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PINK1 Triggers Autocatalytic Activation of Parkin to Specify Cell Fate Decisions

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Summary

Background—The PINK1-Parkin pathway is known to play important roles in regulating mitochondria dynamics, motility, and quality control. Activation of this pathway can be triggered by a variety of cellular stressor signals that cause mitochondrial damage. How this pathway senses different levels of mitochondrial damage and mediates cell fate decisions accordingly is incompletely understood.

Results—Here, we present evidence that PINK1-Parkin has both cytoprotective and pro-apoptotic functions. PINK1-Parkin operates as a molecular switch to dictate cell fate decisions in response to different cellular stressors. Cells exposed to severe and irreparable mitochondrial damage agents such as valinomycin can undergo PINK1-Parkin-dependent apoptosis. The proapoptotic response elicited by valinomycin is associated with the degradation of Mcl-1. PINK1 directly phosphorylates Parkin at Ser⁶⁵ of its Ubl domain and triggers activation of its E3 ligase activity through an autocatalytic mechanism which amplifies its E3 ligase activity towards Mcl-1.

Conclusions—Autocatalytic activation of Parkin bolsters its accumulation on mitochondria and apoptotic response to valinomycin. Our results suggest that PINK1-Parkin constitutes a damage-gated molecular switch that governs cellular context-specific cell fate decisions in response to variable stress stimuli.

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While this manuscript was under review, four publications Koyano et al. *Nature* 510:162-6, Kazlauskaitė et al. *Open Biol* 4:130213 2014; Kazlauskaitė et al. *Biochem J* 460(1):127-39; Kane et al. *JCB* 205:143-53 independently reported the finding of phosphorylation of ubiquitin and Parkin by PINK1.

Introduction

Mutations in PINK1 and Parkin are associated with early-onset familial autosomal recessive Parkinson's disease (PD) [1, 2]. Although the exact molecular mechanism which causes PD is not clearly understood, genetic studies in model organisms coupled with mechanistic studies in mammalian cells suggest that PINK1 acts upstream of Parkin to regulate mitochondrial integrity, dynamics, and motility [3-5].

The level of PINK1 is low in unperturbed mitochondria due to proteolytic degradation of PINK1 by the protease ParL and subsequent retrotranslocation into the cytosol for proteasomal degradation [6]. Upon the loss of mitochondrial membrane potential by decouplers, such as cyanide, the import and degradation of PINK1 are blocked, allowing it to accumulate on the outer mitochondrial membrane [7-9]. Increased expression and activity of PINK1 lead to phosphorylation of mitofusin 2 [10], Parkin [9, 11], Miro [12], and other substrates. Elevated PINK1 activity promotes translocation of Parkin from the cytosol to the mitochondria. In accordance with the significance of Parkin mitochondrial recruitment, many patient derived Parkin mutants are defective in mitochondrial translocation [13, 14]. Parkin is a member of the RING-IBR-RING (RBR) family of ubiquitin E3 ligases with a conserved catalytic cysteine residue analogous to the HECT domain E3s [15]. The auto-ubiquitination activity of Parkin is abolished if this residue is mutated [16, 17]. A diverse set of protein substrates have been shown to be ubiquitinated by Parkin [2], including several proteins localized in the mitochondrial outer membrane such as Mfn1 and Mfn2 [10, 18, 19], Drp1 [20], voltage-dependent anion channel 1 (VDAC1) [21] and Miro [12]. Ubiquitination and degradation of these proteins is linked to mitochondrial fission during mitophagy or mitochondrial motility. Proteomics studies revealed that Parkin may directly or indirectly regulate ubiquitination of more than 100 mitochondrial proteins upon mitochondrial depolarization [22].

Despite these current insights, there are still many outstanding questions that remain to be answered. First, the mechanism by which PINK1 mediates Parkin mitochondrial translocation is still incompletely understood. Second, it remains to be determined how the E3 ligase activity of Parkin is regulated. Biochemical and structure studies revealed that Parkin exists in an autoinhibited conformation due to an interaction between the N-terminal Ubiquitin like domain (Ubl), the repressor element (REP) and the RING1 domain [23]. These observations raise an interesting question about the potential mechanisms that can allosterically activate Parkin *in vitro* and *in vivo*. Finally, the diversity of Parkin substrates begs the question whether there is selective ubiquitination of these substrates and whether ubiquitination of these substrates contribute to cell fate decisions in response to distinct cellular stressor stimuli.

The PINK1-Parkin pathway has also been shown to have cytoprotective activity under various stress conditions [2, 5]. There appears to be distinct Parkin-dependent pathways for preventing cell death depending on the severity of mitochondrial damage and the nature of stress signals. Reactive oxygen species (ROS) can induce PINK1-Parkin dependent repairing damaged mitochondria through the mitochondria derived vesicles (MDVs) trafficking pathway [24]. Upregulation of Parkin-dependent NF- κ B signaling has a

prosurvival role under moderate stress [25]. Mitophagy is the best characterized PINK1-Parkin-dependent pathway for removal of damaged mitochondria [4]. Collectively these results suggest that the PINK1-Parkin nexus can mount different cellular responses depending on the levels of cellular stress signals.

Here we investigated mechanisms by which PINK1-Parkin regulates cell survival. We discovered that beyond its well recognized cytoprotective function, PINK1-Parkin is also required for valinomycin-induced apoptosis. Mechanistically, the pro-apoptotic function of PINK1-Parkin in response to severe apoptotic signal is associated with ubiquitination of anti-apoptotic protein Mcl-1 and activation of the caspase cascade. We reconstituted Parkin-dependent ubiquitination of Mcl-1 *in vitro* and demonstrated that PINK1 allosterically regulates the Parkin E3 ligase activity through phosphorylation of Ser⁶⁵ of Parkin. This phosphorylation event sets off autocatalytic activation of Parkin. Finally, our study showed that ubiquitin is also a substrate for PINK1 and phosphorylated ubiquitin promotes Parkin mitochondrial targeting and afford additional elevation of Parkin activity. Our results reveal a new function of the PINK1-Parkin pathway in cell fate decisions and a Parkin activation cascade in response to diverse stress stimuli.

