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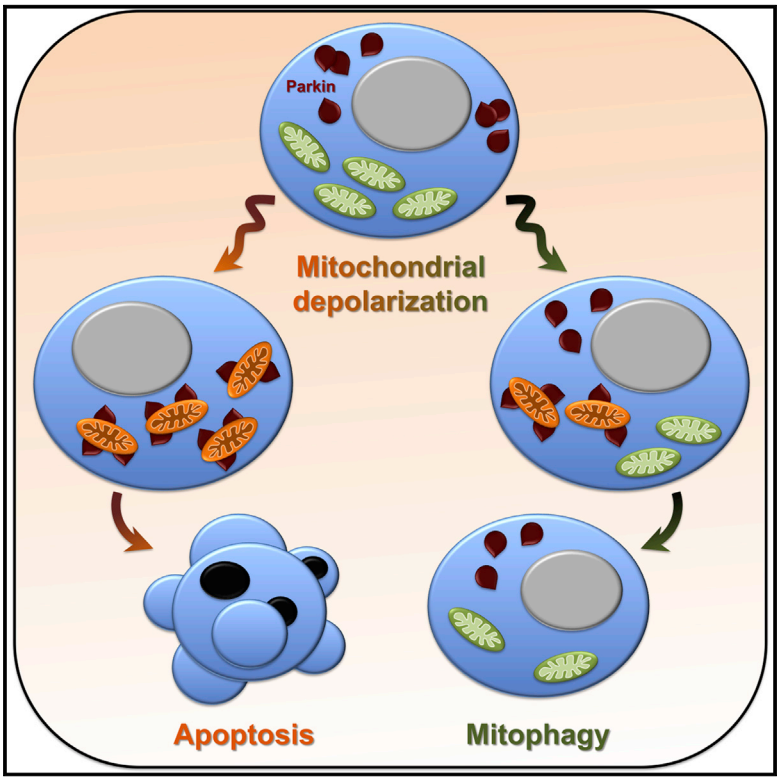
Parkin Sensitizes toward Apoptosis Induced by Mitochondrial Depolarization through Promoting Degradation of Mcl-1

Graphical Abstract



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In Brief

Parkin, the ubiquitin ligase that is mutated in Parkinson's disease, regulates the clearance of damaged mitochondria via selective autophagy (mitophagy). Here, Carroll et al. show that Parkin activation in response to mitochondrial impairment can also result in the elimination of cells containing depolarized mitochondria via apoptosis.

Highlights

Parkin activation can result in mitophagy or apoptosis

Bcl-2 family proteins regulate Parkin-dependent apoptosis

Parkin and PINK1 promote Mcl-1 degradation

Parkin sensitizes toward apoptosis in response to mitochondrial impairment

Carroll et al., 2014, Cell Reports 9, 1538–1553
November 20, 2014 ©2014 The Authors
http://dx.doi.org/10.1016/j.celrep.2014.10.046

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Parkin Sensitizes toward Apoptosis Induced by Mitochondrial Depolarization through Promoting Degradation of Mcl-1

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http://dx.doi.org/10.1016/j.celrep.2014.10.046

SUMMARY

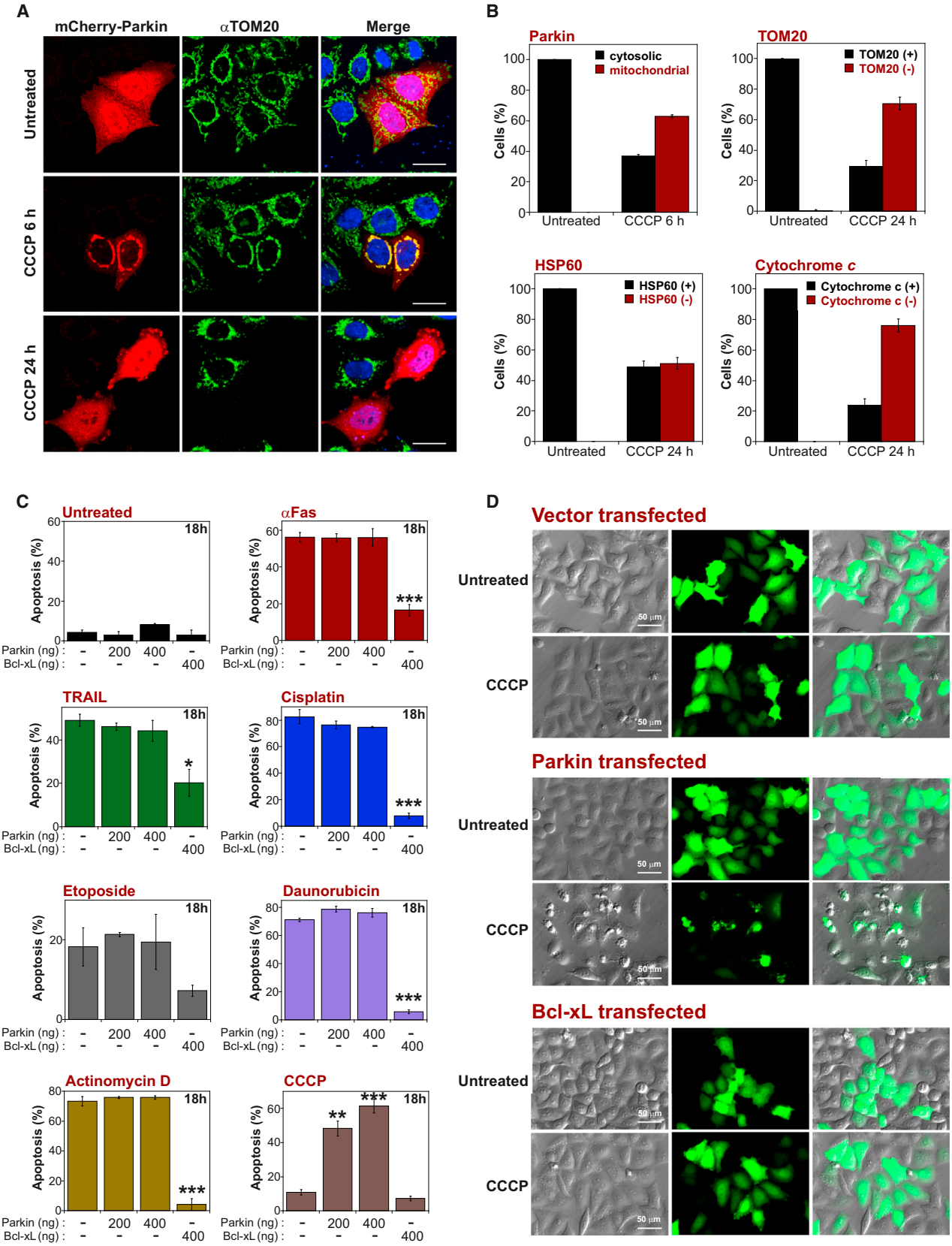
Mitochondrial depolarization promotes Parkin- and PTEN-induced kinase 1 (PINK1)-dependent polyubiquitination of multiple proteins on mitochondrial outer membranes, resulting in the removal of defective mitochondria via mitophagy. Because Parkin mutations occur in Parkinson's disease, a condition associated with the death of dopaminergic neurons in the midbrain, wild-type Parkin is thought to promote neuronal survival. However, here we show that wild-type Parkin greatly sensitized toward apoptosis induced by mitochondrial depolarization but not by proapoptotic stimuli that failed to activate Parkin. Parkin-dependent apoptosis required PINK1 and was efficiently blocked by prosurvival members of the Bcl-2 family or knockdown of Bax and Bak. Upon mitochondrial depolarization, the Bcl-2 family member Mcl-1 underwent rapid Parkin- and PINK1-dependent polyubiquitination and degradation, which sensitized toward apoptosis via opening of the Bax/Bak channel. These data suggest that similar to other sensors of cell stress, such as p53, Parkin has cytoprotective (mitophagy) or cytotoxic modes (apoptosis), depending on the degree of mitochondrial damage.

INTRODUCTION

Parkin is a ubiquitin E3 ligase that is found in mutant form in recessive familial Parkinson's disease, a disorder associated with loss of dopaminergic neurons in the midbrain (Kitada et al., 1998; Shimura et al., 2000). PTEN-induced kinase 1 (PINK1), which shuttles between the cytosol and mitochondria, is also linked with Parkinson's disease and operates in the same pathway as Parkin (Valente et al., 2004). Parkin is normally localized to the cytosol and translocates to depolarized mitochondria in a PINK1-dependent manner (Narendra et al., 2008). In healthy cells, PINK1 is constitutively imported into mitochondria and degraded by mitochondrial proteases but becomes stabilized on the outer membranes of depolarized mitochondria and recruits Parkin to mitochondrial outer membranes (Narendra et al., 2008). Parkin is activated upon PINK1-dependent phosphorylation of ubiquitin, which stabilizes the association of Parkin on mitochondria and activates its Ub ligase activities (Kane et al., 2014; Koyano et al., 2014). Upon activation of Parkin, numerous proteins within the mitochondrial outer membrane become polyubiquitinated, leading to the recruitment of the ubiquitin- and LC3-binding adaptor proteins p62 and NBR1 to mitochondria (Geisler et al., 2010; Narendra et al., 2010). As a consequence, depolarized mitochondria that are decorated with p62 and NBR1 become targeted to autophagosomes and eliminated from the cell via mitophagy (Narendra et al., 2008).

