated with anti-myc (9E10) antibody-conjugated CNBr-activated Sepharose 4B (Pharmacia Biotech, Piscataway, NJ, USA) or control beads. PINK1-IP: HeLa cells stably expressing YFP-Parkin transiently transfected with $PINK1_{\Delta 1\text{-}103}$ or not transfected as a control were lysed in 20 mM HEPES, 100 mM NaCl, 1 mM EDTA pH 7.4 and 1% Triton X-100 in the presence of protease inhibitors (Roche). After sonication and incubation (4°C, 30 min) cell lysates were obtained by centrifugation (20 min, 15 000 × *g*, 4°C). 800 μg lysate protein was used for IP with the Dynabeads® Protein G Immunoprecipitation kit from Invitrogen (Carlsbad, CA, USA) according to manufacturer's protocol. The anti-PINK1 antibody was used at 1:25 dilution. Normal rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, sc-2027) was used as a negative control.

Statistical analysis

Difference among means was analyzed by 2- or 3-way ANOVA followed by a Newman–Keuls post-hoc test. Cell counts were generated for ≥ 100 cells per condition/construct.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

MAF, RdVS, CR, DB, YH, CZ, DMAW, WV and SP designed research; MAF, RdVS, CR, YH, CZ, and YL performed research; MAF, RdVS, CR, DB, YH, CZ, WV, YL and SP analyzed data; MAF, RdVS, CR, YH, CZ, and SP wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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