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# Bcl-2 Family Proteins Participate in Mitochondrial Quality Control by Regulating Parkin/PINK1-Dependent Mitophagy

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## SUMMARY

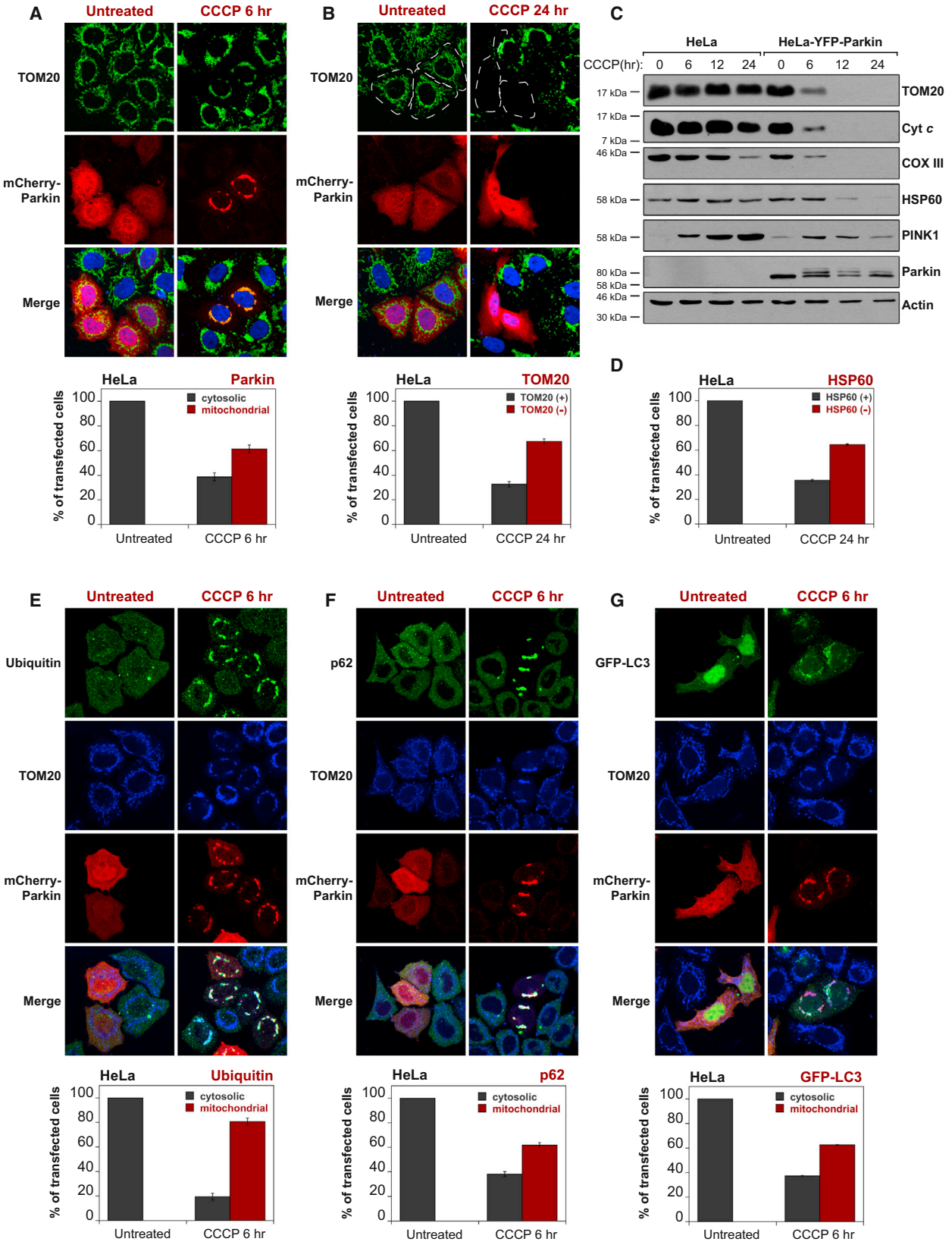
**Mitophagy facilitates the selective elimination of impaired or depolarized mitochondria through targeting the latter to autophagosomes. Parkin becomes localized to depolarized mitochondria in a PINK1-dependent manner and polyubiquitinates multiple mitochondrial outer membrane proteins. This permits ubiquitin-binding proteins (e.g., p62 and NBR1) to target impaired mitochondria to autophagosomes via Atg8/LC3II. Bcl-2 family proteins regulate mitochondrial outer membrane permeabilization during apoptosis and can also influence macroautophagy via interactions with Beclin-1. Here, we show that Parkin-dependent mitophagy is antagonized by prosurvival members of the Bcl-2 family (e.g., Bcl-xL and Mcl-1) in a Beclin-1-independent manner. Bcl-2 proteins suppressed mitophagy through inhibition of Parkin translocation to depolarized mitochondria. Consistent with this, Parkin translocation to mitochondria was enhanced by BH3-only proteins or a BH3-only mimetic. Taken together with their role as regulators of apoptosis-associated mitochondrial permeabilization, as well as mitochondrial fission/fusion dynamics, this suggests that Bcl-2 family proteins act as global regulators of mitochondrial homeostasis.**

## INTRODUCTION

Macroautophagy promotes recycling of long-lived proteins and organelles and involves entrapment of cytoplasmic material within autophagosomes, followed by fusion of autophagosomes with lysosomes (Mizushima, 2007; Chen and Klionsky, 2011). Multiple lysosomal proteases promote degradation of the contents of autolysosomes, thus liberating amino acids and other basic building blocks to be reused within the cell. Autophagy can also be invoked to eliminate damaged organelles through selective ubiquitination followed by targeting of the damaged organelle to autophagosomes via ubiquitin (Ub) and LC3-binding adaptor proteins. Mitophagy is an organelle-specific type of autophagy that facilitates the elimination of damaged or depolarized mitochondria through selective targeting of such mitochondria to the autophagic pathway (Youle and Narendra, 2011; Ashrafi and Schwarz, 2013). However, the molecular events that orchestrate selective recognition and removal of damaged mitochondria are only now emerging.

Parkin is a Ub 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). Due to its role in this neurological condition, the function of Parkin has been the subject of intensive study (Kahle and Haass, 2004). Phosphatase and tensin homolog deleted on chromosome 10-induced kinase 1 (PINK1), which shuttles between the cytosol and mitochondria, has also been linked with Parkinson's disease and operates in the same pathway as Parkin (Valente et al., 2004; Clark et al., 2006; Park et al., 2006; Weihofen et al., 2008). Until recently, the functional role of Parkin and PINK1 remained obscure. However, several studies have shown that Parkin, which is normally localized to the cytosol, translocates to depolarized or impaired mitochondria in a PINK1-dependent manner (Narendra et al., 2008, 2010a; Vives-Bauza et al., 2010). 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 is required for Parkin relocalization to these organelles (Narendra et al., 2008). Several laboratories have reported that Parkin and PINK1 directly interact, and loss of either Parkin or PINK1 completely abrogates mitophagy (Shiba et al., 2009; Um et al., 2009; Narendra et al., 2010a; Vives-Bauza et al., 2010). Parkin is activated upon association with PINK1, and recent studies suggest that PINK1 phosphorylates Parkin at Ser65 (Shiba-Fukushima et al., 2012; Kondapalli et al., 2012), either to trigger its translocation to mitochondria or to activate the Ub ligase activities of the latter.

Upon activation of Parkin on depolarized mitochondria, numerous proteins within the mitochondrial outer membrane become polyubiquitinated, and this triggers translocation of the Ub- and LC3-binding adaptor protein p62 to these organelles (Lee et al., 2010; Narendra et al., 2010b; Okatsu et al., 2010). Thus, p62 appears to act as a bridge to link damaged mitochondria to autophagosomes. While initial studies suggested that p62 was critical for removal of depolarized mitochondria via mitophagy, more recent studies suggest that p62 may be dispensible for this process (Narendra et al., 2010b; Okatsu et al., 2010), possibly due to redundancy with the related Ub- and Atg8/LC3II-binding protein NBR1.