Results

Parkin-dependent mitophagy and apoptotic cellular responses in response to mitochondrial depolarization

It is well established that the PINK1/Parkin pathway is involved in elimination of depolarized mitochondria by mitophagy [26]. As expected, HeLa cells stably expressing fluorescence protein tagged wild type Parkin but not mutant Parkin T240R (seen in Parkinson's patients) undergo mitophagy in the presence of mitochondrial uncoupler protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Figure 1A). The mitophagic response can be demonstrated by the increased colocalization of Parkin and LC3 with the mitochondria marker RFP-Smac-mts (RFP-Smac) - a fusion of the mitochondrial targeting sequence of Smac with RFP (Supplementary Figure 1AB and Supplementary movie 1 and 2). Under these conditions, no significant apoptosis is seen in either cell lines based on RFP-Smac release (Figure 1B).

To further probe specificity of Parkin-induced mitophagy in response to mitochondrial depolarization, we tested mitophagic responses to a panel of mitochondrial damaging agents including rotenone, dinitrophenol, and valinomycin. Surprisingly, we found only valinomycin, a potassium ionophore known to collapse the potassium gradient across the mitochondrial inner membrane and depolarize mitochondria, induces rapid cellular apoptosis in HeLa cells expressing wild type Parkin but not Parkin T240R mutant (Figure 1CD and Supplementary movie 3). Valinomycin resistance is not due to the expression of the Parkin mutant since wild type HeLa cells display identical apoptosis phenotype upon valinomycin treatment (data not shown). Valinomycin induced cell death can be suppressed by the treatment with the caspase inhibitor Z-VAD-FMK, which is consistent with cell death by apoptosis (Supplementary Figure 1GH). Both CCCP and valinomycin induce rapid Venus-Parkin accumulation on mitochondria with similar kinetics (Supplementary Figure

1CDEF). However, despite similar effects on Parkin mitochondrial accumulation, cells exposed to these two depolarizing agents adopt different cell fates.

PINK1 is required for valinomycin-induced Parkin-dependent apoptosis

Because Parkin-dependent mitophagy requires PINK1 stabilization and accumulation on mitochondria [9, 13, 21, 27], we investigated whether PINK1 is also required for Parkin-dependent apoptosis in response to valinomycin using siRNA-mediated silencing of PINK1. Similar to what we observed for CCCP, valinomycin stabilized PINK1 expression (Figure 1F). As expected, PINK1 knockdown blocks Venus-Parkin accumulation on mitochondria and stabilization of PINK1 (Figure 1EF). The levels of apoptosis are significantly reduced in PINK1 knockdown cells (Figure 1EFG). This result indicates that the PINK1-Parkin pathway is involved in both mitophagy and apoptosis in response to the two different stimuli.

Valinomycin specifically suppresses the expression of anti-apoptotic protein Mcl-1 and activates initiator caspase-8 and caspase-9

To address the mechanism of valinomycin-induced apoptosis, we initially assessed the expression of anti-apoptotic proteins and the activation status of several apoptotic hallmark proteins in Parkin wild type and Parkin T240R mutant HeLa cells (Figure 2A). Expression of Parkin T240R is higher than wild type Parkin which may be a result of their differential stability in cells. However, this difference is unlikely to be important for interpreting the experimental results since Parkin T240R is effectively null in valinomycin induced apoptotic responses and serves a better control to demonstrate the specificity of PINK1/Parkin-dependent cellular responses. Interestingly, in cells expressing wild type Parkin, valinomycin treatment slightly elevated PINK1 expression at 2 hr, after which levels declined and suppressed. In contrast, valinomycin strongly induced expression of PINK1 in HeLa cells expressing Parkin T240R mutant (Figure 2A, top panel). The pattern of PINK1 induction in the absence of functional Parkin in HeLa cells is reminiscent to CCCP treatment described previously [26]. Among the three well characterized Bcl2 proteins, only Mcl-1 is significantly down regulated by valinomycin treatment in a Parkin-dependent manner. No changes in Bcl-xL or Bcl2 were observed. Valinomycin treatment causes activation of initiator caspase-8 and caspase-9 within 4 hr in HeLa cells expressing the wild type but not in cells expressing the mutant Parkin (Figure 2A). In agreement with strong apoptotic activation by valinomycin, effector caspase-3, caspase-7, and PARP are cleaved in a Parkin-dependent manner (Figure 2A). To determine whether suppression of Mcl-1 is due to Parkin-dependent ubiquitin-mediated degradation, HeLa cells with or without Parkin expression were treated with valinomycin in the presence or absence of proteasome inhibitor MG132. High molecular weight Mcl-1 only presents in cells expressing Parkin and accumulates in the presence of MG132 (Figure 2B, lane 8). This finding suggests Mcl-1 is likely ubiquitinated by Parkin upon treatment with valinomycin. Activation of apoptosis by valinomycin is associated with suppression of Mcl-1 and activation of the intrinsic and extrinsic apoptotic initiator caspases.

If Mcl-1 suppression is linked to differences in cell fates upon exposure to CCCP and valinomycin, we would expect that CCCP should not cause significant Mcl-1 degradation.

Immunoblotting analysis revealed that CCCP only causes a slight drop in Mcl-1 and remains stabilized after six hours as opposed to steady decline of Mcl-1 in response to valinomycin (Figure 2C and D). Conversely, if Mcl-1 level is a key determinant of cell fate decision, lowering Mcl-1 should be sufficient to alter the CCCP response. To test this hypothesis, we knocked down expression of Mcl-1 by shRNA (Figure 2E). Shown in Figure 2F, HeLa cells with lower endogenous Mcl-1 undergo apoptosis in response to CCCP. These data suggests that Parkin catalyzes selective degradation of specific substrates in response to specific stimuli. Suppression of Mcl-1 by Parkin is critical for differential cell fate decisions in response to CCCP and valinomycin.