Because Parkinson's disease is associated with progressive loss of dopaminergic neurons, it is widely assumed that Parkin has a cell-death-inhibitory function. However, few studies have addressed this issue explicitly, and there is a paucity of information concerning how Parkin connects to the well-characterized cell-death machinery. It has been suggested that Parkin blocks cell death in response to a variety of stimuli (Darios et al., 2003; MacCormac et al., 2004; Yang et al., 2005; Berger et al., 2009; Ekholm-Reed et al., 2013; Sun et al., 2013; Wang et al., 2013). However, many of these studies utilized proapoptotic triggers (such as etoposide, ceramide, staurosporine, thapsigargin, death receptor ligands) that do not activate Parkin. Furthermore, a recent study suggested that Parkin suppresses apoptosis through promoting NFκB-dependent transcriptional upregulation of the mitochondrial inner membrane protein, Opa1, thereby antagonizing apoptosis-associated cytochrome *c* release (Müller-Rischart et al., 2013). Furthermore, the latter report also suggested, rather surprisingly, that Parkin inhibits apoptosis in a PINK1-independent manner (Müller-Rischart et al., 2013). However, although Opa1 has been implicated in apoptosis-associated cristae junction remodeling, this event is not a major checkpoint in apoptosis and overexpression or knockdown of Opa1 does not greatly alter the threshold for apoptosis (Sheridan et al., 2008). Instead, it has been well established that members of the Bcl-2 family are the major determinants of cell survival in response to stresses that impact on the mitochondrial pathway to apoptosis (Martin et al., 2012). Thus, how Parkin influences cell-death decisions requires further clarification.

Here, we have explored the role of Parkin as a regulator of apoptosis in response to mitochondrial depolarizing agents,



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which are well established to promote Parkin activation during mitophagy (Narendra et al., 2008; Vives-Bauza et al., 2010; Van Humbeeck et al., 2011). In sharp contrast to the claims that Parkin suppresses apoptosis, here we report that Parkin dramatically sensitized cells toward apoptosis induced by mitochondrial depolarization-inducing agents, whereas mutant Parkin failed to do so. Moreover, under the same conditions, Parkin had no discernable effect on apoptosis induced by a range of other proapoptotic stimuli that failed to activate this E3 ligase. Parkin-dependent apoptosis was associated with PINK1/Parkin-dependent degradation of Mcl-1 and overexpression of the latter, as well as other prosurvival Bcl-2 family proteins, suppressed Parkin-dependent cell death. These data challenge the view that Parkin is invariably an inhibitor of apoptosis and suggest, instead, that its role may be to facilitate the repair (via mitophagy) or elimination (via apoptosis) of cells carrying impaired or dysfunctional mitochondrial networks. Thus, similar to the DNA damage sensor p53, which promotes either DNA repair or apoptosis in response to genomic damage, Parkin activation may have two distinct outcomes (mitophagy or apoptosis) depending on the degree of mitochondrial impairment. The failure of Parkin-dependent mitophagy and/or apoptosis may therefore permit the accumulation of cells carrying defective mitochondrial networks.

RESULTS

Parkin and PINK1 Promote Ubiquitination and Removal of Depolarized Mitochondria

To explore the role of Parkin as a regulator of cell death, we initially confirmed that wild-type Parkin could promote mitophagy in response to mitochondrial depolarization. HeLa cells lack detectable Parkin but express endogenous PINK1 and exhibit robust mitophagic clearance of mitochondria upon expression of Parkin in these cells (Hollville et al., 2014). As previously reported by several laboratories, depolarization of mitochondria using the protonophore CCCP led to rapid translocation of transfected Parkin from the cytosol to mitochondria within a few hours of CCCP treatment (Figure 1A). Coincident with Parkin translocation, mitochondrial networks in CCCP-treated cells collapsed around the perinuclear region, as revealed by TOM20 staining (Figure 1A). Parkin translocation to mitochondria was followed, within 24 hr, by complete loss of multiple mitochondrial markers, such as TOM20, HSP60, and cytochrome *c* (Figures 1B, S1A, and S1B). Consistent with previous results (Narendra et al., 2008, 2010; Vives-Bauza et al., 2010; Matsuda et al., 2010; Geisler et al., 2010), depolarized mitochondria in Parkin-expressing cells became heavily decorated with ubiquitin and the ubiquitin-binding adaptor protein p62 (Figures S1C and S1D). GFP-LC3 also relocalized to depolarized mitochondria within Parkin-transfected cells (Figure S1E). Thus, mitochondrial depolarization in HeLa cells promoted rapid recruitment of Parkin to these organelles, followed by ubiquitination of mitochondrial substrates, decoration of depolarized mitochondrial networks with the ubiquitin-binding proteins p62 and LC3, culminating in the elimination of these organelles via mitophagy.

Parkin Fails to Suppress Apoptosis in Response to Diverse Proapoptotic Stimuli

HeLa cells are sensitive to numerous proapoptotic stimuli, including those that engage the extrinsic death receptor pathway (Fas, TRAIL, TNF) as well as the intrinsic or mitochondrial pathway (cytotoxic drugs, DNA damage as well as many other stimuli) to apoptosis (Cullen et al., 2013). To explore whether Parkin suppressed apoptosis routed through either the extrinsic or intrinsic pathways, we transfected cells with either empty vector, or a cDNA encoding wild-type Parkin, along with a GFP expression plasmid to mark transfected cells. As a positive control, we used the well-characterized antiapoptotic protein, Bcl-xL, which blocks apoptosis induced by numerous stimuli (Sheridan et al., 2008). After 24 hr, cells were then treated with a diverse panel of proapoptotic stimuli. However, as shown in Figures 1C and 1D, Parkin failed to provide any protection toward apoptosis induced by Fas, TRAIL, cisplatin, etoposide, daunorubicin, or actinomycin D. In sharp contrast, Bcl-xL exhibited robust antiapoptotic effect toward all of the proapoptotic stimuli used (Figures 1C and 1D). Surprisingly, however, expression of Parkin dramatically sensitized toward apoptosis induced by the mitochondrial depolarizer CCCP (Figures 1C and 1D).

Parkin Selectively Sensitizes toward Mitochondrial Depolarization-Initiated Apoptosis

To ask whether the increased sensitivity toward mitochondrial depolarization-induced apoptosis was reproducible in other cell types, we repeated the preceding experiments in two additional cell lines. As illustrated in Figures 2A and 2B, Parkin failed to exhibit any antiapoptotic activity toward a diverse panel of extrinsic or intrinsic proapoptotic stimuli in either MCF-7 or SH-SY5Y cells. However, once again, there was a clear sensitization toward apoptosis induced by mitochondrial

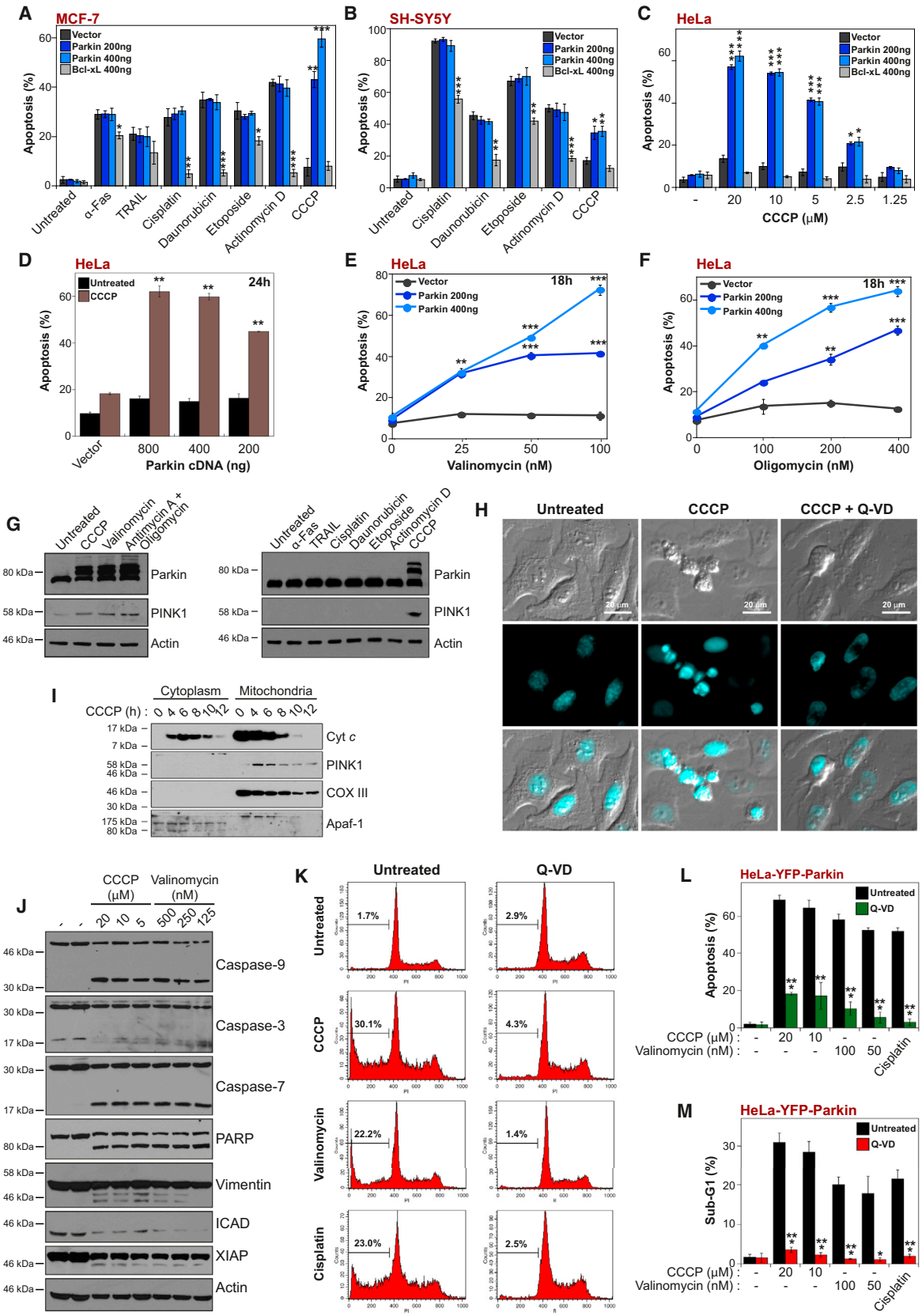
Figure 1. Parkin Promotes Removal of Depolarized Mitochondria but Fails to Suppress Apoptosis in Response to Diverse Proapoptotic Stimuli

(A) HeLa cells were transfected with a plasmid encoding mCherry-Parkin (400 ng) for 24 hr and treated with CCCP (10 μM) for 6 or 24 hr. Mitochondria were immunostained for TOM20 (green), and nuclei were stained with Hoechst (blue). Cells were analyzed by confocal microscopy. Scale bars represent 20 μm.