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Bcl-2 family proteins regulate entry into apoptosis by influencing the permeability of the mitochondrial outer membrane through controlling the assembly of multimeric BAX/BAK channels (Chipuk et al., 2006). BAX/BAK oligomerization permits the efflux of mitochondrial intermembrane space proteins, such as cytochrome *c*, from mitochondria. The latter event is critical for the formation of the Apaf-1/Caspase-9 apoptosome, which unleashes a cascade of caspase activation events that culminate in apoptosis. Apart from this key role, studies have also shown that prosurvival members of the Bcl-2 family can regulate macroautophagy through binding to the autophagy regulator Beclin-1 and blocking its participation in the triggering of autophagosome formation (Pattingre et al., 2005; Erlich et al., 2007). However, it is not known whether Beclin-1 is required for mitophagy. Bcl-2 family members have also been implicated in the regulation of mitochondrial fission and fusion dynamics (reviewed in Youle and van der Bliek, 2012; Autret and Martin, 2009), processes that have been implicated in the quarantining of impaired mitochondria during mitophagy (Twig et al., 2008; Gomes et al., 2011; Rambold et al., 2011). Thus, several members of the Bcl-2 family are localized to mitochondria, affect mitochondrial fission and fusion dynamics, bind to Beclin-1, and regulate mitochondrial permeabilization, an event that leads to mitochondrial depolarization. Because of the multiplicity of events surrounding mitophagy that Bcl-2 family proteins have also been implicated in, we wondered whether members of this family could also regulate Parkin/PINK1-dependent mitophagy.

Here, we show that Parkin-dependent elimination of depolarized mitochondria can be inhibited by prosurvival Bcl-2 family members, such as Bcl-xL and Mcl-1. However, we found no evidence for the involvement of Beclin-1 in Parkin-dependent mitophagy. Instead, Bcl-2 proteins suppressed mitophagy through binding to Parkin/PINK1 complexes and inhibiting Parkin translocation to depolarized mitochondria, thereby blocking Parkin-dependent ubiquitination of mitochondrial substrates and downstream events. Thus, Bcl-2 family proteins participate in the maintenance of healthy mitochondrial networks through their ability to regulate Parkin/PINK1-dependent mitophagy. We propose that Bcl-2 proteins act as global regulators of mitochondrial network integrity by regulating mitochondrial permeabilization during apoptosis, steady-state mitochondrial fission/fusion dynamics, and the removal of impaired mitochondria via mitophagy.

## RESULTS

### Parkin/PINK-1 Promote Elimination of Depolarized Mitochondria

As previously reported by several laboratories, depolarization of mitochondria using the protonophore CCCP led to rapid translocation of mCherry-Parkin from the cytosol to mitochondria within several hours of 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, COXIII, and cytochrome *c* (Figures 1B–1D; Figure S1A available online). In CCCP-treated cells lacking mitochondrial markers, Parkin was again found in the cytosol (Figure 1B). Parkin was absolutely required for mitochondrial elimination in response to CCCP, as cells transfected with empty vector failed to eliminate these organelles, as did untransfected cells adjoining those transfected with Parkin complementary DNA (cDNA) (Figure 1B). Consistent with previous results (Narendra et al., 2008, 2010b; Vives-Bauza et al., 2010; Matsuda et al., 2010; Geisler et al., 2010), depolarized mitochondria in Parkin-expressing cells became heavily decorated with Ub (Figure 1E) and were also labeled with the Ub- and LC3-binding adaptor proteins p62 (Figure 1F) and NBR1 (Figure S1B). Furthermore, we also detected relocalization of green fluorescent protein (GFP)-LC3 to depolarized mitochondria within Parkin-transfected cells (Figure 1G). Thus, depolarization of mitochondria promoted recruitment of Parkin to mitochondria; ubiquitination of mitochondrial substrates; and decoration of depolarized mitochondrial networks with the Ub-binding proteins p62, NBR1, and LC3, followed by elimination of these organelles via mitophagy.

### Bcl-2 Family Members Inhibit Parkin/PINK1-Dependent Mitophagy

Impaired mitochondria are thought to undergo separation from the remainder of the mitochondrial network during mitophagy through enhanced mitochondrial fission or decreased mitochondrial fusion. Consequently, proteins that regulate mitochondrial fission/fusion dynamics have been implicated as regulators of mitophagy (Twig et al., 2008; Gomes et al., 2011; Rambold et al., 2011). Studies from several laboratories have also implicated members of the Bcl-2 family as regulators of mitochondrial

**Figure 1. Parkin and PINK-1 Promote Elimination of Depolarized Mitochondria**

(A and B) HeLa cells were transfected with mCherry-Parkin cDNA (400 ng) and treated with CCCP (10 μM) for 6 hr (A) or 24 hr (B). Mitochondria were immunostained for TOM20 (green). Colocalization between Parkin and mitochondria (A) and the absence of the mitochondrial marker TOM20 (B) were scored among mCherry-Parkin-positive cells by confocal microscopy.

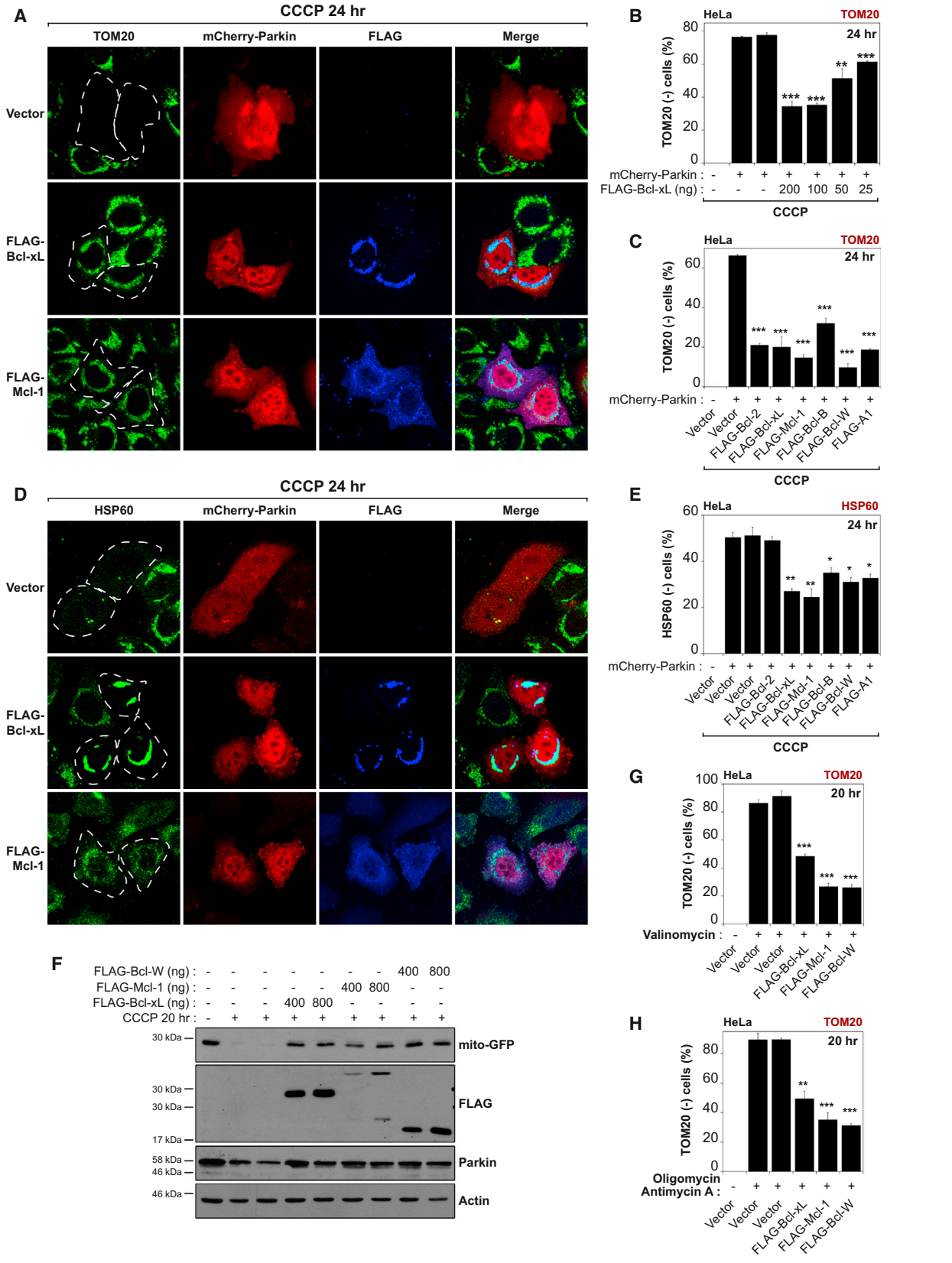
(C) HeLa and HeLa cells stably expressing YFP-Parkin were treated with CCCP (10 μM). Whole-cell lysates were analyzed for TOM20, cytochrome *c*, COX III, HSP60, PINK1, and Parkin contents by immunoblotting.