Valinomycin induces PINK1- and Parkin-dependent apoptotic response in MEFs

To determine whether the endogenous PINK1 or Parkin is required for valinomycin-induced apoptosis in another cell system, we first investigated the response to valinomycin in MEFs derived from the parental and PINK1 knockout mice as well as MEFs from Parkin knockout mice [28]. Using time-lapse live cell imaging, we monitored cell apoptotic responses during the time course of valinomycin exposure by a caspase-3 activity sensor NucView 488 (Biotium). DEVD-NucView™ 488 caspase-3 substrate is a cell membrane permeable fluorescence sensor. Upon cleavage by active caspase-3, the DNA-binding dye is released and migrates to the cell nucleus to stain the nucleus bright green. The nuclei and their condensation state were also scored independently by Hoechst 33258 fluorescence using an automated MetaXpress application module. As seen in HeLa cells, parental wild type MEFs are more prone to valinomycin induced apoptosis than PINK1 or Parkin null MEFs (Figure 3AB). To further demonstrate that the endogenous PINK1 has a pro-apoptotic role in valinomycin induced apoptotic response, we knocked down the expression of PINK1 in wild type MEFs using shRNA. A mammalian non-targeting shRNA (NT shRNA) was used as a control. Depletion of PINK1 was confirmed by immunoblotting (Figure 3C). Treatment with valinomycin induced stronger apoptosis in control MEFs (Figure 3CD). Treatment with valinomycin also suppresses Mcl-1 expression in the control MEFs (Figure 3E). However, down regulation of Mcl-1 is partially abrogated in PINK1 knockdown MEF cells (Figure 3E). These results suggest that valinomycin induces PINK1 and Parkin-dependent suppression of Mcl-1 and apoptosis in MEFs.

We tested whether valinomycin sensitivity can be rescued in PINK1 or Parkin null MEFs by stably expressing the two genes. The human versions of these genes were used because commercial antibodies against mouse Parkin are largely ineffective. Nevertheless, stable expression of human PINK1 and Parkin fully restores the defects of valinomycin response in MEFs that are deficient for either gene (Figure 3AB). Immunoblotting confirmed similar pattern of regulation of caspases and Bcl2 proteins seen in HeLa cells (Supplementary Figure 2A) and Z-VAD-FMK sensitivity (Supplementary Figure 2BC). These findings indicate that the PINK1-Parkin pathway controls cell fate determination in response to valinomycin.

PINK1 phosphorylates a highly conserved Ser⁶⁵ residue in Parkin ubiquitin-like (Ubl) domain and ubiquitin

To further probe the biochemical mechanisms by which PINK1 and Parkin regulate cellular apoptosis, we investigated how the PINK1 and Parkin activity controls Mcl-1 ubiquitination *in vitro*. Our initial attempt was to use recombinant PINK1 to drive the pathway. Despite intensive efforts, we were unable to detect robust kinase activity of human PINK1. Previous studies by Muqit and colleagues showed that an insect orthologue of PINK1 (TcPINK1) from *Tribolium castaneum* is catalytically active [29]. In agreement with the previous report, we find TcPINK1 can phosphorylate human Parkin¹⁻¹⁰⁸ and Ser⁶⁵ is most likely the site targeted by TcPINK1 as no phosphorylation can be detected when Ser⁶⁵ was mutated to Ala (Figure 4A, lane 1 versus 2). Despite robust phosphorylation of recombinant human Parkin¹⁻¹⁰⁸ by TcPINK1 *in vitro*, full length human Parkin was reported to be a poor substrate for TcPINK1 [11]. In light of the recent success with the full length rat Parkin (rParkin) protein in structural studies [23], we tested whether recombinant rat Parkin may be a better substrate for TcPINK1 because rParkin is well folded. Shown in Figure 4B, rParkin is robustly phosphorylated by TcPINK1. Mutation of catalytic Cys at 431 to Ser does not affect its phosphorylation. However, mutation of Ser⁶⁵ to Ala in the Ubl domain of rParkin largely eliminates its phosphorylation (Figure 4B, lanes 7-9). Since the Ubl domain of Parkin shares significant sequence and structure homology with ubiquitin and Ser⁶⁵ is conserved and solvent exposed (Supplementary Figure 3ABC), we tested whether ubiquitin may also be a substrate for PINK1. As seen with Parkin Ubl, TcPINK1 can phosphorylate wild type ubiquitin and this phosphorylation is significantly reduced when Ser at 65 is mutated to Ala or Asp (Supplementary Figure 4A), suggesting Ser⁶⁵ is the primary phosphorylation site of ubiquitin. These results indicate that PINK1 phosphorylates Parkin and ubiquitin primarily at a single Ser residue (Ser⁶⁵).

Mcl-1 is a substrate for Parkin E3 ubiquitin ligase

To demonstrate that Mcl-1 is a substrate for Parkin, we reconstituted Parkin-dependent Mcl-1 ubiquitination using highly purified ubiquitin, Uba1(E1), UbcH7 (E2), rat Parkin, and Mcl-1 (Figure 4C) and incubated for 3 hr at room temperature (Figure 4D). Polyubiquitination of Mcl-1 is more robust with a Parkin^{W403A} (Figure 4D lane 7 versus 8), a mutant that has been shown to inactivate the REP element and to elevate its autoubiquitination activity [23]. A double mutant with the substitution of catalytic Cys 431 to Ser has no activity (Figure 4D, lane 9). Omission of any component required in the ubiquitin transfer reaction abrogated polyubiquitination of Mcl-1, suggesting this reaction is highly specific (Figure 4D, lanes 1-6). This finding indicates Mcl-1 is a substrate for Parkin *in vitro*.

Activation of Parkin E3 ubiquitin ligase activity by PINK1 through phosphorylation of Parkin Ubl at Ser⁶⁵

Given that Parkin Ser⁶⁵ is phosphorylated by PINK1, we analyzed the effects of Parkin phosphorylation on its auto-ubiquitination and Mcl-1 ubiquitination activity. Shown in Figure 4E, GST-rParkin is phosphorylated by PINK1 and this phosphorylation is absent in GST-rParkin^{S65A} as detected by a Parkin phospho-Ser⁶⁵ antibody (Figure 4E, lane 2 versus