(B) HeLa cells were transfected and treated as in (A). Mitochondria were immunostained for TOM20, HSP60, or cytochrome *c*. TOM20-negative, HSP60-negative, or cytochrome *c*-negative cells were counted among mCherry-Parkin-positive cells.

(C and D) HeLa cells were transfected with a plasmid encoding eGFP (50 ng) along with the indicated amount of Parkin or Bcl-X_L cDNA for 24 hr. Cells were treated with anti-Fas (200 ng/ml), TRAIL (100 ng/ml), cisplatin (50 μM), etoposide (100 μM), daunorubicin (5 μM), actinomycin D (5 μM), or CCCP (10 μM) for 18 hr. Cell death was quantified in GFP-positive cells based on cell morphology by phase and fluorescence microscopy (C), and images were acquired (D).

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of a minimum of 100 cells. Statistical significance was assessed by two-tailed paired Student's-t test. Asterisk(s) indicate significance, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$. See also Figure S1.



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depolarization (CCCP) in both cell lines (Figures 2A and 2B). Of the cells that failed to die after exposure to mitochondrial depolarization agents after 24 hr, the majority of these displayed features of mitophagy (Figures S2A and S2B). However, the surviving cell population also underwent apoptosis after prolonged (48–72 hr) exposure to CCCP or valinomycin (Figure S2A).

Of note, the sensitizing effects of Parkin toward mitochondrial depolarization-induced apoptosis were highly selective and were not observed in response to any of the other proapoptotic stimuli used (Figures 1C, 2A, and 2B). Furthermore, the proapoptotic effects of Parkin were readily observed over a wide range of CCCP concentrations (Figures 2C and S2B), as well as a range of Parkin plasmid concentrations (Figure 2D). We also explored whether apoptosis induced by other mitochondrial depolarizing agents was also enhanced in the presence of Parkin. As can be seen from Figures 2E and S2B, Parkin also greatly increased sensitivity toward apoptosis induced by valinomycin, as well as the combination of oligomycin and antimycin A (Figure 2F). It is noteworthy that the only stimuli that elicited Parkin-dependent increases in apoptosis share in common the ability to trigger Parkin activation and PINK1 stabilization (Figure 2G, left panel), as well as Parkin translocation to mitochondria (Figures S2C–S2G). In contrast, treatment with Fas, TRAIL, cisplatin, daunorubicin, etoposide, or actinomycin D failed to activate Parkin or stabilize PINK1 (Figure 2G, right panel). Thus, expression of Parkin sensitized three independent cell lines toward apoptosis induced by mitochondrial depolarization, but not other proapoptotic stimuli.

Parkin-dependent cell death in response to mitochondrial depolarization exhibited stereotypical features of apoptosis, such as chromatin condensation and nuclear fragmentation (Figure 2H), was accompanied by mitochondrial cytochrome *c* release (Figures 2I, S2H, and S2I), activation of multiple proapoptotic caspases and proteolysis of caspase substrates (Figure 2J), as well as DNA fragmentation (Figure 2K). Furthermore, Parkin-dependent apoptosis induced by mitochondrial depolarization was readily attenuated with the polycaspase inhibitor Q-VD (Figures 2H, 2K–2M, S2J, and S2K).

Collectively, these data demonstrate that not only did Parkin fail to protect against apoptosis initiated by multiple proapoptotic stimuli, but this E3 ligase greatly sensitized toward mitochondrial-depolarization-induced apoptosis, a condition that activates Parkin.

Mitochondrial Depolarization-Induced Apoptosis Is Enhanced by Parkin in a PINK1-Dependent Manner

PINK1 is stabilized on mitochondrial outer membranes in response to mitochondrial depolarization (Vives-Bauza et al., 2010; Figure 3A). However, PINK1 stabilization, on its own, was not sufficient to sensitize toward apoptosis induced by CCCP (Figures 3A and 3B). The latter result strongly suggests that apoptosis in this context is Parkin-dependent and that accumulation of PINK1 on the mitochondrial outer membrane is insufficient.

To explore whether Parkin-dependent apoptosis required PINK1, we used small interfering RNA (siRNA)-mediated knockdown of the latter. As shown in Figure 3C, PINK1 was very efficiently silenced using specific siRNAs, and this completely blocked the translocation of Parkin to mitochondria in response to CCCP treatment, as expected. We then explored the effects of PINK1 knockdown on mitochondrial depolarization-induced apoptosis and, as can be seen from Figure 3D, silencing of PINK1 expression reversed the Parkin-dependent sensitization to apoptosis seen in response to CCCP or valinomycin treatment. Thus, mitochondrial depolarization-induced apoptosis requires both Parkin and PINK1, with neither alone being sufficient.

Figure 2. Parkin Enhances Mitochondrial Depolarization-Initiated Apoptosis

(A and B) MCF-7 (A) and SH-SY5Y (B) were transfected with a plasmid encoding eGFP (50 ng) along with the indicated amount of Parkin or Bcl-X_L cDNA for 24 hr. Cells were treated with anti-Fas (200 ng/ml), TRAIL (100 ng/ml), cisplatin (50 μM), daunorubicin (5 μM), etoposide (100 μM), actinomycin D (5 μM), or CCCP (10 μM) for 24 hr. Cell death was counted among GFP-positive cells based on cell morphology.

(C) HeLa cells were transfected as in (A) and were then treated with the indicated dose of CCCP for 18 hr. Cell death was scored in GFP-positive cells based on cell morphology.

(D) HeLa cells were transfected with a plasmid encoding eGFP (50 ng) along with the indicated amount of Parkin cDNA for 24 hr. Cells were treated or not with CCCP (10 μM) for 24 hr, and cell death was counted among GFP-positive cells based on cell morphology.

(E and F) HeLa cells were transfected with a plasmid encoding eGFP (50 ng) along with Parkin cDNA for 24 hr. Cells were treated with the indicated dose of valinomycin (E) or with the indicated dose of oligomycin and antimycin A (500 nM) (F) for 18 hr. Cell death was counted among GFP-positive cells based on cell morphology.

(G) HeLa-YFP-Parkin cells were treated with CCCP (10 μM), valinomycin (50 nM), or antimycin A (500 nM) + oligomycin (100 nM) for 18 hr (left panel) or with anti-Fas (200 ng/ml), TRAIL (100 ng/ml), cisplatin (50 μM), daunorubicin (5 μM), etoposide (100 μM), antinomycin D (5 μM), or CCCP (10 μM) for 12 hr (right panel). PINK1 stabilization and Parkin activation was analyzed by western blotting.

(H) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) in the presence or absence of Q-VD-OPh (10 μM) for 14 hr. Nuclei were stained with Hoechst (2 μM, blue), and nuclear fragmentation was imaged by phase and fluorescence microscopy. Scale bars represent 20 μm.

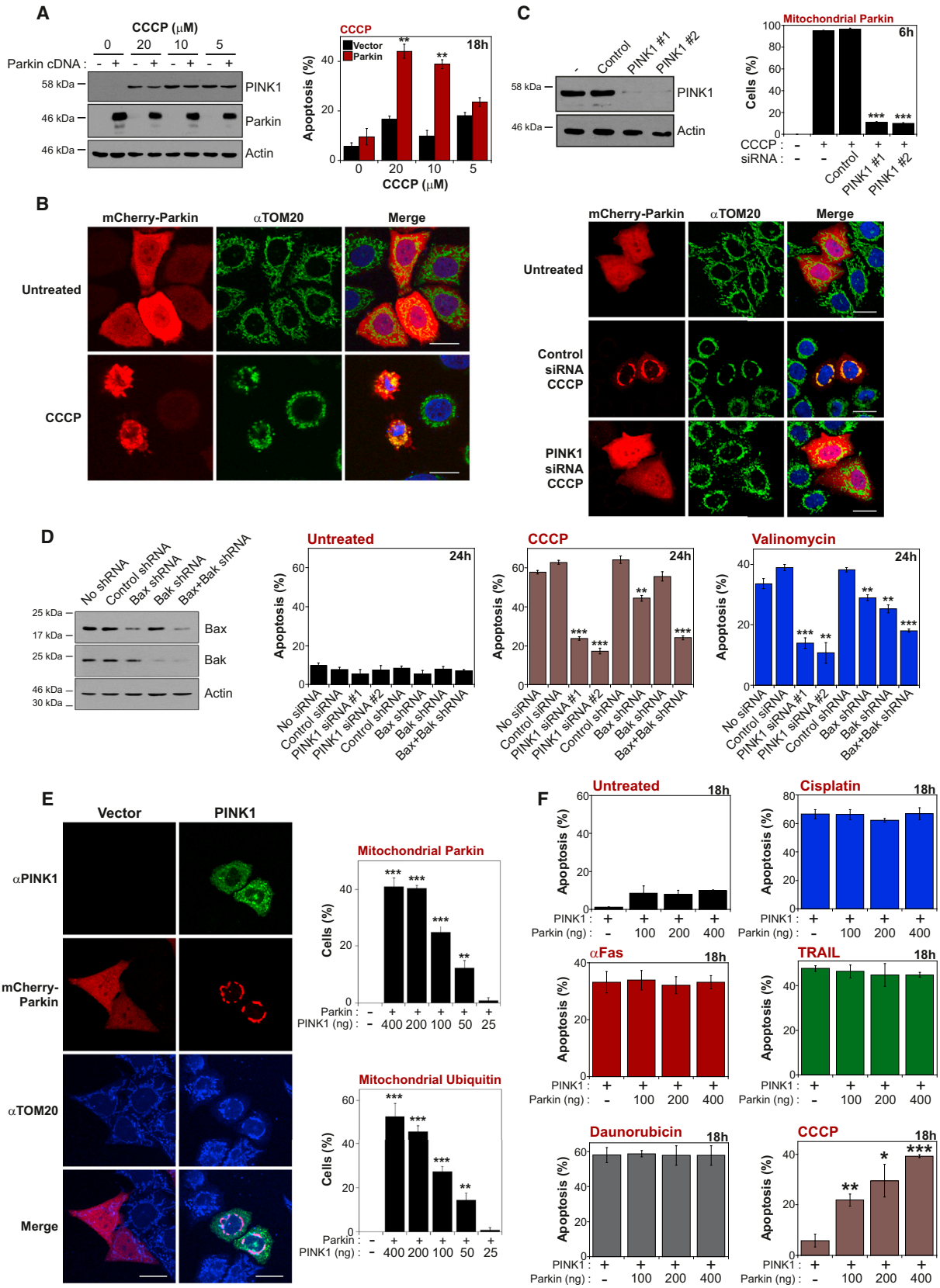
(I) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for the indicated period of time before mitochondrial and cytoplasmic fractions were prepared and analyzed by western blotting.