(D) HeLa cells were transfected and treated as in (B). Mitochondria were immunostained for HSP60. MCherry-Parkin-positive cells were quantified for the absence of the mitochondrial marker HSP60.

(E and F) HeLa cells were transfected with mCherry-Parkin cDNA (200 ng) and treated with CCCP (10 μM) for 6 hr. Cells were coimmunostained for TOM20 (blue) and Ub (E) or p62 (F) (green). Colocalization between Ub (E) or p62 (F) and mitochondria was scored among mCherry-Parkin-positive cells.

(G) Cells were transfected with plasmids encoding mCherry-Parkin (200 ng) and GFP-LC3 (400 ng) and treated as in (E) and (F). Cells were immunostained for TOM20 (blue). Colocalization between GFP-LC3 and mitochondria was quantified among mCherry-Parkin-positive cells.

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of 100 cells per treatment. See also Figure S1.

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fission and fusion (Karbowski et al., 2006; Delivani et al., 2006; Sheridan et al., 2008), as well as regulators of macroautophagy (Pattingre et al., 2005; Elgendy et al., 2011). Thus, we wondered whether members of the Bcl-2 family could also influence clearance of depolarized mitochondria via Parkin/PINK1-dependent mitophagy. To explore this possibility, we transiently overexpressed Bcl-2 and its close relatives (Bcl-xL, Mcl-1, Bcl-B, Bcl-W, and A1), along with Parkin followed by CCCP treatment, and assessed clearance of depolarized mitochondria in the presence or absence of Bcl-2 family proteins. As Figure 2 illustrates, overexpression of Bcl-xL, as well as other Bcl-2 family members, potently inhibited the elimination of mitochondria in Parkin-expressing cells, as assessed by detection of mitochondrial TOM20 (Figures 2A–2C), HSP60 (Figures 2D and 2E), or mitochondrially targeted GFP (Figure 2F).

Inhibition of Parkin-dependent mitophagy by Bcl-xL, Mcl-1, and Bcl-W was also observed when mitochondria were depolarized with valinomycin (Figure 2G), as well as due to treatment with a combination of oligomycin and antimycin A (Figure 2H). Similar results were also observed in HeLa cells stably expressing yellow fluorescent protein (YFP)-Parkin (Figures S2A and S2B), as well as mCherry-Parkin-transfected SKOV-3 (Figures S2C and S2D) and HaCaT cells (Figure S2E). Thus, Parkin/PINK1-dependent elimination of depolarized mitochondria can be antagonized by prosurvival members of the Bcl-2 family.

It is interesting that Bcl-2 itself did not consistently inhibit mitophagy (Figure 2E); however, we were unable to reliably assess this due to degradation of Parkin upon overexpression of Bcl-2 (Figures S2F and S2G).

## Bcl-2 Proteins Do Not Inhibit Mitochondrial Depolarization or PINK1 Stabilization

One explanation for the ability of prosurvival Bcl-2 family proteins to inhibit mitophagy was the possibility that the latter interfered with depolarization of mitochondria, or with stabilization of PINK1 on the mitochondrial outer membrane as a consequence of loss of membrane potential. However, staining of mitochondria with the potential-sensitive dye mitoTracker revealed that, under conditions of Bcl-2 family protein overexpression, mitochondria were still depolarized in response to CCCP treatment (Figure 3A). In agreement with this, depolarization-induced stabilization of endogenous PINK1 was also unaffected in cells overexpressing Bcl-2 family proteins (Figure 3B). Thus, Bcl-2 proteins do not inhibit mitophagy through interfering with mitochondrial depolarization or stabilization of the Parkin receptor, PINK1, on depolarized mitochondria.

## Bcl-2 Family Binding Partners, Beclin-1, BAX, and BAK Are Dispensible for Mitophagy

Previous studies have shown that prosurvival Bcl-2 family proteins directly interact with Beclin-1, a protein involved in the initiation of macroautophagy under certain conditions (Liang et al., 1999; Pattingre et al., 2005; Funderburk et al., 2010; Elgendy et al., 2011). Thus, we explored whether Beclin-1 was a possible point of intersection between Bcl-2 proteins and the mitophagy machinery. However, as Figures 3C–3G demonstrate, knockdown of Beclin-1 using two independent small interfering RNAs (siRNAs) failed to attenuate CCCP/Parkin-induced mitophagy. Thus, Parkin-dependent clearance of impaired mitochondria appears to be Beclin-1-independent. In contrast, knockdown of PINK1 robustly inhibited Parkin-dependent mitophagy under the same conditions (Figures 3C–3G).

BAX and BAK are critical binding partners for the prosurvival Bcl-2 proteins in the context of apoptosis (reviewed in Chipuk et al., 2010) and have also been implicated in mitochondrial fission and fusion (Karbowski et al., 2006; Sheridan et al., 2008; Cleland et al., 2011; Hoppins et al., 2011). Because mitochondrial fission has been implicated in the quarantining of impaired mitochondria prior to their removal via mitophagy (Twig et al., 2008; Gomes et al., 2011; Rambold et al., 2011), we also explored whether BAX or BAK were required for Parkin-dependent mitophagy. However, knockdown of BAX or BAK, or both, failed to suppress Parkin-dependent mitophagy (Figures 3H and 3I), thereby ruling out a role for these proteins as the targets of Bcl-2 family proteins during mitophagy. In contrast, knockdown of BAX or BAX/BAK robustly suppressed apoptosis induced by daunorubicin (Figure 3J). Thus, while Beclin-1, BAX, and BAK were dispensible for Parkin-induced mitophagy, PINK1 was found to be essential for this process.

## Bcl-2 Family Proteins Antagonize Ubiquitination of Depolarized Mitochondria and Downstream Events

To identify the point at which Bcl-2 family proteins intersect with the mitophagy machinery, we explored whether decoration of

**Figure 2. Bcl-2 Family Proteins Antagonize Parkin-Dependent Mitophagy**

(A) HeLa cells, transfected with plasmids encoding mCherry-Parkin (200 ng) and FLAG-Bcl-xL or FLAG-Mcl-1 (400 ng), were treated with CCCP (10 μM) for 24 hr. Cells were then immunostained for TOM20 (green) and FLAG (blue).

(B) HeLa cells were transfected with mCherry-Parkin cDNA (400 ng), along with the indicated amount of FLAG-Bcl-xL plasmid, and treated as in (A). Mitochondria were immunostained for TOM20. The percentage of TOM20-negative cells among mCherry-Parkin-positive cells was then scored.

(C) HeLa cells were transfected with mCherry-Parkin cDNA (400 ng) and the indicated FLAG-Bcl-2 construct (400 ng). Cells were processed and analyzed as in (B).

(D and E) HeLa cells were transfected and treated as in (C). Mitochondria were immunostained for HSP60, and mCherry-Parkin-positive cells were scored for the absence of mitochondria.

(F) HeLa cells were transfected with plasmids encoding mitochondrially targeted GFP (mito-GFP, 200 ng) and Parkin (200 ng) along with the indicated amount of FLAG-Bcl-2 construct. Cells were treated with CCCP (5 μM) for 20 hr and whole-cell lysates were analyzed by immunoblotting.

(G and H) HeLa cells, transfected with mCherry-Parkin cDNA (400 ng) and the indicated FLAG-Bcl-2 construct (800 ng), were treated with valinomycin (10 nM) (G) or oligomycin (25 nM) and antimycin A (250 nM) (H) for 20 hr. Cells were processed and analyzed as in (B).