3). Phosphorylation of Parkin greatly increases its auto- and trans-ubiquitination activity by the early appearance of high molecular weight Parkin conjugates and ubiquitinated Mcl-1 (Figure 4E, lanes 5 -6 versus lanes 7-8). GST-rParkin^{S65A} is inactive in the presence or absence of PINK1 (Figure 4E, lane 3-4 versus 9-10). Since PINK1 also phosphorylates ubiquitin at Ser⁶⁵ (Supplementary Figure 4A), we also tested the effect of this phosphorylation on Parkin E3 ligase activity. Ubiquitin was first incubated with PINK1 or kinase dead PINK1 for 2 hr to allow it to be phosphorylated. Phosphorylated ubiquitin and control ubiquitin were used as inputs in the reconstituted Mcl-1 ubiquitination reaction by Parkin *in vitro*. In the reaction with phosphorylated ubiquitin, ubiquitinated Mcl-1 showed up earlier than the control reaction (Supplementary Figure 4B, lanes 1-3 versus lanes 4-6) as well as the auto-ubiquitinated Parkin bands. Consistent with the positive effect of ubiquitin phosphorylation, phosphomimetic ubiquitin^{S65D} stimulates Parkin auto-ubiquitination more effectively than ubiquitin^{S65A} with Ser⁶⁵ phosphorylated rParkin or unphosphorylated rParkin (Supplementary Figure 4C). These results indicate that phosphorylation of Parkin by PINK1 contributes to its activation *in vitro*. The effect of ubiquitin Ser⁶⁵ phosphorylation on Parkin mitochondrial recruitment was tested by comparing HA-Ub and HA-Ub^{S65A} in HeLa cells expressing Venus-Parkin. Expression of HA-Ub^{S65A} but not HA-Ub caused a significant reduction in Parkin recruitment to mitochondria in response to valinomycin treatment (Supplementary Figure 4D and E). This result points to a potential role for ubiquitin phosphorylation in Parkin recruitment to mitochondria.

Phosphorylation of Parkin Ser⁶⁵ is required for valinomycin induced apoptotic responses

To address the significance of Parkin Ser⁶⁵ phosphorylation in Parkin pro-apoptotic signaling in response to valinomycin, we stably expressed mTurquoise-Parkin^{S65A} in Parkin null MEF and HeLa cells. The mitochondrial recruitment behavior of mTurquoise-Parkin is identical to Venus-Parkin (data not shown). While valinomycin induces wild type Parkin mobility shift (Figure 5A), Parkin^{S65A} is largely unresponsive to valinomycin treatment. mTurquoise-Parkin^{S65A} is also defective in mitochondrial recruitment in response to valinomycin (Figure 5B and 5C). MEF cells expressing mTurquoise-Parkin^{S65A} are resistant to valinomycin induced cell death (Figure 5B and C), suggesting phosphorylation of Parkin Ser⁶⁵ is required for initiating Parkin recruitment to mitochondria and transducing apoptotic signal.

Autocatalysis is a key mechanism for Parkin activation and mitochondrial recruitment

Inefficient accumulation of Parkin^{S65A} on mitochondria could be due to its inability to associate with mitochondria or lack of catalytic activity to generate a positive feedback that is required for rapid and efficient activation of Parkin. Since Ser⁶⁵ phosphorylated Parkin has robust auto-ubiquitination activity, we reasoned that the defect of Parkin^{S65A} in mitochondrial recruitment could be rescued by the presence of wild type Parkin *in trans*. We tested this hypothesis by expressing Venus-Parkin and mTurquoise-Parkin^{S65A} independently or together in the same HeLa cells. As expected, Venus-Parkin is recruited to mitochondria while mTurquoise-Parkin^{S65A} is not when expressed independently upon valinomycin treatment (Figure 5D). In contrast, when both were co-expressed in the same cell, we found that mTurquoise-Parkin^{S65A} is efficiently recruited and indistinguishable from Venus-Parkin at 2 hr (Figure 5E and F). Detailed kinetics analysis revealed that there

was an approximately 10 min delay in the early phase of accumulation for mTurquoise-Parkin^{S65A} and both approached similar levels at approximately 110 min (Figure 5G). This result suggests that catalytically active Parkin converts Parkin^{S65A} to the wild type Parkin phenotype in terms of mitochondrial recruitment.

One hypothesis that can explain the rescue of Parkin^{S65A} by wild type Parkin is that Parkin activation is through an autocatalytic mechanism. To test this hypothesis, we set up an *in vitro* ubiquitination assay using a combination of wild type and mutant Parkin proteins (Figure 6AB). An untagged rParkin^{S65A} was purified to mix with either GST-rParkin or GST-rParkin^{S65A} at 3:1 ratio for the ubiquitination reaction after incubation with TcPINK1 to allow phosphorylation of GST-rParkin to occur. With a three-fold reduction of GST-rParkin, the rate of Parkin auto-ubiquitination slowed down (Figure 6A, lanes 6-8 versus Figure 4E, lanes 5-6). When nonphosphorylatable untagged rParkin^{S65A} was included in the reaction, polyubiquitination of rParkin^{S65A} occurred judging by the disappearance of the substrate (Figure 6A, top panel, lane 3-5). No reaction was seen in the control with GST-rParkin^{S65A} with rParkin^{S65A} (Figure 6A, lane 9-11), suggesting neither protein has catalytic activity in the presence of TcPINK1. With the Parkin pSer65 antibody, we can see the autoubiquitination rate of GST-rParkin increased dramatically in the presence of rParkin^{S65A} (Figure 6A, lanes 3-5 versus 6-8). This is unlikely due to the stimulation of GST-rParkin phosphorylation by rParkin^{S65A} since the overall phosphorylation of GST-rParkin stayed the same (Figure 6A, lane 3-5). This result indicates that Parkin activation likely occurs via an autocatalytic mechanism. PINK1 phosphorylation of Parkin at Ser⁶⁵ provides the initial spark for the nonlinear amplification of Parkin activity.