(J) HeLa-YFP-Parkin cells were treated with the indicated dose of CCCP or valinomycin for 24 hr. Caspases and caspase substrates were then analyzed by western blotting.

(K) HeLa-YFP-Parkin cells were treated with CCCP (10 μM), valinomycin (50 nM), orcisplatin (50 μM) in the presence or absence of Q-VD-OPh (10 μM) for 20 hr. DNA content was analyzed by flow cytometry.

(L and M) HeLa-YFP-Parkin cells were treated with the indicated dose of CCCP or valinomycin or with cisplatin (50 μM) in the presence or absence of Q-VD-OPh (10 μM) for 14 (L) or 20 hr (M). Cell death was scored based on cell morphology (L), and DNA content of cells was analyzed by flow cytometry (M).

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of a minimum of 100 cells. Statistical significance was assessed by two-tailed paired Student's-t test. Asterisk(s) indicate significance, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$. See also Figure S2.



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PINK1-Induced Parkin Translocation to Mitochondria Selectively Enhances Apoptosis Induced by Mitochondrial Depolarization

Parkin is constitutively cytosolic and inactive in the absence of mitochondrial depolarization (Figures 1A and 2G). Thus, the failure of Parkin to modulate apoptosis induced by cytotoxic stimuli other than mitochondrial depolarizers may be related to its inactive (cytosolic) state, as mitochondrial depolarizers were the only proapoptotic stimuli capable of activating Parkin and promoting its translocation to mitochondria (Figures 2G and S2C–S2G). Although Parkin activation is typically achieved through mitochondrial depolarization, which stabilizes PINK1 on mitochondrial outer membranes, this can also be achieved through overexpression of PINK1, which is sufficient to trigger Parkin translocation to mitochondria (Figure 3E). Thus, we next explored whether PINK1 overexpression-mediated activation of Parkin could modulate apoptosis induced by diverse proapoptotic stimuli. Despite being activated and relocalized to mitochondria through overexpression of PINK1 (Figure 3E), Parkin failed to block or enhance apoptosis induced by death receptor ligands such as Fas, TRAIL, or apoptosis induced by cytotoxic drugs (Figure 3F). Once again, CCCP-induced apoptosis was dramatically enhanced in the presence of Parkin/PINK1 (Figure 3F).

PINK1-Mediated Parkin Activation Is Sufficient to Promote Apoptosis in the Absence of Mitochondrial Depolarization

Because PINK1 overexpression was sufficient to promote Parkin activation and translocation to mitochondria, even under conditions where mitochondria remained polarized (Figure 3E), we wondered whether Parkin activation in the absence of mitochondrial depolarization was sufficient to promote apoptosis. Coexpression of Parkin in the presence or absence of PINK1 over a range of concentrations revealed that the combination of Parkin/PINK1 was sufficient to promote apoptosis in HeLa, MCF-7, HEK293T, and SH-SY5Y cells (Figures 4A–4C). PINK1/Parkin-dependent cell death was readily inhibited using the polycaspase inhibitor Q-VD (Figure S3A) and exhibited stereotypical features of apoptosis such as chromatin compaction and extensive plasma membrane blebbing (Figures 3C and S3B), as well as caspase activation (Figure S3C), caspase substrate proteolysis (Figure S3C), and DNA fragmentation (Figures S3D and S3E).

We also explored whether the Ub ligase activity of Parkin was required for CCCP-induced apoptosis by using two different Parkin mutants (Figure 4D), one of which ($Parkin^{C431F}$) failed to translocate to depolarized mitochondria, whereas the $Parkin^{R275W}$ mutant did so but failed to promote mitochondrial ubiquitination (Figures S3F–S3I). As illustrated in Figure 4E, both Parkin mutants were impaired in sensitizing toward CCCP or valinomycin-induced apoptosis, despite being as efficiently expressed as wild-type Parkin (Figure S3G).

Silencing of Endogenous Parkin or PINK1 Protects against Apoptosis Induced by Mitochondrial Depolarization but Not Other Stimuli

Because the experiments described thus far used Parkin overexpression approaches, we next asked whether endogenous Parkin was required for mitochondrial depolarization-induced apoptosis. SH-SY5Y cells naturally express Parkin and PINK1 (Figure 4F) and are susceptible to apoptosis induced by mitochondrial depolarization (Figure 4G). However, upon knockdown of endogenous Parkin or PINK1, SH-SY5Y cells were protected against CCCP- and valinomycin-induced apoptosis, but not apoptosis induced by cisplatin (Figure 4G). Similarly, HEK293T cells were also protected against mitochondrial depolarization-associated apoptosis through knockdown of endogenous Parkin or PINK1 (Figure 4H). These data provide further support for the idea that Parkin and PINK1 can promote apoptosis in response to acute mitochondrial depolarization.

Figure 3. Mitochondrial Depolarization-Induced Apoptosis Is Enhanced by Parkin in a PINK1-Dependent Manner

(A) HeLa cells were cotransfected with a plasmid encoding eGFP (50 ng) and either empty vector (200 ng) or Parkin vector (200 ng) for 24 hr. Cells were then treated with the indicated dose of CCCP for 18 hr. PINK1 stabilization was analyzed by western blotting (left panel), and cell death was counted among GFP-positive cells based on cell morphology by phase and fluorescence microscopy (right panel).

(B) HeLa cells were transfected with a plasmid encoding mCherry-Parkin (400 ng) for 24 hr and treated with CCCP (10 μM) for 12 hr. Mitochondria were immunostained for TOM20 (green) and nuclei were stained with Hoechst (blue). Cell morphology was assessed by confocal microscopy. Scale bars represent 20 μm.

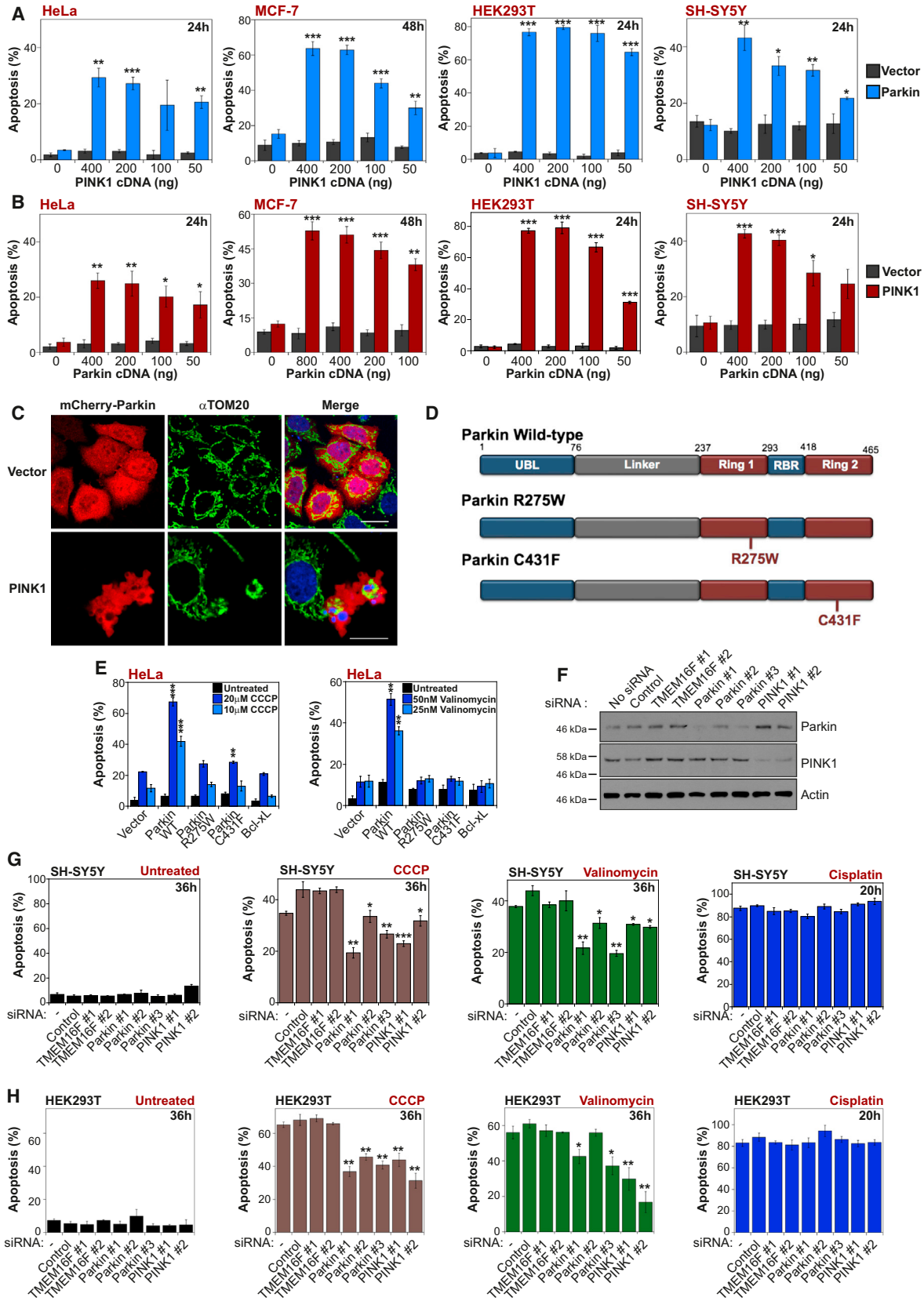
(C) HeLa cells were transfected with nontargeting control siRNA or siRNA targeted against PINK1 for 24 hr. Cells were then transfected with a plasmid encoding mCherry-Parkin (200 ng) and incubated for a further 24 hr before treatment with CCCP (10 μM) for 6 hr. Knockdown efficiency was analyzed by western blotting (top left panel). Cells were also immunostained for mitochondria (TOM20, green), and nuclei were stained with Hoechst (blue). Colocalization between mCherry-Parkin and mitochondria was quantified by confocal microscopy among mCherry-Parkin-positive cells (top right and bottom panel). Scale bars represent 20 μm.