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

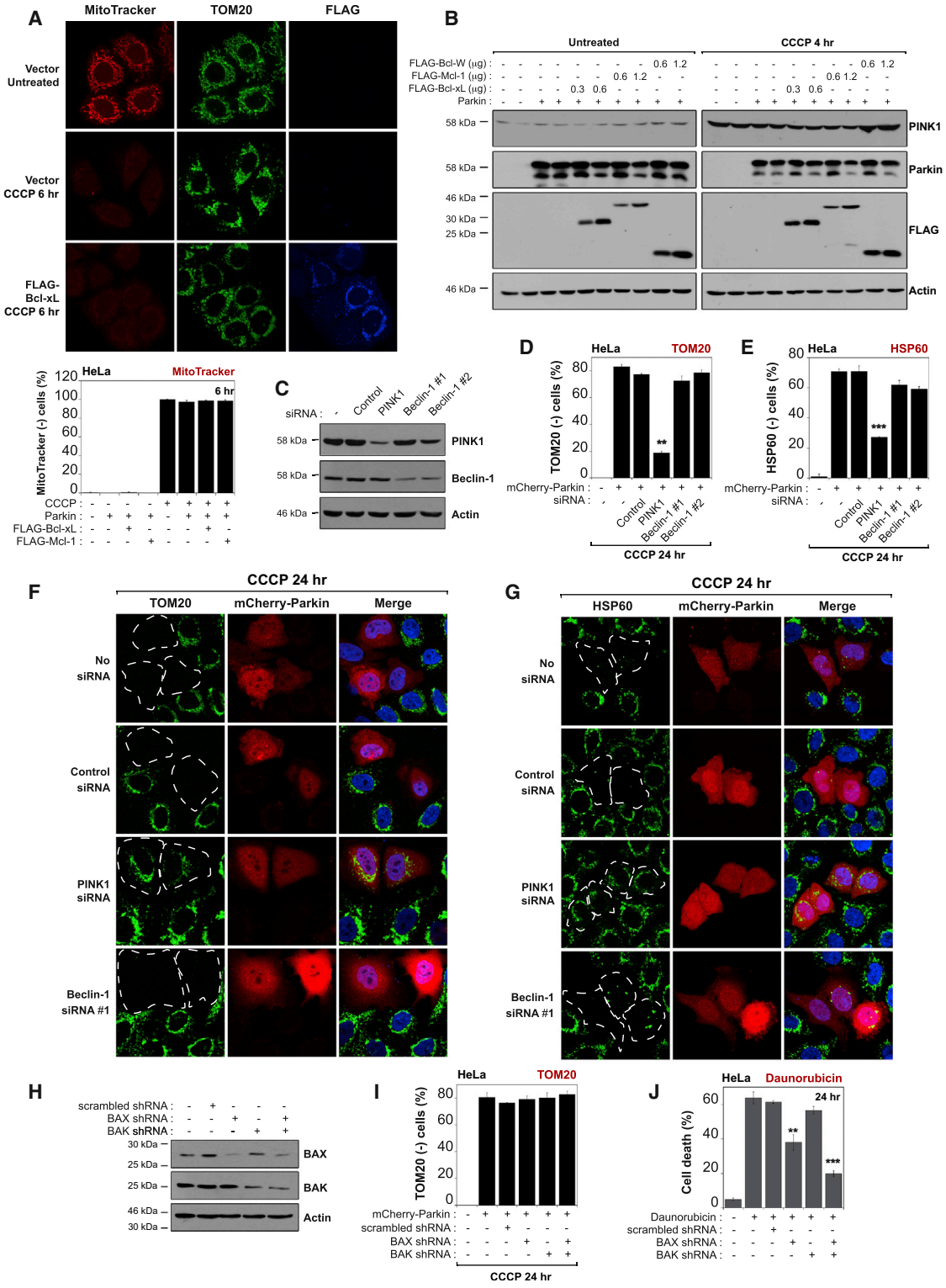


**Figure 3. Bcl-2- Binding Proteins Beclin-1, BAX, and BAK Are Dispensable for Parkin-Dependent Mitophagy**

(A) HeLa cells were transfected with plasmids encoding Parkin (200 ng) and FLAG-Bcl-xL or FLAG-Mcl-1 (400 ng) and treated with CCCP (10 μM) for 6 hr. Cells were stained with MitoTracker red (50 nM) and immunostained for TOM20 (green) and Bcl-xL or Mcl-1 (FLAG, blue). MitoTracker-negative cells were scored. (B) SDS-PAGE analysis of endogenous PINK1 expression. HeLa cells were transfected with Parkin cDNA (100 ng), along with the indicated amount of FLAG-Bcl-2 construct. Cells were treated with CCCP (5 μM) for 4 hr before analysis by immunoblotting.

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depolarized mitochondria with Ub, p62, NBR1, or GFP-LC3 were impaired in Parkin-transfected cells expressing Bcl-xL or other members of the Bcl-2 family. As Figure 4 shows, decoration of depolarized mitochondria in Parkin-expressing cells with Ub (Figure 4A), p62 (Figure 4B), or GFP-LC3 (Figure 4C) was all markedly inhibited upon coexpression of Bcl-xL, Mcl-1, and other members of the Bcl-2 family, except Bcl-2 itself. Similarly, translocation of the Ub and LC3II adaptor, NBR1, was also impaired by overexpression of prosurvival Bcl-2 family proteins (Figure S3). This suggested that prosurvival Bcl-2 proteins inhibit Parkin/PINK1-dependent mitophagy at an early stage in the process, prior to the decoration of depolarized mitochondria with Ub and Ub adaptor proteins involved in targeting mitochondria to autophagosomes.

## Parkin Translocation to Depolarized Mitochondria Is Attenuated by Prosurvival Bcl-2 Family Proteins

Because Parkin-dependent ubiquitination of mitochondria and its downstream sequelae were blocked by Bcl-xL and other Bcl-2 family proteins, we explored whether the PINK1-dependent translocation of Parkin to mitochondria was affected by Bcl-2 family members. Indeed, during the preceding experiments, we noticed that, in CCCP-treated cells, Parkin was frequently observed in the cytoplasm, rather than on mitochondria, in cells coexpressing Bcl-2 family proteins (Figures 4A–4C). This was readily appreciated when CCCP-treated cells were stained for Parkin along with prosurvival Bcl-2 family proteins. As Figure 5A and Figure S4A demonstrate, whereas Parkin was clearly cytoplasmic in cells overexpressing Bcl-xL or Mcl-1, Parkin was sharply localized to mitochondria in neighboring (Bcl-xL- or Mcl-1-negative) cells.

Thus, we performed time course analyses of Parkin translocation after CCCP treatment in cells expressing Bcl-xL versus empty vector. As Figures 5B and 5C illustrate, Parkin rapidly translocated to mitochondria within 1 hr after CCCP treatment; however, this was attenuated in the presence of Bcl-xL. Similarly, we readily observed impaired translocation of Parkin to depolarized mitochondria in cells overexpressing other prosurvival Bcl-2 family proteins (Figures 5D–5E). We also observed delayed Parkin translocation in the presence of prosurvival Bcl-2 family proteins upon depolarization of mitochondria with valinomycin (Figure 5F) or the combination of oligomycin/antimycin A (Figure 5G). Furthermore, Bcl-xL, Mcl-1, and Bcl-W also inhibited PINK1-dependent Parkin translocation in response to mitochondrial depolarization in HaCaT (Figure S4B and S4C) and SKOV-3 cells (Figure S4D).

It is interesting that we also observed spontaneous Parkin translocation, in the absence of mitochondrial depolarization, upon overexpression of PINK1 in A549, HeLa, SKOV-3, and HaCaT cells, and this was also inhibited through coexpression of Bcl-2 family proteins (Figures S4E–S4I).

## Parkin Translocation to Mitochondria Is Enhanced by BH3-Only Proteins or a BH3-Mimetic

Because endogenous prosurvival Bcl-2 proteins can be neutralized through expression of BH3-only proteins, we also explored whether the latter could have an impact on Parkin translocation to depolarized mitochondria. To investigate this, we transiently overexpressed a panel of BH3-only proteins along with Parkin, followed by treatment of cells with CCCP. These experiments were carried out in the presence of the poly-caspase inhibitor zVAD-fmk to block apoptosis mediated by BH3-only proteins. As Figures 5H and 5I show, coexpression of Bad, Bim, Noxa, or Puma all enhanced translocation of Parkin to depolarized mitochondria. Furthermore, treatment of cells with the BH3-only mimetic ABT-737, which enhanced apoptosis through neutralization of endogenous prosurvival Bcl-2 family proteins (Figure S4J), also enhanced Parkin translocation in response to CCCP treatment (Figure 5J). Similar results were also observed in HeLa cells stably expressing YFP-Parkin (Figures 5K and S4K).