Autocatalytic activity but not mitochondrial recruitment of Parkin is required for Parkin-dependent apoptotic response to valinomycin

Previous studies showed that mitochondrial expression of linear ubiquitin chain promotes mitochondrial targeting of Parkin^{C431S} mutant that is defective in ubiquitin E3 ligase activity in response to CCCP[16]. To test whether mitochondrial recruitment of Parkin is sufficient for triggering apoptotic response in the presence of valinomycin, we stably expressed mitochondrial targeted linear ubiquitin chain Tom70-4xUbi in Parkin^{-/-}MEF cells along with Venus-Parkin^{C431S}. Immunoblotting showed that Parkin^{C431S} had an extra high molecular weight band, which was independent of valinomycin treatment or Tom70-4xUbi expression (Supplementary Figure 5AB). Upon treatment with valinomycin, Venus-Parkin^{C431S} accumulates on mitochondria in 40% of cells expressing both proteins compared to less than 5% cells expressing Venus-Parkin^{C431S} alone at 90 min (Supplementary Figure 5CD). Meanwhile, valinomycin induced apoptosis is indistinguishable between these two cell lines similar to the cell line expressing Venus-Parkin^{S65A} whereas Venus-Parkin showed dramatic apoptotic response (Supplementary Figure 5EF). Thus in the absence of catalytic activity, Parkin recruitment to mitochondria does not switch cellular responses. This result suggests that Parkin mitochondrial targeting alone is insufficient to trigger valinomycin-induced apoptosis and in line with the notion that Parkin autocatalytic activation and ubiquitination of Mcl-1 are required for mediating the apoptotic responses.

Discussion

Here we show that the PINK1-Parkin pathway can mediate different cell fates in response to different cell stressor stimuli. We uncovered a new pro-apoptotic function of PINK1 and Parkin when cells are exposed to valinomycin in addition to its well documented role in mitophagy and cytoprotection. The PINK1-Parkin dependent apoptotic response is associated with suppression of Mcl-1. PINK1 phosphorylates Parkin at Ser⁶⁵ and unleashes the autocatalytic activity of Parkin and ubiquitination of Mcl-1, which contributes to irreversible molecular switches resulting in apoptotic responses. In addition, we reveal that PINK1 can also phosphorylate ubiquitin at Ser⁶⁵. These two posttranslational events bolster Parkin accumulation on mitochondria and apoptotic response to valinomycin. Our results suggest that PINK1-Parkin constitutes a damage-gated molecular switch that governs cellular context-specific cell fate decisions in response to variable stress stimuli.

PINK1 as a molecular gauge for cellular stress

The anti-apoptotic and cytoprotective function of PINK1 against cell stressors has been well recognized although the exact mechanism is still debatable. For example, PINK1 is known to antagonize MG132-induced cell death [30-32]. The pro-survival function of PINK1 has been attributed to the inhibition of BAX translocation by cytoplasmic PINK1 activity [32] or phosphorylation of Bcl-xL by mitochondrial PINK1 [30, 31]. Some of the protective activity of PINK1 is independent of Parkin or its mitochondria recruitment as PINK1 without mitochondria targeting signal retains anti-apoptotic activity in response to MG132 or MPTP [32, 33]. Here we demonstrate that PINK1 can also mediate pro-apoptotic signals and this function requires Parkin. To reconcile this apparent paradoxical role of PINK1 in cell death regulation, we have to consider the type of cellular damage each cellular stressor inflicts. McLelland *et al.* recently showed that excessive production of reactive oxygen species (ROS) by antimycin A causes mild oxidative damage which generates Mitochondria-derived vesicles (MDVs) in PINK1 and Parkin-dependent manner. These vesicles contain oxidized cargo that is transported to lysosomes for disposal [24] (Figure 7). This pathway is distinct from PINK1-Parkin dependent mitophagy which is triggered by the dissipation of ψ_m as a result of exposure to decouplers such as CCCP and irreversible damage of mitochondria. In many cellular contexts, mitophagy or autophagy may act as a rescue mechanism that the cell uses to escape from cell death [34]. With the treatment of valinomycin, the severity of mitochondria or organelle damage is likely heightened to a threshold beyond repair due to severe stress. Under these conditions, the PINK1-Parkin pathway executes apoptotic program. Hence the cell fate determination dictated by PINK1 or Parkin can be stimulus specific.

Stimuli specific degradation of substrates by PINK1-Parkin pathway

We found that valinomycin and CCCP treatment caused different cell fates via the same PINK1/Parkin activation. The levels and kinetics of Parkin mitochondrial recruitment are comparable (Supplementary Figure 1). This result suggests cell fate decisions are AND-gates since Parkin mitochondrial recruitment alone is insufficient. Indeed, we discovered that the endogenous Mcl-1 level set by Parkin recruitment followed by ubiquitination specifies mitophagy vs. apoptosis decision in response to CCCP or valinomycin. Therefore

targeting Parkin to mitochondria does not lead indiscriminate substrate ubiquitination. There are additional stimuli specific changes that are in play. Our study identified a key determinant in Mcl-1 level and an AND-gate in cell fate decisions. Future studies are needed to uncover stimuli specific determinant that makes Mcl-1 susceptible to Parkin ubiquitination. Regardless the specificity factor of Mcl-1 degradation, our results suggest that PINK1-Parkin pathway serves as damage-gated molecular switch to specify distinct cell fate decisions (Figure 7).

An autocatalytic mechanism for the PINK1-Parkin cascade

Mechanistically we linked valinomycin induced apoptotic response to ubiquitination and degradation of Mcl-1 by Parkin. Mcl-1 is an unstable protein and its steady state levels are regulated by several ubiquitin E3 ligases including Mule [35], SCF^{Fbw7}[36] and SCF^{TrCP}[37]. Here we provide an example of signal-regulated Mcl-1 E3 ligase in Parkin. Using a reconstituted *in vitro* system, we demonstrated that two PINK1-dependent phosphorylation events drive amplification of its auto-ubiquitination and transubiquitination activity. PINK1 directly phosphorylates Ser⁶⁵ of Parkin in the Ubl domain and ubiquitin at Ser⁶⁵. This sets off an autocatalytic cycle for activating Parkin auto-ubiquitination and transubiquitination of Mcl-1. While we still do not know precisely how Ser⁶⁵ phosphorylation to enable Parkin to adopt an active conformation, we can speculate that phosphorylation in Ubl domain of Parkin may unmask the E2 docking site [23]. It is a mystery why phosphorylated ubiquitin can promote its activity. In essence, Ser⁶⁵ phosphorylated ubiquitin could be derived from phosphorylation of auto-ubiquitinated Parkin by PINK1 or conjugation of free ubiquitin phosphorylated by PINK1 or both. Future experiments are needed to determine to what degree ubiquitin is phosphorylated in cells upon PINK1 activation. We show that a Parkin mutant defective in PINK1 phosphorylation can stimulate wild type Parkin E3 activity *in trans*. This result suggests that Parkin is a preferred substrate for itself and a small fraction of Parkin phosphorylation by PINK1 can trigger nonlinear amplification of E3 ligase activity. The positive feedback generated by Parkin auto-ubiquitination and autocatalysis is likely an underlying mechanism for irreversible cell fate determination. This model provides one explanation why wild type Parkin can rescue the defects of Parkin Ser⁶⁵ mutant and robust Parkin accumulation on mitochondria. It will be interesting to determine whether this mechanism can operate locally in a spatial constrained manner.