(D) HeLa cells were transfected with nontargeting control siRNA, siRNA targeted against PINK1, or plasmids encoding GFP-tagged shRNA targeted against control (scrambled) or against Bax or Bak (500 ng) for 24 hr. Cells were then transfected with a plasmid encoding Parkin (200 ng) and incubated for a further 24 hr. Knockdown efficiency was analyzed by western blotting (left panel). Cells were treated with CCCP (10 μM) or valinomycin (50 nM) for 24 hr, and cell death was scored based on cell morphology.

(E) HeLa cells were transfected with a plasmid encoding mCherry-Parkin (200 ng) along with the indicated amount of PINK1 cDNA for 24 hr. Cells were immunostained for PINK1 (green) or ubiquitin and mitochondria (TOM20, blue). Colocalization between mitochondria and mCherry-Parkin (top right panel) or ubiquitin (bottom right panel) was analyzed and scored by confocal microscopy. Scale bars represent 20 μm.

(F) HeLa cells were transfected with plasmids encoding eGFP (50 ng) and PINK1 (100 ng) along with the indicated amount of Parkin cDNA for 24 hr. Cells were then treated with cisplatin (50 μM), anti-Fas (200 ng/ml), TRAIL (100 ng/ml), daunorubicin (5 μM), or CCCP (10 μM) for 18 hr. Cell death was scored among GFP-positive cells based on cell morphology.

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of a minimum of 100 cells. Statistical significance was assessed by two-tailed paired Student's-t test. Asterisk(s) indicate significance, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$.



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Parkin-Dependent Apoptosis Is Blocked by Bcl-2 Family Members and Is Associated with Enhanced Bax Activation

Two major pathways to apoptosis have been identified, one of which (the intrinsic pathway) promotes caspase activation and apoptosis through permeabilization of mitochondrial outer membranes via activation of the Bax/Bak channel (reviewed in Martin et al., 2012). Opening of the Bax/Bak channel is positively regulated by BH3-only members of the Bcl-2 family and suppressed by prosurvival Bcl-2 family members, such as Bcl-xL, Mcl-1, and Bcl-2 (Willis et al., 2007). Of note, we have recently reported that translocation of Parkin to mitochondria in response to mitochondrial depolarization is impaired through overexpression of members of the prosurvival subset of the Bcl-2 family, thereby inhibiting mitophagy downstream (Hollville et al., 2014). Thus, we wondered whether Parkin-dependent apoptosis could also be inhibited by prosurvival Bcl-2 family proteins.

To explore this, we coexpressed Parkin in the presence or absence of Bcl-xL, Bcl-2, Mcl-1, Bcl-B, or A1, followed by exposure of cells to CCCP or valinomycin. As can be seen from Figures 5A and 5B, Bcl-2 family proteins robustly protected from Parkin-dependent apoptosis, suggesting that Parkin activates the intrinsic pathway to apoptosis. Because the intrinsic pathway promotes activation of the Bax/Bak channel to facilitate cytochrome *c* release, we next explored whether Parkin led to enhanced Bax activation in response to mitochondrial depolarizing agents. Thus, we stained CCCP-treated Parkin-transfected HeLa cells with an antibody (6A7) specific for the active form of Bax. As Figures 5C and 5D illustrate, Parkin greatly sensitized toward Bax activation in response to mitochondrial depolarization. To explore further the role of the Bax/Bak channel in Parkin-dependent apoptosis, we silenced the expression of endogenous Bax, Bak, or both, followed by exposure to CCCP or valinomycin. As Figure 5E shows, knockdown of Bax or Bak, or both, robustly protected from Parkin/PINK1-dependent apoptosis in response to mitochondrial depolarization.

Parkin and PINK1 Promote Selective Degradation of Mcl-1 in Response to Mitochondrial Depolarization

Because prosurvival members of the Bcl-2 family are localized to mitochondrial outer membranes and set a threshold for Bax/Bak activation, we considered the possibility that active Parkin might promote degradation of one or more Bcl-2 family proteins to promote opening of the Bax/Bak channel, leading to apoptosis. Therefore, we monitored the stability of the major prosurvival Bcl-2 family proteins expressed in HeLa cells (Mcl-1, Bcl-xL, Bcl-2, and Bcl-W) in response to mitochondrial depolarization. As Figure 5F shows, Mcl-1 was selectively and rapidly degraded upon CCCP treatment, in tandem with Parkin activation and PINK1 stabilization, whereas the stability of other prosurvival Bcl-2 family members was largely unaffected under the same conditions. Moreover, knockdown of PINK1 completely reversed CCCP-induced degradation of Mcl-1 and blocked the activation of Parkin (Figure 5F). Furthermore, addition of the proteasome inhibitor MG132 blocked the degradation of Mcl-1 seen upon treatment with CCCP (Figure 5G). We also explored the fate of Bax and Bak in response to mitochondrial depolarization. As Figure 5H illustrates, whereas Bax expression levels remained stable in response to mitochondrial depolarization, Bak was also degraded in tandem with Mcl-1. However, the decline in Bak expression levels appeared to be a consequence of caspase activation, because this effect was reversed with the polycaspase inhibitor zVAD-fmk, whereas Mcl-1 degradation was only minimally affected under the same conditions (Figure 5H).

Collectively, these data suggested that in response to mitochondrial depolarization, Parkin/PINK1 promote the selective degradation of the prosurvival Bcl-2 family member Mcl-1, which may explain the increased sensitivity of Parkin-expressing cells to apoptosis.

Parkin and PINK1 Promote Polyubiquitination and Degradation of Mcl-1

The preceding results suggested that activated Parkin promotes Mcl-1 degradation in response to mitochondrial depolarization. To explore this further, we looked for evidence of Mcl-1

Figure 4. PINK1-Mediated Parkin Activation Is Sufficient to Promote Apoptosis in the Absence of Mitochondrial Depolarization

(A) HeLa, MCF-7, HEK293T, and SH-SY5Y cells were transfected with plasmids encoding eGFP (50 ng) and Parkin (200 ng) along with the indicated amount of PINK1 cDNA for 24 or 48 hr. Cell death was counted among GFP-positive cells based on cell morphology.

(B) HeLa, MCF-7, HEK293T, and SH-SY5Y cells were transfected with plasmids encoding eGFP (50 ng) and PINK1 (200 ng) along with the indicated amount of Parkin cDNA for 24 or 48 hr. Cell death was counted among GFP-positive cells based on cell morphology.

(C) HeLa cells were transfected with a plasmid encoding mCherry-Parkin (400 ng) along with PINK1 vector (200 ng) for 24 hr. Mitochondria were immunostained for TOM20 (green), and nuclei were stained with Hoechst (blue). Cell morphology was assessed by confocal microscopy. Scale bars represent 20 μm.

(D) Diagram representing the location of point mutations associated with Parkinson's disease in the Parkin gene (*PARK2*).