In contrast, we failed to observe significant enhancement of mitochondrial Parkin translocation upon overexpression of the unconventional BH3-containing proteins, BNIP3, Nix, or Beclin-1 (Figures S4L–S4N).

## Knockdown of Endogenous Prosurvival Bcl-2 Family Proteins Sensitize toward Depolarization-Induced Parkin Translocation to Mitochondria

Because the preceding experiments utilized overexpression approaches, we also explored whether knockdown of endogenous Bcl-2 family proteins could modulate Parkin translocation to mitochondria. As shown in Figures 6A and 6B, whereas knockdown of individual prosurvival Bcl-2 family proteins sensitized toward apoptosis, this had little effect on CCCP-induced Parkin translocation to mitochondria (Figures 6C and 6D). However, knockdown of multiple prosurvival Bcl-2 proteins enhanced CCCP-induced Parkin translocation as well as mitophagy (Figures 6C–6G), suggesting a degree of redundancy in this regard. Furthermore, we also found that knockdown of certain Bcl-2 protein pairs (e.g., Bcl-xL/Bcl-2; Bcl-xL/Bcl-B) was also sufficient to

(C) HeLa cells were transfected with the indicated siRNA for 48 hr before analysis by immunoblotting.
(D and E) HeLa cells were transfected with the indicated siRNA along with mCherry-Parkin cDNA (200 ng). After 24 hr of CCCP treatment (10 μM), mitochondria were immunostained for TOM20 (D) or HSP60 (E). The percentage of TOM20-negative (D) or HSP60-negative (E) cells among mCherry-Parkin-positive cells was scored.
(F and G) Confocal images of HeLa cells transfected, treated, and immunostained as in (D) and (E).
(H) HeLa cells were transfected with a plasmid encoding GFP-tagged shRNA targeted against control (scrambled) or against BAX or BAK (500 ng) for 48 hr before analysis by immunoblotting.
(I) HeLa cells were transfected with plasmids encoding the indicated shRNA (500 ng) and mCherry-Parkin (200 ng) for 48 hr. After 24 hr of CCCP treatment (10 μM), mitochondria were immunostained for TOM20. TOM20-negative cells were counted among mCherry-Parkin-positive cells.
(J) HeLa cells were transfected with a plasmid encoding the indicated shRNA (500 ng) for 48 hr followed by treatment with daunorubicin (2 μM) for 24 hr. Apoptosis was assessed based on standard morphological criteria.
Results shown are representative of at least three independent experiments. Error bars indicate sd of triplicate counts of 100 cells. Statistical significance was assessed by two-tailed paired Student's t test. Asterisks indicate significance: *$p \leq 0.01$; **$p \leq 0.001$; ***$p \leq 0.0001$.

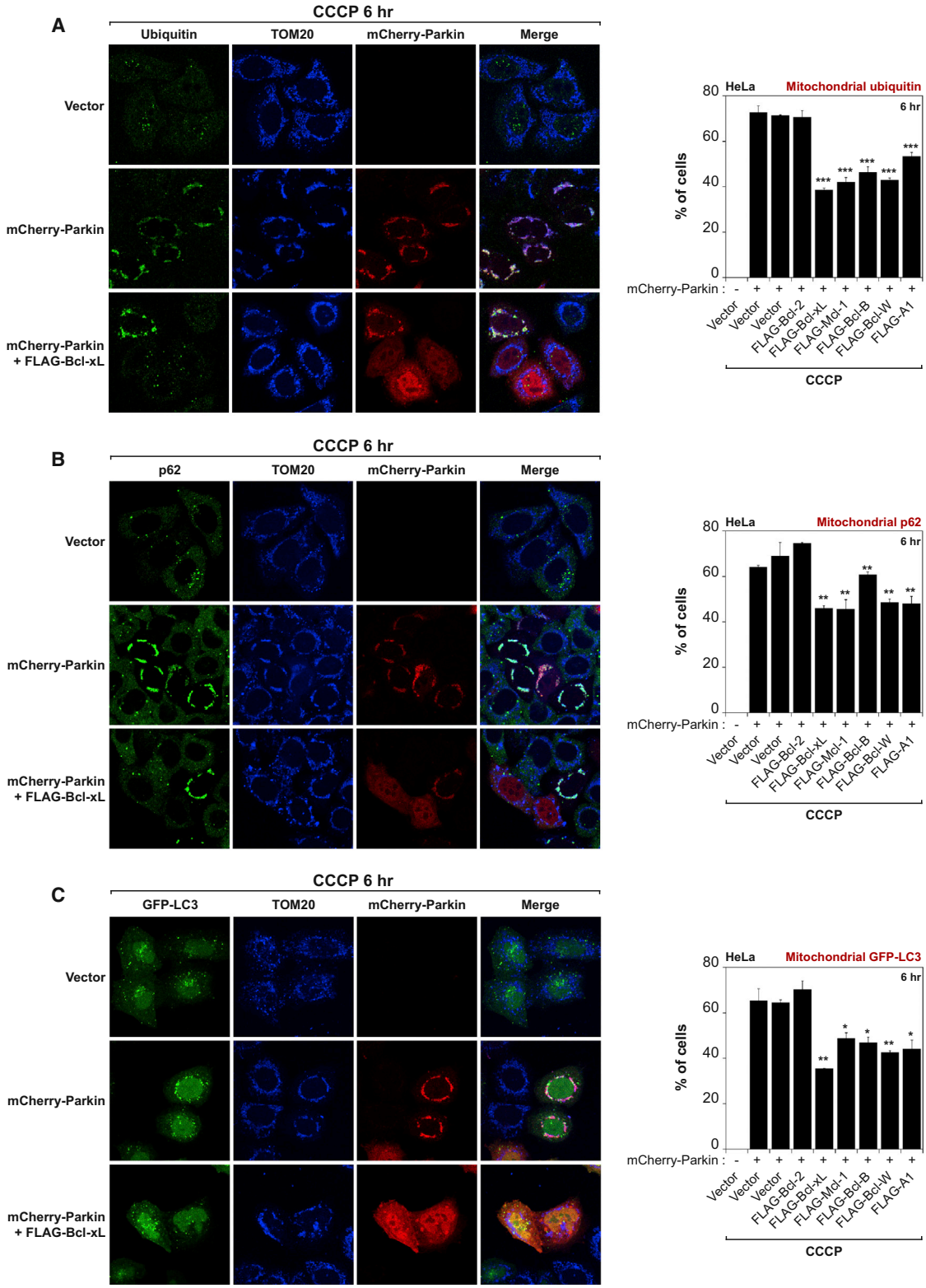


**Figure 4. Bcl-2 Family Proteins Block Parkin-Dependent Decoration of Mitochondria with Ub, p62, and LC3**

(A and B) HeLa cells were transfected with mCherry-Parkin cDNA (200 ng) and the indicated FLAG-Bcl-2 construct (400 ng). Cells, treated with CCCP (10 μM) for 6 hr, were coimmunostained for mitochondria (TOM20, blue) and Ub (green) (A) or p62 (green) (B). Colocalization between Ub (A) or p62 (B) and mitochondria was analyzed by confocal microscopy and scored among mCherry-Parkin-positive cells.

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enhance CCCP-induced Parkin translocation and mitochondrial ubiquitination (Figures 6H–6J).

In contrast, knockdown of endogenous BH3-only proteins, while protecting against apoptosis induced by cisplatin, Fas, or TRAIL (Figures S5A and S5D), failed to influence CCCP-induced translocation of Parkin to mitochondria in two different cell lines (Figures S5B and S5C).