Experimental Procedures

Cell culture, constructs and antibodies

The source of cell lines, recombinant DNA constructs and antibodies are listed in supplemental experimental procedures.

***In vitro* kinase assay**

Recombinant wild-type and D359A (kinase-dead) in the background of MBP-TcPINK1 and the substrate His₆-Sumo-Parkin (1-108) were purified and the kinase reaction were performed as described [11].

***In Vitro* ubiquitination assay**

Purification of ubiquitin E1, E2 (UbcH7) and reagents for *in vitro* ubiquitination assay was described previously [38]. Wild-type or mutant Parkin proteins were purified from *E. coli* as described previously [23]. Human Mcl-1 (1-327 aa) was subcloned in pET15b and purified by Ni-NTA affinity chromatography. The ubiquitination assays were performed by incubating 1 μ M His₆-Mcl-1 with 0.5 μ M E1, 5 μ M UbcH7 (E2), 0.7 μ M rParkin E3 complex, 20 μ M ubiquitin (Sigma), and 1 μ l 20 \times energy regeneration system (10 mM ATP, 20mM HEPES pH 7.4, 10 mM MgOAc, 300 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase) in a final volume of 20 μ l. The reactions were incubated at 37°C for indicated time, terminated by boiling in 2 \times SDS protein sample buffer and analyzed by SDS-PAGE and immunoblotting with indicated antibodies.

Cell death assays

Three independent cell death assays were used. Detailed methods of RFP-Smac release, NucView caspase-3 biosensor and Hoechst 33258 staining as well as live cell imaging are described in supplementary experimental procedures.

Supplementary Material

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Highlights

- PINK1-Parkin operates as a damaged gated molecular switch for cell fate decisions.
- Parkin catalyzes ubiquitination of selective substrates in response to specific stimuli.
- Parkin is the E3 ligase for Mcl-1 and mediates valinomycin-induced apoptosis.
- PINK1 triggers autocatalytic activation of Parkin by phosphorylating Ser⁶⁵ of Parkin.

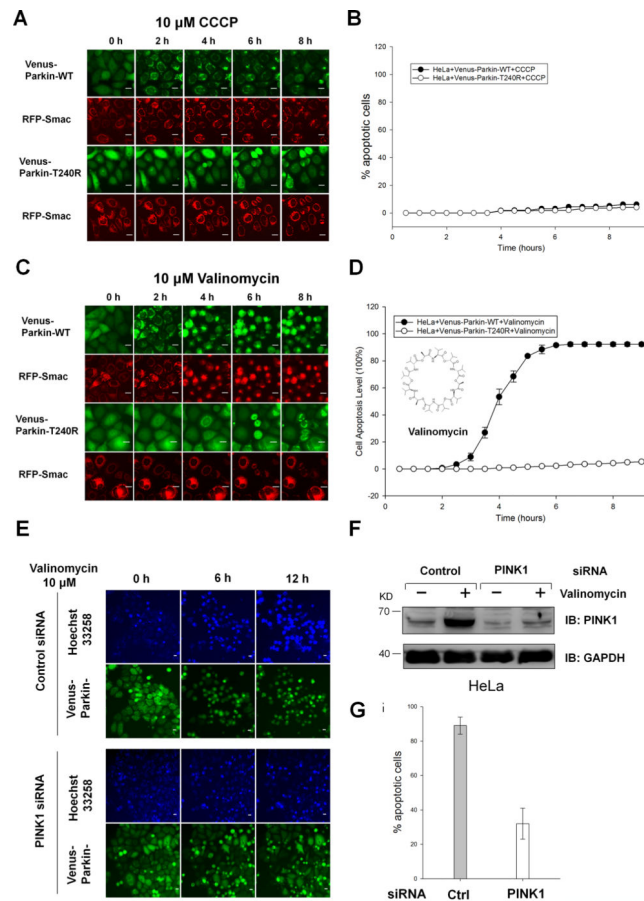


Figure 1. Valinomycin triggers Parkin mitochondrial recruitment and apoptosis in a PINK1/Parkin-dependent manner

(A-D) HeLa cells expressing RFP-Smac-mts and either Venus-Parkin-WT or Venus-Parkin-T240R were treated with 10 μ M CCCP (A and B) or 10 μ M Valinomycin (C and D) in a 9-hour time course. Scale bar, 10 μ m

(A) CCCP induced mitochondrial recruitment of Parkin-WT, but not Parkin-T240R. Neither Parkin-WT nor Parkin-T240R triggered RFP-Smac release from the mitochondria. (B) Apoptotic cell death was quantified by counting cells positive for RFP-Smac release. CCCP did not trigger significant RFP-Smac release in either cell lines. (C) Valinomycin induced mitochondrial recruitment of Parkin-WT, but not Parkin-T240R. (D) Quantitation of cell death by counting cells positive for RFP-Smac release in valinomycin treated cells.

(E-G) HeLa cells expressing Venus-Parkin-WT were transfected with Control or PINK1 siRNA and treated with 10 μ M valinomycin in a 12-hour time course. Apoptotic cell death was visualized by staining with Hoechst 33258 which enters all cells regardless cell health. The average intensity of DNA labeling significantly increases in apoptotic cells as nuclei condense during apoptosis. Quantitation of cell death was performed using automated cell heath application module in MetaXpress software. Representative images are shown. Scale bar, 10 μ m.

(E) PINK1-dependent apoptotic cell death was visualized by fluorescent microscopy. Cells were incubated with valinomycin for 1.5h prior to image capture.

(F) WT and PINK1 knockdown cells in response to valinomycin were immunoblotted using an antibody for PINK1.

(G) Apoptotic cell death was quantified by counting cells positive for cells with condensed chromatin at 12h. More than five hundred cells were scored. Error bars represent standard deviation.