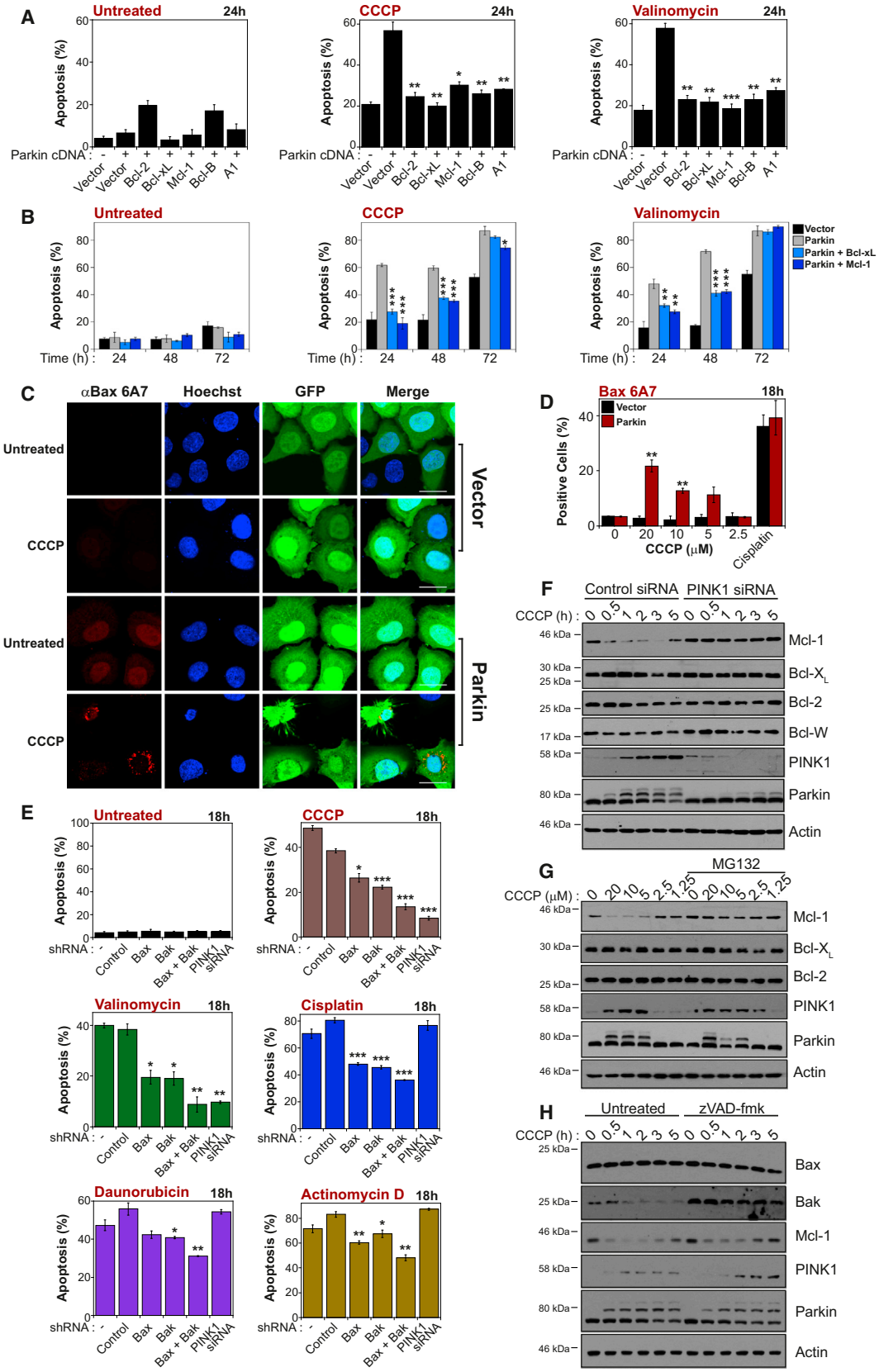
(E) HeLa cells were transfected with a plasmid encoding eGFP (50 ng) along with Parkin wild-type (WT), Parkin R275W, Parkin C431F, or Bcl-X_L cDNA (200 ng) for 24 hr. Cells were treated with the indicated dose of CCCP or valinomycin for 18 hr. Cell death was counted among GFP-positive cells based on cell morphology.

(F) SH-SY5Y cells were transfected with nontargeting control siRNA, siRNA targeted against an irrelevant protein (TMEM16F), or siRNA targeted against Parkin or PINK1 for 48 hr. Knockdown efficiency was analyzed by western blotting.

(G) SH-SY5Y cells were transfected with nontargeting control siRNA, siRNA targeted against an irrelevant protein (TMEM16F), or siRNA targeted against Parkin or PINK1 for 48 hr. Cells were then treated with CCCP (20 μM) or valinomycin (200 nM) for 36 hr or cisplatin (50 μM) for 20 hr. Cell death was scored based on cell morphology.

(H) HEK293T cells were transfected with nontargeting control siRNA, siRNA targeted against an irrelevant protein (TMEM16F), or siRNA targeted against Parkin or PINK1 for 48 hr. Cells were then treated with CCCP (10 μM) or valinomycin (50 nM) for 36 hr or cisplatin (50 μM) for 20 hr. Cell death was scored based on cell morphology.

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of a minimum of 100 cells. Statistical significance was assessed by two-tailed paired Student's-t test. Asterisk(s) indicate significance, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$. See also Figure S3.



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ubiquitination upon CCCP treatment. As Figures 6A and 6B demonstrate, in response to mitochondrial depolarization in HeLa cells stably expressing Parkin, we detected high-molecular-weight forms of Mcl-1, consistent with ubiquitination of the latter, which were enhanced through addition of the proteasome inhibitor, MG132. Mitochondrial depolarization-induced appearance of Mcl-1 high-molecular-weight species was greatly attenuated through knockdown of PINK1, which blocked activation of Parkin (Figure 6C), suggesting that Parkin may promote polyubiquitination and degradation of Mcl-1 upon mitochondrial depolarization. To explore this further, we transfected Parkin-expressing HeLa cells with His-tagged ubiquitin and pulled down ubiquitin conjugates in the presence and absence of CCCP to explore whether Mcl-1 was polyubiquitinated under the latter conditions. As Figure 6D demonstrates, ubiquitin adducts of Mcl-1 were readily detected in response to mitochondrial depolarization-induced Parkin activation. In addition, knockdown of PINK1 attenuated polyubiquitination of Mcl-1 in response to mitochondrial depolarization (Figure 6E).

We also asked whether transient expression of Parkin could promote Mcl-1 degradation. As Figure 6F illustrates, coexpression of Mcl-1 with Parkin led to a dramatic reduction in Mcl-1 expression levels, which was greatly enhanced through addition of CCCP. Furthermore, Parkin overexpression also led to the detection of Mcl-1 high-molecular-weight species upon CCCP treatment, consistent with ubiquitination of the latter (Figures 6G and 6H), and pull-down of ubiquitin conjugates in the presence of Parkin revealed that Mcl-1 ubiquitination was enhanced in the presence of this E3 ligase upon CCCP treatment (Figure 6I).

Collectively, these results strongly suggest that Mcl-1 is a direct substrate for Parkin-mediated polyubiquitination and degradation in response to mitochondrial depolarization.

Knockdown of Mcl-1 Sensitizes toward Mitochondrial Depolarization-Induced Apoptosis

Because the preceding results suggested that Mcl-1 underwent selective Parkin/PINK1-dependent degradation in response to mitochondrial depolarization, we wondered whether knockdown of Mcl-1 was sufficient to sensitize toward mitochondrial depolarization-induced apoptosis. As controls, we also knocked down other members of the prosurvival subset of the Bcl-2 family (Bcl-2, Bcl-B, and Bcl-W). As Figure 7A shows, knockdown of Mcl-1 in Parkin-expressing HeLa cells greatly sensitized toward apoptosis induced by mitochondrial depolarizing agents. In contrast, knockdown of Bcl-2, Bcl-B, and Bcl-W had much more modest effects by comparison (Figure 7A). Similarly, knockdown of Mcl-1 in SH-SY5Y cells also greatly sensitized toward apoptosis induced by mitochondrial depolarization (Figure 7B).

Altogether, these data suggest that Parkin activation in response to mitochondrial depolarization sensitizes toward apoptosis through lowering the threshold for opening of the mitochondrial Bax/Bak channel, at least in part through degradation of the prosurvival Bcl-2 family member Mcl-1.

DISCUSSION

Here, we have shown that, rather surprisingly, Parkin sensitized toward apoptosis induced by mitochondrial depolarization. This effect was highly selective and was not observed in response to a range of additional proapoptotic stimuli that failed to activate Parkin, including initiators of the extrinsic pathway to apoptosis (Fas, TRAIL), as well as initiators of the intrinsic pathway (cisplatin, etoposide, daunorubicin, actinomycin D). Interestingly, Parkin mutants recapitulating the mutations found in Parkinson's disease were greatly impaired in their ability to sensitize toward apoptosis. Parkin-dependent apoptosis was PINK1-dependent and was associated with activation of the Bax/Bak channel. Furthermore, mitochondrial depolarization triggered rapid and selective polyubiquitination and degradation of the prosurvival Bcl-2 family protein Mcl-1, which was blocked through inhibition of Parkin activation via knockdown of PINK1. These observations suggest, somewhat heretically, that Parkin may be a proapoptotic protein that polices mitochondrial integrity and is capable of either initiating repair of mitochondrial

Figure 5. Parkin/PINK1-Induced Cell Death Is Regulated by Bcl-2 Family Proteins

(A) HeLa cells were cotransfected with plasmids encoding eGFP (50 ng) and Parkin (200 ng) along with plasmids encoding the indicated Bcl-2 family member (600 ng) for 24 hr. Cells were treated with CCCP (10 μM) or valinomycin (50 nM) for 24 hr, and cell death was scored among GFP-positive cells based on cell morphology.

(B) HeLa cells were transfected as in (A). Cells were then treated with CCCP (5 μM) or valinomycin (50 nM) for 24, 48, or 72 hr. Cell death was scored among GFP-positive cells based on cell morphology.

(C and D) HeLa cells were cotransfected with plasmids encoding eGFP (50 ng) and Parkin (400 ng) for 24 hr. Cells were then treated with the indicated dose of CCCP or with cisplatin (50 μM) for 18 hr. Cells were immunostained for the active form of Bax (anti-Bax 6A7, red) and stained for nuclei (Hoechst, blue). Cells were analyzed by confocal microscopy, and GFP-positive cells were scored for 6A7 positivity. Scale bars represent 20 μm.

(E) HeLa cells were transfected with plasmids encoding GFP-tagged shRNA targeted against control (scrambled) or against Bax or Bak (500 ng) or with siRNA targeted against PINK1 for 24 hr. Cells were then transfected with Parkin cDNA (200 ng) and incubated for a further 24 hr before treatment with CCCP (10 μM), valinomycin (50 nM), cisplatin (50 μM), daunorubicin (5 μM), or actinomycin D (5 μM) for 18 hr. Cell death was scored among GFP-positive cells based on cell morphology.

(F) HeLa-YFP-Parkin cells were transfected with nontargeting control siRNA or siRNA targeted against PINK1 (siRNA #2) for 48 hr. Cells were treated with CCCP (10 μM) for the indicated period of time. Bcl-2 family proteins expression was analyzed by western blotting.

(G) HeLa-YFP-Parkin cells were treated with the indicated dose of CCCP in the presence or absence of MG132 (10 μM) for 1 hr. Bcl-2 family proteins expression was analyzed by western blotting.

(H) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) in the presence or absence of zVAD-fmk (10 μM) for the indicated period of time. Bcl-2 family protein expression was analyzed by western blotting.

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of a minimum of 100 cells. Statistical significance was assessed by two-tailed paired Student's t test. Asterisk(s) indicate significance, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$.