### Bcl-2 Family Members Interact with the Parkin/PINK1 Complex

The preceding experiments revealed that members of the Bcl-2 family could influence translocation of Parkin to depolarized mitochondria, thereby having an impact on Parkin-dependent events such as decoration of mitochondria with Ub, p62, NBR1, and LC3 and their removal by mitophagy. This strongly suggested that members of the Bcl-2 family are involved at an early stage in the sequence that results from depolarization of mitochondria, stabilization of PINK1 on mitochondrial outer membranes, and formation of the Parkin/PINK1 complex. One possibility was that Bcl-2 family proteins directly antagonized these events through direct interactions with Parkin, PINK1, or both. Thus, we performed coimmunoprecipitation experiments with Bcl-xL, Mcl-1, and Bcl-W to ask whether these could interact with Parkin. As Figure 7A shows, we readily detected an interaction between Parkin and all three Bcl-2 family proteins, regardless of whether mitochondria were depolarized or not. We carried out similar immunoprecipitation experiments with PINK1 and also found that we could detect interactions between the latter and overexpressed Bcl-2 family proteins (Figure 7B). Coexpression of Parkin, PINK1, and Bcl-2 family proteins also coimmunoprecipitated both Parkin and PINK1 with Bcl-2 proteins (Figure 7C, left panel), although Parkin capture was diminished upon treatment with CCCP, suggesting that PINK1 might compete with Parkin for binding to Bcl-2 family proteins (Figure 7C, right panel). Reciprocal immunoprecipitation experiments with GFP-Parkin suggested that the interaction between Parkin and PINK1 was diminished through coexpression with Bcl-xL or Mcl-1 (Figure 7D).

We also performed immunoprecipitation experiments at endogenous levels of Bcl-2 family expression to confirm that the aforementioned results were not due to promiscuous interactions due to overexpression. As Figure 7E shows, immunoprecipitation of endogenous Mcl-1 or Bcl-xL coimmunoprecipitated endogenous Parkin, which was enhanced upon treatment with CCCP. However, we failed to detect direct immunoprecipitation of PINK1 under the same conditions (Figure 7E), suggesting that the interaction seen under overexpression conditions was nonspecific or indirect via endogenous Parkin.

Thus, prosurvival Bcl-2 family proteins may interfere with the stable recruitment of Parkin to mitochondria, through direct binding to Parkin, thereby repressing formation of the Parkin/PINK1 complex and setting a threshold for elimination of impaired mitochondria via mitophagy.

## DISCUSSION

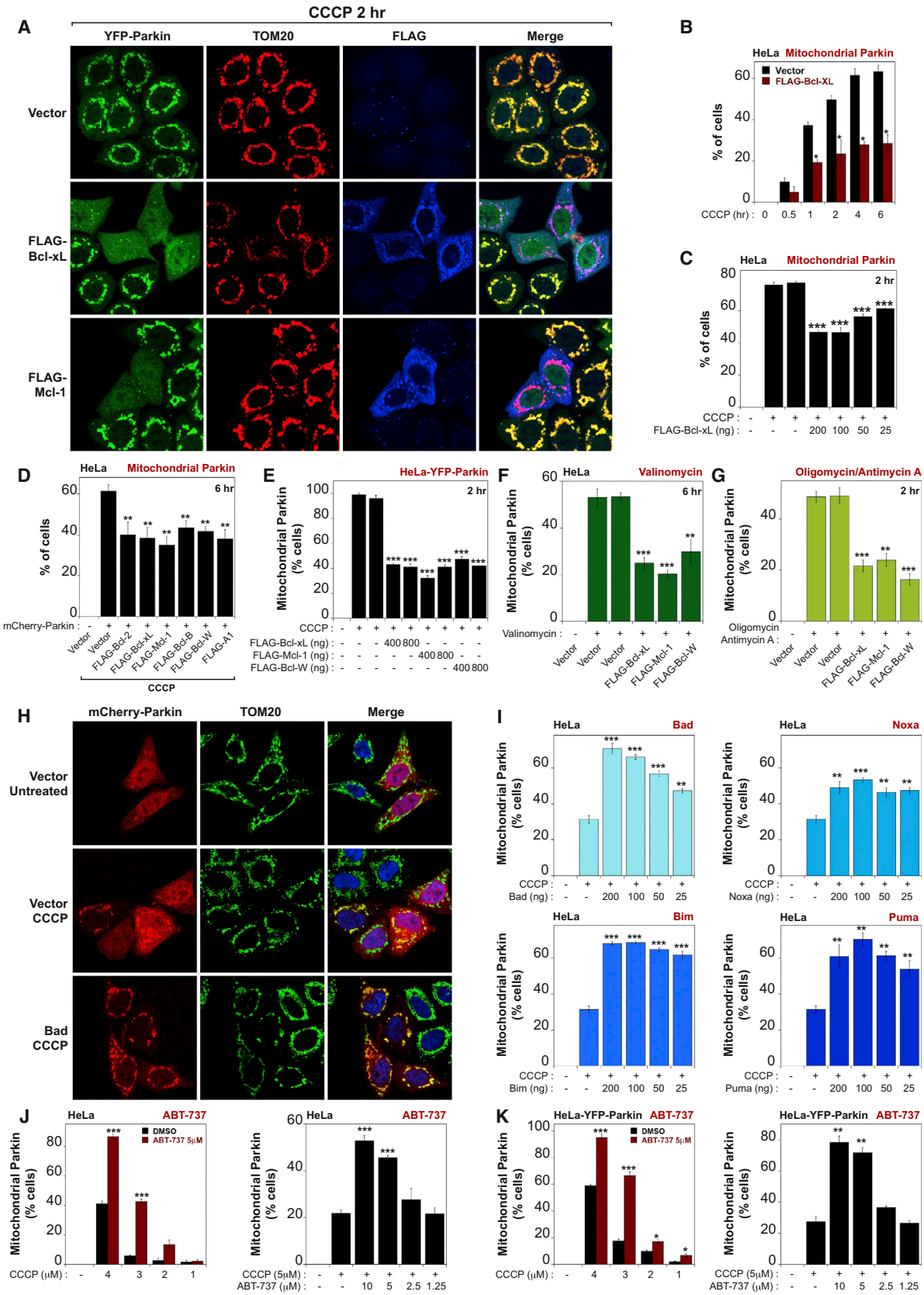
Here, we have shown that members of the Bcl-2 family can regulate mitophagy through influencing the recruitment of Parkin to depolarized mitochondria. Bcl-xL, Mcl-1, and other members of the prosurvival subset of the Bcl-2 family were found to directly interact with Parkin and to impede its stable recruitment to depolarized mitochondria, thereby raising the threshold for clearance of the latter via mitophagy. Conversely, BH3-only proteins, such as Bad, Bim, Puma, and Noxa, as well as a BH3-only mimetic, were capable of accelerating Parkin recruitment to impaired mitochondria, most likely through neutralization of endogenous Bcl-2 family proteins. BAX and BAK did not appear to be involved in this process, as knockdown of the latter failed to affect Parkin translocation or mitophagic clearance of these organelles. Collectively, these data suggest that, in addition to their role as regulators of mitochondrial outer membrane permeabilization during apoptosis and mitochondrial network dynamics, members of the Bcl-2 family can also regulate the clearance of impaired mitochondria via mitophagy.

Because Parkin is localized to the cytosol and prosurvival Bcl-2 proteins are localized predominantly to mitochondrial outer membranes, it would seem counterintuitive that the latter can block translocation of Parkin to depolarized mitochondria. However, one way in which this may occur is through Bcl-2 proteins interfering with the stable recruitment of Parkin to its mitochondrial receptor(s). This may operate in a manner similar to how Bcl-xL has been reported to block recruitment of BAX (which is also cytosolic) to mitochondria, with the latter continuously interacting with mitochondrial membranes but undergoing retrotranslocation to the cytosol by Bcl-xL (Edlich et al., 2011). In a similar vein, Parkin may be continuously retrotranslocated to the cytosol via interactions with mitochondria-localized Bcl-2 family proteins (Figure S5E). Alternatively, because there is evidence that the initial pool of Parkin that is recruited to depolarized mitochondria may seed a feed-forward amplification loop to promote further Parkin recruitment via Parkin-Parkin interactions (Lazarou et al., 2013), Bcl-2 family members may block this feed-forward amplification loop by binding to the initial pool of mitochondria-localized Parkin, blocking further recruitment (Figure S5F).

The removal of dysfunctional mitochondria is required to maintain a healthy mitochondrial network, and this process must be restricted to impaired mitochondria. Prosurvival Bcl-2 family proteins may contribute to setting a threshold for ongoing Parkin-mediated removal from the mitochondrial network, thereby protecting healthy mitochondria from Parkin-mediated ubiquitination and removal. However, the role

(C) HeLa cells were transfected with plasmids encoding GFP-LC3 (400 ng), mCherry-Parkin (200 ng), and the indicated FLAG-Bcl-2 construct (400 ng). Cells were treated as in (A) and (B) and immunostained for TOM20 (blue). Colocalization between GFP-LC3 and mitochondria was scored among mCherry-Parkin-positive cells.