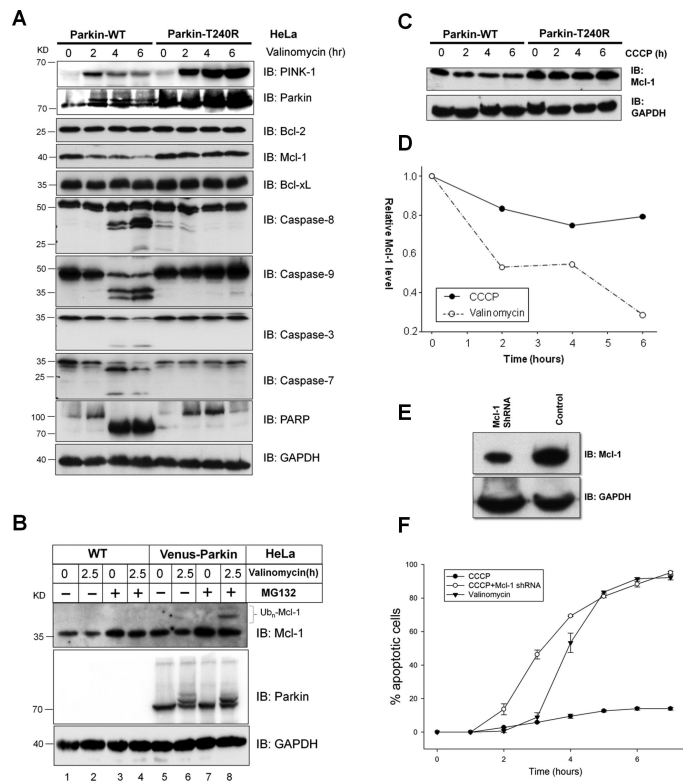


Figure 2. Valinomycin induces Parkin-dependent suppression of Mcl-1 and activation of apoptotic initiator caspases

(A) HeLa cells expressing Parkin-WT or Parkin-T240R were subjected to 10 μ M valinomycin in a 6-hour time course and collected at 2-hour intervals. Pro-apoptotic and anti-apoptotic proteins were monitored by western blotting using antibodies for their respective proteins. Upon valinomycin treatment, cells expressing Parkin-WT exhibited a depletion of Mcl-1 levels, activation of Caspase-8 and -9, and cleavage of Caspase-3, -7 and PARP. The corresponding apoptotic response was not detected in cells expressing Parkin-T240R.

(B) HeLa cells with or without Parkin expression were treated with 10 μ M valinomycin and/or 10 μ M MG132 for 2.5 hr. The response was monitored by western blotting using an antibody for Mcl-1 and Parkin. Cells expressing Parkin degrade Mcl-1 when treated with valinomycin and accumulated Ub-Mcl-1 when treated simultaneously with the proteasome inhibitor MG132.

(C) Effect of CCCP on Mcl-1 levels. The indicated cell lines were treated with 10 μ M CCCP in a 6-hour time course. Endogenous Mcl-1 was blotted with anti-Mcl-1 with GAPDH as a loading control. (D) Mcl-1 levels from (A) and (C) was plotted.

(E) Suppression of endogenous Mcl-1 expression by shRNA. Stable knockdown HeLa cells expression Venus-Parkin and RFP-Smac were obtained by infection with shRNA for Mcl-1.

(F) CCCP induces apoptosis in HeLa cells with reduced Mcl-1 expression. Apoptotic cell death was quantified by counting cells positive for RFP-Smac release.

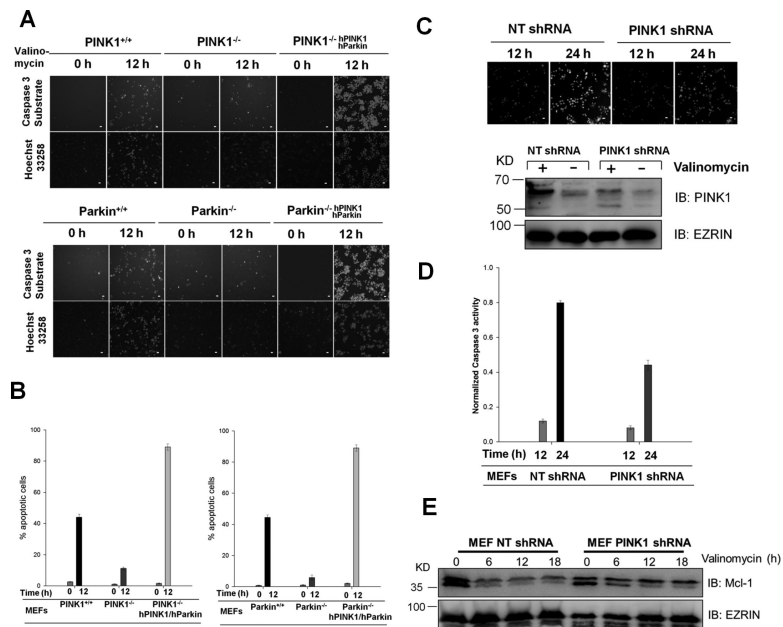


Figure 3. Valinomycin-induced apoptosis requires PINK1 and Parkin

(A) and (B) Wild type MEFs, PINK1 or Parkin null MEFs or MEF nulls reconstituted with human PINK1 and Parkin were stained with NucView caspase-3 sensor and Hoechst 33258 and treated with 10 μ M valinomycin for 12 hr. Apoptotic cell death was visualized with fluorescent microscopy (A) and quantified by measuring the percentage of apoptotic nuclei (B). Scale bar, 10 μ m. (C) Wild type MEF cells were stably introduced with nontargeting or PINK1 shRNA and treated with 10 μ M valinomycin for 24 hrs. PINK1 expression in control and knockdown cells was measured by western blot. Apoptosis was monitored by caspase-3 sensor and quantified. (D) and (E) PINK1 knockdown attenuates valinomycin-induced apoptosis and suppressed Mcl-1 degradation.