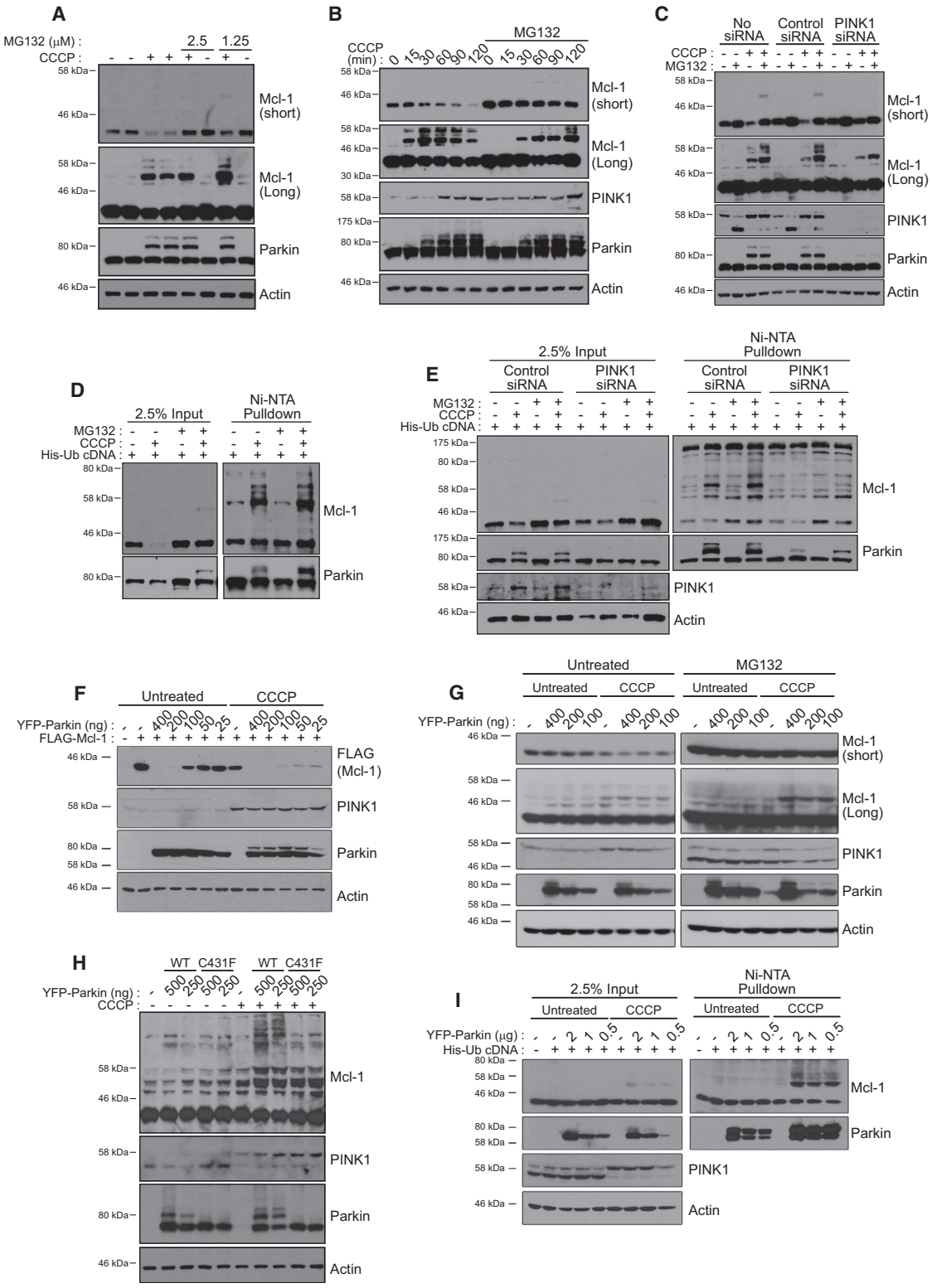

Figure 6. Parkin Acts as an E3 Ligase for Mcl-1

(A) HeLa-YFP-Parkin cells were either left untreated or treated with CCCP (10 μM) in the presence or absence of the indicated dose of MG132 for 1 hr. Mcl-1 expression was analyzed by western blotting.

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networks via mitophagy or promoting the elimination of critically damaged cells via apoptosis.

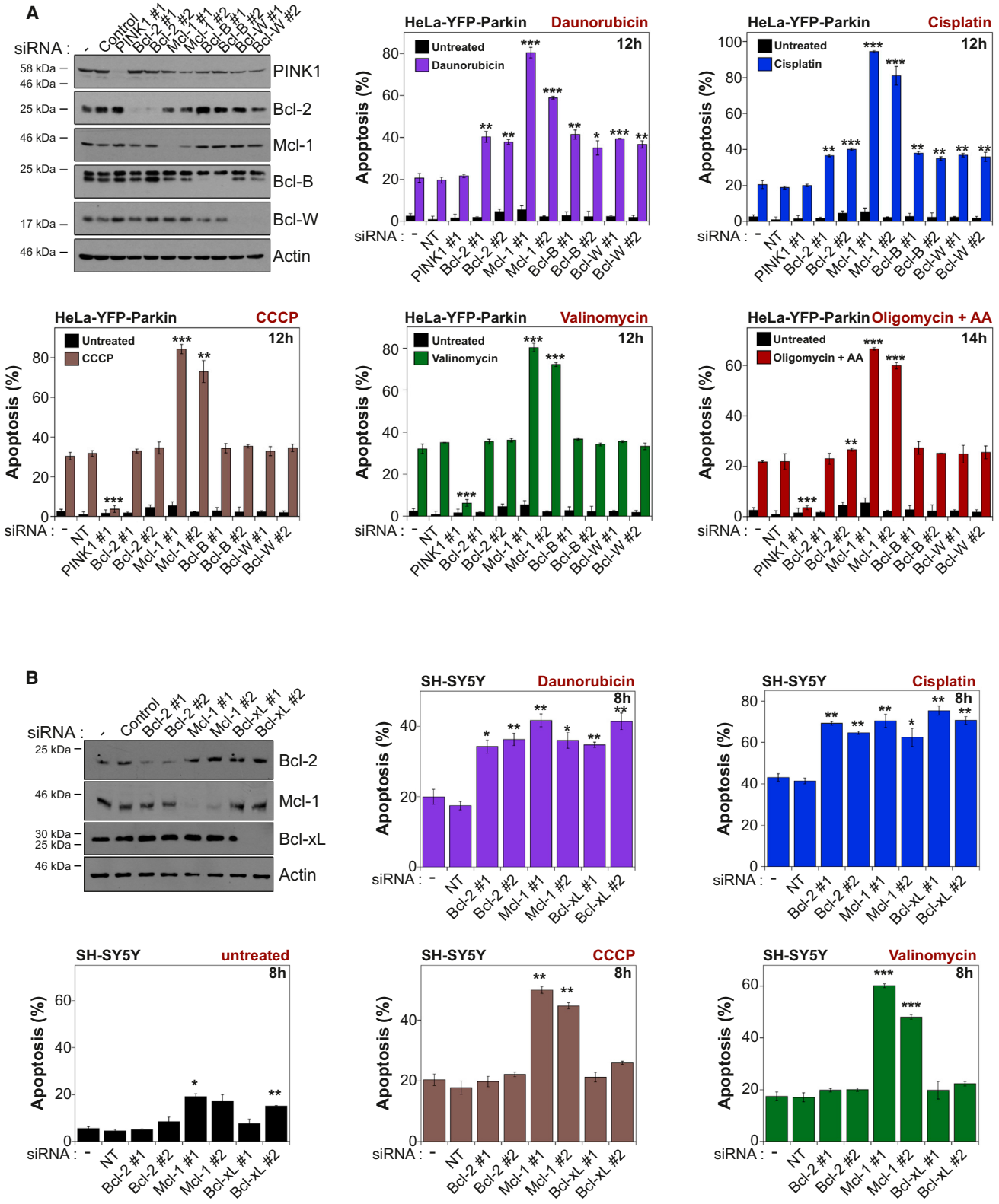
Although it is frequently assumed that Parkin is a suppressor of apoptosis, few studies have explored the mechanistic basis for the apparent cytoprotective activity of this ubiquitin ligase (Darios et al., 2003; MacCormac et al., 2004; Yang et al., 2005; Berger et al., 2009; Ekholm-Reed et al., 2013; Sun et al., 2013; Wang et al., 2013). Johnson and colleagues have reported that Parkin-mediated ubiquitination of Bax provides a protective effect against etoposide (Johnson et al., 2012). However, as we have shown here, etoposide failed to activate Parkin. Müller-Rischart et al. (2013) also reported that induction of OPA1 expression by Parkin mediates Parkin antiapoptotic function in response to staurosporine. However, it is relevant to note that none of these studies demonstrated that Parkin ubiquitin ligase activity was stimulated under the conditions used. In agreement with established models of mitophagy (Narendra et al., 2008), we have found that PINK1-mediated activation of the ubiquitin ligase activity of Parkin is absolutely required for the proapoptotic function of the latter. Therefore, although the idea that Parkin can suppress apoptosis would apparently correlate with the loss of dopaminergic neurons in Parkinson's disease, there is very little mechanistic evidence to support this model. An alternative model is that the Parkin-dependent removal of cells carrying defective mitochondria may enable the early replacement of dysfunctional neurons, thereby preventing their accumulation. The latter effect may be critical to prevent neural networks becoming populated with defective neurons that might lead to neurodegeneration upon reaching a tipping point where large numbers of dysfunctional cells die en mass. Pruning of individual neurons, in a Parkin-dependent manner, as mitochondrial defects are detected may enable the efficient replacement of defective neurons, thereby preventing the accumulation of large numbers of injured cells and the loss of a whole network.