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

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of Bcl-2 family members in this process may be especially important under stress conditions where mitochondrial damage is acute. Although we found evidence of redundancy among members of the Bcl-2 family with respect to the regulation of mitophagy, individual Bcl-2 family members may play a greater or lesser role in regulating Parkin activation in particular cell types, depending on their relative expression levels in particular tissues. Redundancy of function is also seen within the Bcl-2 family in the context of cell death control, with relative tissue expression levels playing a role in dictating the relative importance of particular Bcl-2 family members in specific tissues. In the context of mitophagy, it is relevant to note that Bcl-W has been implicated to play an important role in the maintenance of mitochondrial network function and integrity in neurons (Liu and Shio, 2008; Courchesne et al., 2011). Furthermore, Bcl-xL has also been implicated in the regulation of mitochondrial biomass in neurons (Berman et al., 2009), which may be related to the ability of the latter to regulate mitochondrial turnover via mitophagy. It is also noteworthy that previous studies have suggested that Bcl-2 family members interact with Parkin or PINK1 (Chen et al., 2010; Johnson et al., 2012; Arena et al., 2013). Parkin has been shown to monoubiquitinate Bcl-2, enhancing Bcl-2-Beclin-1 interaction (Chen et al., 2010). However, given that we could not find any requirement for Beclin-1 in mitophagy, the significance of the latter observation for mitophagy is unclear. PINK1 has also been reported to phosphorylate Bcl-xL in response to mitochondrial depolarization (Arena et al., 2013); however, the implications of this phosphorylation event for mitophagy are currently unclear.

Accumulating evidence suggests that the role of Bcl-2 family proteins in mitochondrial as well as cellular homeostasis is much broader than previously appreciated (reviewed in Autret and Martin, 2009). It is now well established that members of the extended Bcl-2 family play a central role in dictating the onset of apoptosis through regulating the permeability of the mitochondrial outer membrane (Chipuk et al., 2010). A network of interactions between BH3-only proteins, BAX and BAK, and the prosurvival members of the Bcl-2 family set a threshold for BAX/BAK oligomerization within the mitochondrial outer membrane, effectively dictating the integrity of the entire mitochondrial network. In addition to this, Bcl-2 family members have also been repeatedly implicated in mitochondrial fission and fusion dynamics (reviewed in Martinou and Youle, 2011; Delivani et al., 2006; Sheridan et al., 2008). In this context, it is relevant to note that the ability to quarantine depolarized mitochondria from the remainder of the mitochondrial network, via increased fission or decreased fusion, may be an important feature of mitophagy (Twig et al., 2008; Gomes et al., 2011; Rambold et al., 2011). Mcl-1 has also been implicated as a regulator of mitochondrial respiration (Perciavalle et al., 2012). The atypical BH3-only proteins BNIP3 and Nix can regulate autophagy as well as mitophagy in erythrocytes (reviewed in Zhang and Ney, 2009). Furthermore, several Bcl-2 family members have also been implicated as regulators of macroautophagy, through their ability to bind to Beclin-1 (Pattingre et al., 2005; Elgendy et al., 2011).

It is striking that many of the processes with which members of the Bcl-2 family have been linked revolve around mitochondrial function and integrity. This suggests that Bcl-2 proteins may function as global regulators of mitochondrial homeostasis in response to cell stress and can regulate several aspects of mitochondrial function, including: mitochondrial permeability (via BAX/BAK pore formation), mitochondrial connectivity (via effects on fission/fusion dynamics), mitochondrial turnover (via mitophagy), and ATP synthesis (via effects on the respiratory chain). It is also highly compelling that perturbation of many of these processes individually can affect the others. For example, inhibition of autophagy is well known to sensitize toward apoptosis; conversely, inhibition of apoptosis increases the likelihood of autophagy (reviewed by Maiuri et al., 2007). Similarly, mitochondrial networks fragment during apoptosis, and enforced

**Figure 5. Translocation of Parkin to Depolarized Mitochondria Is Antagonized by Prosurvival Bcl-2 Proteins and Accelerated by BH3-Only Proteins**

(A) Confocal images of HeLa-YFP-Parkin cells transfected with FLAG-Bcl-xL or FLAG-Mcl-1 cDNA (500 ng). Cells were treated with CCCP (5 μM) for 2 hr and coimmunostained for Bcl-xL or Mcl-1 (FLAG, blue) and TOM20 (red).

(B) HeLa cells were transfected with plasmids encoding mCherry-Parkin (200 ng) and FLAG-Bcl-xL (400 ng). Cells were treated with CCCP (10 μM) and immunostained for mitochondria (TOM20). Colocalization between Parkin and mitochondria was scored among mCherry-Parkin-positive cells.

(C) HeLa cells were transfected with mCherry-Parkin cDNA (400 ng) and the indicated amount of FLAG-Bcl-xL plasmid. Cells were treated, immunostained, and analyzed as in (B).

(D) HeLa cells were transfected with mCherry-Parkin cDNA (200 ng) along with the indicated FLAG-Bcl-2 construct (400 ng). Cells were treated, immunostained, and analyzed as in (B).

(E) HeLa-YFP-Parkin cells, transfected with the indicated amount of FLAG-Bcl-2 construct, were treated and immunostained as in (A). Cells presenting YFP-Parkin colocalized with mitochondria were scored.

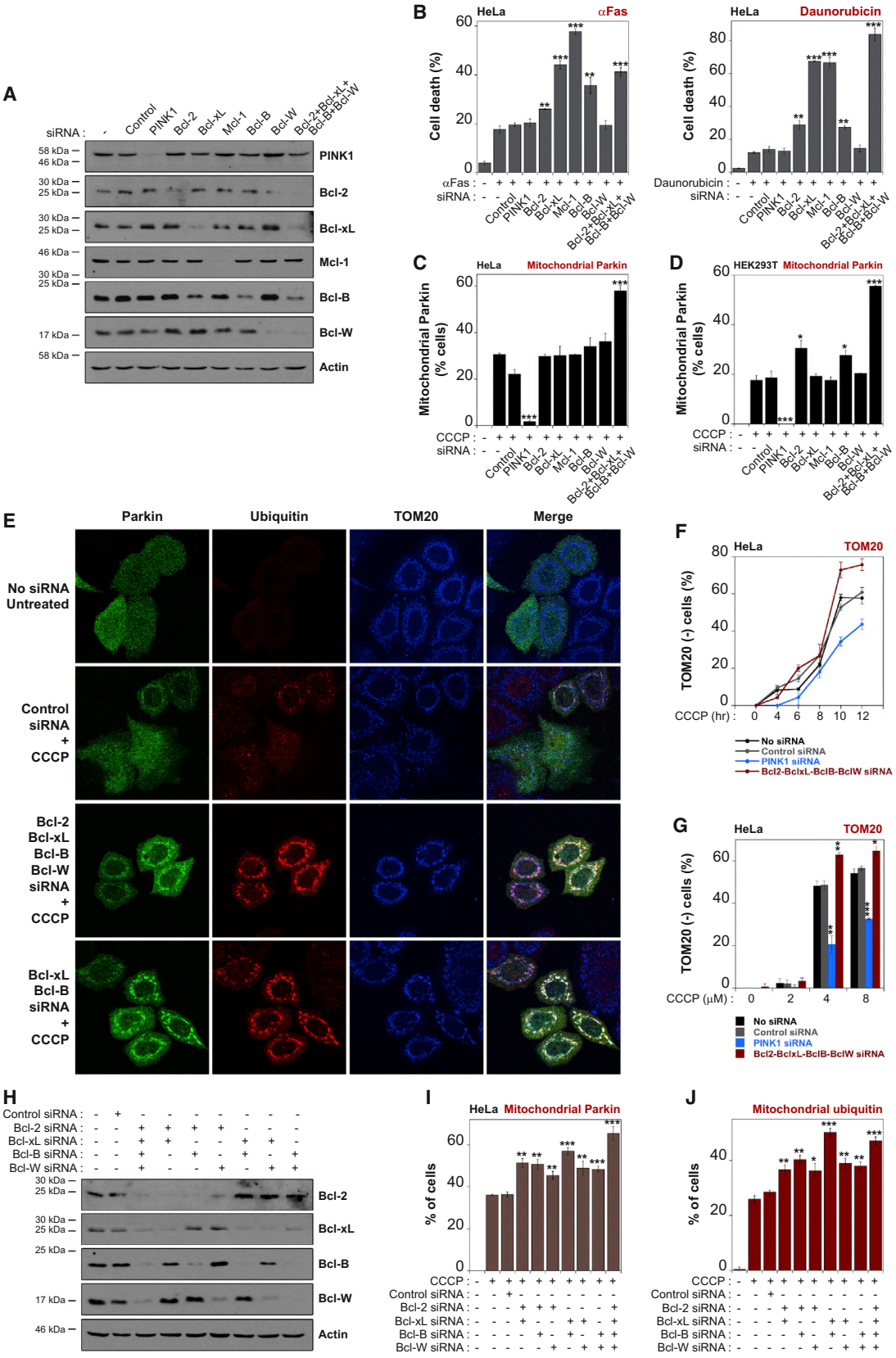
(F and G) HeLa cells were transfected with mCherry-Parkin cDNA (400 ng) and the indicated FLAG-Bcl-2 construct (800 ng). Cells, treated with valinomycin (10 nM) for 6 hr (F) or with oligomycin (50 nM) and antimycin A (500 nM) for 2 hr (G), were immunostained for TOM20. Colocalization between Parkin and mitochondria was scored among mCherry-Parkin-positive cells.