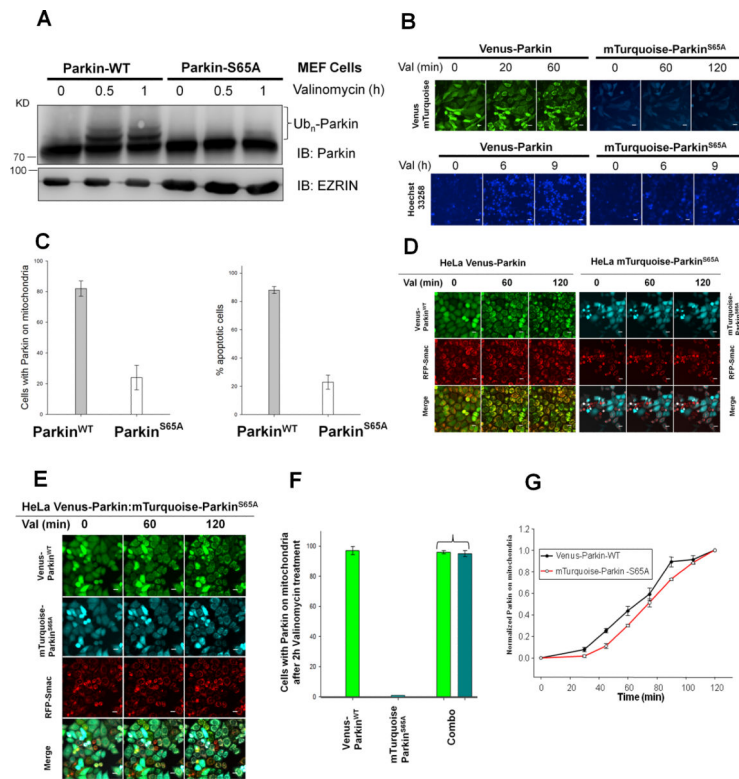


Figure 5. Phosphorylation of Parkin Ser⁶⁵ is required for valinomycin-induced apoptosis PINK1^{-/-} Parkin^{-/-} MEF^{hPINK1} cells expressing Venus-Parkin or mTurquoise-Parkin^{S65A} were treated with valinomycin for indicated time. (A) Immunoblotting analysis of Parkin and Parkin^{S65A} response to valinomycin treatment. (B) Parkin mitochondrial recruitment is perturbed by Ser⁶⁵ mutation. Cells expressing mTurquoise-Parkin^{S65A} are resistance to valinomycin in apoptosis. (C) Quantitation of (B). (D) Parkin mitochondrial recruitment upon treatment with valinomycin in HeLa cells expressing either Venus-Parkin or mTurquoise-Parkin^{S65A} or (E) both Venus-Parkin and mTurquoise-Parkin^{S65A}. Scale bar, 10 μ m. (F) Quantitation of Venus-Parkin or mTurquoise-Parkin^{S65A} accumulation on mitochondria. (G) Time course of Venus-Parkin and mTurquoise-Parkin^{S65A} mitochondrial recruitment in response to valinomycin treatment in double positive HeLa cells.

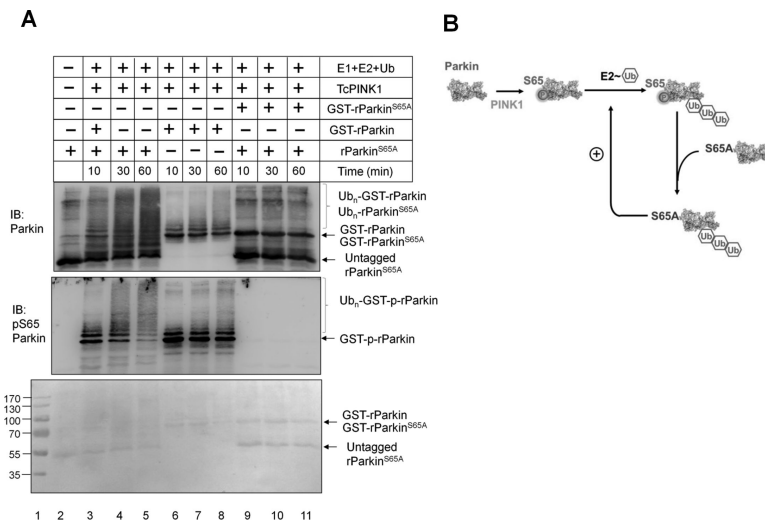


Figure 6. Autocatalytic activation of Parkin by PINK1

(A) *In vitro* ubiquitination of wild type and mutant Parkin through autocatalysis. 0.15 μ M GST-rParkin or GST-rParkin^{S65A} was mixed with 0.45 μ M rParkin^{S65A} prior to incubation with 0.5 μ M TcPINK1 for phosphorylation at 30°C for 60 min. Ubiquitination reactions were monitored by immunoblotting with anti-Parkin or anti-pSer⁶⁵ Parkin antibodies. (B) Schematic diagram of the autocatalytic ubiquitination reaction.

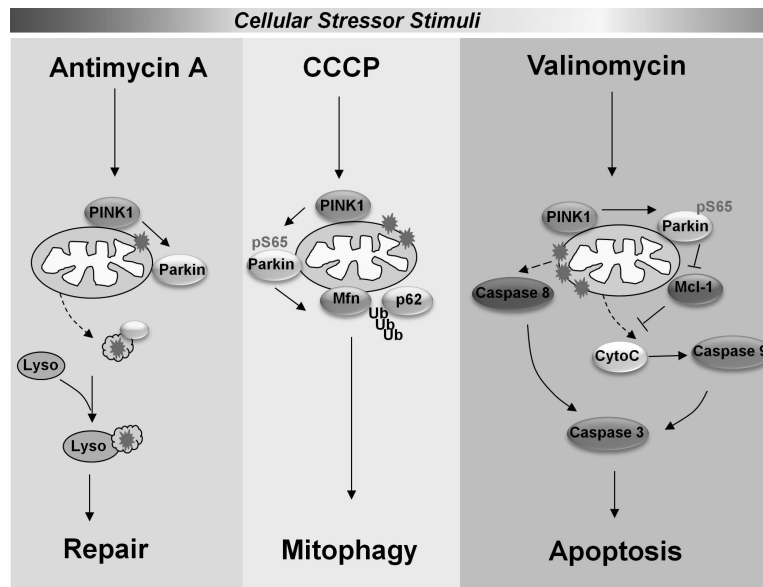


Figure 7. Model depicting the tandem action of PINK1-Parkin as a damaged-gated molecular switch for cell fate specification.