Although the data reported here would appear contradictory to the currently proposed models, Parkin proapoptotic activity has been sporadically noted (Lee et al., 2012; Morrison et al., 2011). Moreover, Mcl-1 was recently described as a substrate for Parkin in response to mitochondrial depolarizers (Sarraf et al., 2013). Furthermore, a very recent study also provides evidence to support the idea that Parkin and PINK1 can collaborate to promote apoptosis (Zhang et al., 2014). The latter study, which utilizes Parkin- or PINK1-null mouse embryonic fibroblasts, complements the findings of the present study very well and suggests that deregulated Parkin activation, in response to severe mitochondrial depolarization, can result in cell death (Zhang et al., 2014).

We have recently shown that members of the prosurvival subset of the Bcl-2 family can regulate the onset of Parkin-dependent mitochondrial clearance through suppressing Parkin translocation to depolarized mitochondria (Hollville et al., 2014). Here, we show that prosurvival members of the Bcl-2 family also suppress Parkin-dependent apoptosis through antagonizing Parkin function. Thus, there is a reciprocal interplay between Parkin and Bcl-2 family members that sets a threshold for Parkin-dependent mitophagy, or Parkin-dependent apoptosis.

Parkin may act as a sensor for mitochondrial damage or dysfunction, becoming recruited to damaged mitochondria to promote their removal via mitophagy, or if mitochondrial impairment is extensive, to promote death of the injured cell via apoptosis. The functional outcome of Parkin activation may be dose dependent, with mitophagy occurring at low thresholds of Parkin activation and apoptosis at higher levels. Limited or transient mitochondrial depolarization may engage Parkin-dependent mitophagy, leading to mitochondrial network repair and cell survival. However, robust or sustained activation of Parkin may lead to the opposite outcome of apoptosis to facilitate removal of the damaged cell. This is reminiscent of how many biological sensors operate. For example, the tumor suppressor protein, p53, is involved in monitoring DNA integrity and is activated in response to DNA damage, thereby promoting DNA repair (Kastan et al., 1991). However, in response to extensive DNA damage, p53 activation promotes the elimination of cells via apoptosis (Lowe et al., 1993). Recent studies suggest that this effect is related to the degree of DNA damage that promotes p53 activation, with low levels of damage triggering the repair function of p53 and higher levels of damage unleashing its proapoptotic activity (Purvis et al., 2012; Chen et al., 2013). Similarly, Myc activation can lead to cell proliferation or apoptosis

(B) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for the indicated period of time in the presence or absence of MG132 (10 μM). Mcl-1 expression was analyzed by western blotting.
(C) HeLa-YFP-Parkin cells were transfected with nontargeting control siRNA or siRNA targeted against PINK1 (siRNA #1) for 48 hr. Cells were treated with CCCP (10 μM) in the presence or absence of MG132 (10 μM) for 1 hr. Mcl-1 expression was analyzed by western blotting.
(D) HeLa-YFP-Parkin cells were transfected with a plasmid encoding His-tagged ubiquitin (5 μg) for 24 hr. Cells were then treated with CCCP (10 μM) in the presence or absence of MG132 (10 μM) for 1 hr. His-tagged ubiquitin was then pulled down, and Mcl-1 ubiquitination was analyzed by western blotting.
(E) HeLa-YFP-Parkin cells were transfected with nontargeting control siRNA or siRNA targeted against PINK1 (siRNA #1) for 24 hr. Cells were then transfected with a plasmid encoding His-tagged ubiquitin and incubated for a further 24 hr. Cells were treated with CCCP (10 μM) in the presence or absence of MG132 (10 μM) for 1 hr. His-tagged ubiquitin was pulled down, and Mcl-1 ubiquitination was analyzed by western blotting.
(F) HeLa cells were transfected with a plasmid encoding FLAG-Mcl-1 (200 ng) and the indicated amount of YFP-Parkin cDNA for 24 hr. Cells were then treated with CCCP (10 μM) for 4 hr. Mcl-1 expression was analyzed by western blotting.
(G) HEK293T cells were transfected with the indicated amount of YFP-Parkin plasmid. Cells were then treated with CCCP (10 μM) in the presence or absence of MG132 (10 μM) for 1.5 hr. Mcl-1 expression was analyzed by western blotting.
(H) HEK293T cells were transfected with the indicated amount of YFP-Parkin WT or YFP-Parkin C431F construct for 24 hr. Cells were then treated with MG132 (10 μM) in the presence or absence of CCCP (10 μM) for 2 hr. Mcl-1 expression was analyzed by western blotting.
(I) HEK293T cells were transfected with the indicated amount of YFP-Parkin plasmid along with His-tagged ubiquitin cDNA (2 μg). Cells were then treated with MG132 (10 μM) in the presence or absence of CCCP (10 μM) for 2 hr. His-tagged ubiquitin was pulled down, and Mcl-1 ubiquitination was analyzed by western blotting.
Results shown are representative of at least three independent experiments.



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depending on the context and Ras activation can promote cell division, cell senescence, or cell death (Evan et al., 1992; Serrano et al., 1997; Elgendy et al., 2011).

In conclusion, here we have shown that Parkin selectively promotes apoptosis in response to mitochondrial depolarization in a PINK1-dependent manner. These data challenge the view that Parkin is an inhibitor of apoptosis and suggest, instead, that its role may be to facilitate the elimination of cells carrying large numbers of impaired or dysfunctional mitochondria.

EXPERIMENTAL PROCEDURES

Cell Culture

HeLa cells, HeLa cells stably expressing YFP-Parkin, and MCF-7 were cultured in RPMI supplemented with 5% fetal calf serum (Sigma-Aldrich) and L-glutamine (Gibco). HEK293T cells were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum. SH-SY5Y were cultured in Dulbecco's minimum essential medium supplemented with 15% fetal calf serum, L-Glutamine, nonessential amino acids, and sodium pyruvate. Cells were maintained in 5% CO_2 at 37°C.

Tranfections and Gene Silencing

HeLa, MCF-7, and SH-SY5Y cells were seeded at 10^5 cells/well in 6-well plates 24 hr prior to transfection. Cells were transfected with Genejuice (Merck Millipore) for 24 hr. HEK293T cells were seeded at 10^5 cells/well in 6-well plates 24 hr prior transfection and were transfected according to the standard calcium phosphate precipitation method. For siRNA transfections, HeLa and HeLa-YFP-Parkin cells (6–10 × 10^5) were nucleofected with 200 pmol of each siRNA in nucleofection buffer (5 mM KCl, 15 mM $MgCl_2$, 20 mM HEPES, 150 mM Na_2HPO_4 [pH 7.2]) using Amaxa Nucleofector (program I-013). Cells (2 × 10^5 cells/well) were plated in 6-well plates. SH-SY5Y cells were seeded at 5 × 10^4 cells/well in 6-well plates 24 hr prior transfection with 200 nM of siRNA using Lipofectamine 2000 (Invitrogen, Life Technologies). HEK293T were seeded at 10^5 cells/well in 6-well plates 24 hr prior transfection with 200 pmol of siRNA according to the standard calcium phosphate precipitation method.

Western Blot Analysis

Whole-cell lysates were prepared with 100 μl of SDS-PAGE sample buffer on ice. Samples were boiled for 10 min, ran on 10% or 12% SDS-PAGE gels, and transferred onto nitrocellulose membranes. After blocking and incubation with primary and secondary antibodies, immunoreactions were visualized with SuperSignal West Pico (Thermo Scientific) and exposure to autoradiography films.

Sub-G1 Analysis

HeLa-YFP-Parkin cells were seeded at 10^5 cells/well in 6-well plates 48 hr prior treatment. Cells were fixed with 1 ml ice-cold 70% ethanol overnight at −20°C. Fixed cells were pelleted at 750 g for 5 min and incubated in 1 ml of PBS for 30 min at room temperature. Cells were pelleted 750 g for 5 min and incubated in 500 μl of PBS containing 100 μg/ml RNase A for 30 min at room temperature. Cells were stained with 50 μg/ml PI before analysis with BD Biosciences FACSCalibur.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.046.

AUTHOR CONTRIBUTIONS

R.G.C. and E.H. designed experiments, analyzed data, generated the figures, and wrote the figure legends. S.J.M. conceived the study, designed and analyzed experiments, supervised the study, and wrote the paper. S.J.M. obtained the funding to support the study.

ACKNOWLEDGMENTS

We thank Dr. Stephen Tait for provision of GFP-Parkin-expressing HeLa cells, Dr. Richard Youle for provision of mCherry-Parkin and YFP-Parkin plasmids, Dr. Dennis J. Selkoe for provision of PINK1-FLAG plasmid, and Dr. Maria Soengas for provision of shRNA plasmids targeted against Bax and Bak. The S.J.M. laboratory is supported by PI (08/IN.1/B2031) and SRC (07/SRC/B1144) grants from Science Foundation Ireland. S.J.M. is a Science Foundation Ireland Principal Investigator.

Received: July 24, 2014
Revised: September 12, 2014
Accepted: October 16, 2014
Published: November 13, 2014

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Figure 7. Knockdown of Mcl-1 Sensitizes toward Mitochondrial Depolarization-Induced Apoptosis

(A) HeLa-YFP-Parkin cells were transfected with nontargeting control siRNA or siRNA targeted against the indicated Bcl-2 family member for 48 hr. Knockdown efficiency was analyzed by western blotting (top left panel). Cells were treated with daunorubicin (5 μM), cisplatin (50 μM), CCCP (10 μM), or valinomycin (50 nM) for 12 hr or with oligomycin (500 nM) and antimycin A (500 nM) for 14 hr. Cell death was scored based on cell morphology.

(B) SH-SY5Y cells were transfected with nontargeting control siRNA or siRNA targeted against the indicated Bcl-2 family member for 48 hr. Knockdown efficiency was analyzed by western blotting (top left panel). Cells were treated with daunorubicin (5 μM), cisplatin (50 μM), CCCP (20 μM), or valinomycin (100 nM) for 8 hr. Cell death was scored based on cell morphology.

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of a minimum of 100 cells. Statistical significance was assessed by two-tailed paired Student's t test. Asterisk(s) indicate significance, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$.

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