(H) Confocal images of HeLa cells transfected with plasmids encoding mCherry-Parkin (200 ng) and Bad (100 ng) for 8 hr in the presence of zVAD-fmk (20 μM). Cells were treated with CCCP (5 μM) for 1.5 hr and immunostained for mitochondria (TOM20, green).

(I) HeLa cells were transfected with mCherry-Parkin cDNA (200 ng), along with the indicated amount of BH3-only construct for 8 hr in the presence of zVAD-fmk (20 μM). Cells were treated and immunostained as in (H), and colocalization between Parkin and mitochondria was scored among mCherry-Parkin-positive cells.

(J and K) HeLa cells transfected with mCherry-Parkin cDNA (400 ng) (J) or HeLa-YFP-Parkin cells (K) were pretreated with ABT-737 for 1 hr and treated with CCCP for 45 min. Mitochondria were immunostained for TOM20, and cells presenting Parkin associated with mitochondria were scored.

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of 100 cells. Statistical significance was assessed by two-tailed paired Student's t test. Asterisks indicate significance: *$p \leq 0.01$; **$p \leq 0.001$; ***$p \leq 0.0001$. See also Figure S4.

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mitochondrial fusion has been reported to delay apoptosis (reviewed in Autret and Martin, 2009). Furthermore, mitochondrial networks undergo fusion during macroautophagy (Gomes et al., 2011) and mitochondrial fission has been shown to facilitate mitophagy (Twig et al., 2008; Gomes et al., 2011; Rambold et al., 2011).

It is perhaps highly significant that Bcl-2 family proteins have been implicated as regulators of all of the processes described earlier, suggesting an overarching role for members of this family as regulators of mitochondrial "fitness." Thus, Bcl-2 family proteins may police mitochondrial network integrity at a global level, coordinating the isolation (via increased fission or decreased fusion) of impaired mitochondria and their removal (via mitophagy) to minimize the likelihood of complete depolarization of mitochondria and consequent escape of cytochrome *c*, which would have disastrous consequences for the cell (i.e., apoptosis). On the other hand, in response to severe stress, other members of the Bcl-2 family can directly depolarize mitochondria, via BAX/BAK activation, fragment the mitochondrial network, and permit the escape of mitochondrial intermembrane space proteins to facilitate the elimination of the cell via apoptosis.

In conclusion, the ability of Bcl-2 proteins to regulate diverse aspects of mitochondrial function, including the removal of impaired mitochondria via Parkin/PINK1-dependent mitophagy, as we have shown here, suggests that the Bcl-2 family act as global regulators of mitochondrial network homeostasis, rather than simply as regulators of apoptosis.

## EXPERIMENTAL PROCEDURES

### Immunostaining

Cells were grown on coverslips before transfection and treatment with CCCP, valinomycin, or oligomycin and antimycin A. Cells were washed in PBS, fixed with 3% paraformaldehyde in PBS for 10 min, permeabilized with 0.15% Triton X-100 in PBS for 15 min, and blocked in 2% BSA in PBS for 30 min. Primary antibodies were used at 1:200 for 1 hr at room temperature, followed by secondary antibodies at 1:1,000 for 1 hr at room temperature. Final washing included incubation with 500 nM Hoechst (Sigma-Aldrich) for 10 min. Cells were mounted with Slow Fade (Molecular Probes), and observed on a laser scanning confocal microscope (Olympus FV1000) using a 488 nm Argon laser (green fluorescence), a 543 nm HeNe laser (red fluorescence), and a 405 nm LD laser. Confocal images were acquired with a UPlanSApo 60×/1.35 NA oil objective lens (zoom, 2.5) using Fluoview 1000 V.1 application software. Quantification of mitophagy, Parkin translocation, mitochondrial Ub, p62 or GFP-LC3 was estimated by counting a minimum of 3 × 100 cells for each treatment.

### Western Blot Analysis

Following CCCP treatment or transfection with siRNA or small hairpin RNA (shRNA) (48 hr), whole-cell lysates were prepared with 100 μl of SDS-PAGE sample buffer. Samples were boiled for 10 min, ran on 10% or 12% SDS-PAGE gels, and transferred on to 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.

### Immunoprecipitation Assays

HEK293T cells, transfected for 24 hr, were treated with CCCP and lysed at $10^7$ cells per milliliter with 1% NP40 lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40 for FLAG immunoprecipitations) or 0.2% NP40 lysis buffer (20 mM HEPES [pH 7.9], 1 mM dithiothreitol [DTT], 0.2% Nonidet P-40 for GFP immunoprecipitations) containing 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μM MG132. Lysates were incubated for 15 min under rotation at 4°C. Following centrifugation at 15,000 × g for 15 min at 4°C, clarified lysates were precleared with 20 μl of agarose-coupled protein A/G (Santa Cruz Biotechnology) and 1 μg of mouse immunoglobulin G (Sigma-Aldrich). Precleared lysates were subjected to immunoprecipitation using 2 μg of the appropriate antibody and 30 μl of agarose-coupled protein A/G at 4°C for 4 hr. Complexes were washed three times in lysis buffer. Immunoprecipitates were then analyzed by immunoblotting. For endogenous immunoprecipitation, HEK293T cells were lysed at 5 × $10^7$ cells per milliliter with 1% NP40 lysis buffer containing 1 mM EDTA, 1 mM DTT, and 10% glycerol. Immunoprecipitation was performed using 4 μg of the appropriate antibody preconjugated to 20 μl of agarose-coupled protein A/G at 4°C for 4 hr.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.06.001.

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**Figure 6. Knockdown of Bcl-2 Family Proteins Enhances Parkin Recruitment to Depolarized Mitochondria and Mitophagy**

(A) HeLa cells were transfected with the indicated siRNA for 48 hr before analysis by immunoblotting.

(B) HeLa cells transfected as in (A) were treated with anti-Fas antibody (200 ng/ml) for 10 hr or daunorubicin (1 μM) for 12 hr. Apoptosis was assessed based on standard morphological criteria.

(C) HeLa cells were transfected with the indicated siRNA along with Parkin cDNA (100 ng) for 48 hr. Cells, treated with CCCP (4 μM) for 1 hr, were coimmunostained for mitochondria (TOM20) and Parkin. Colocalization between Parkin and mitochondria was scored.

(D) HEK293T cells were transfected as in (C) and treated with CCCP (10 μM) for 45 min. Cells were processed and analyzed as in (C).

(E) Confocal analysis of HeLa cells transfected and treated as in (C). Cells were coimmunostained for Parkin (green), Ub (red), and TOM20 (blue).

(F and G) HeLa cells, transfected with the indicated siRNA along with Parkin cDNA (100 ng) for 48 hr, were treated with CCCP (4 μM) for the indicated period of time (F) or with the indicated dose of CCCP for 10 hr (G). Following coimmunostaining for mitochondria (TOM20) and Parkin, TOM20-negative cells were scored among Parkin-positive cells.

(H) HeLa cells were transfected with the indicated siRNA for 48 hr before analysis by immunoblotting.

(I and J) HeLa cells were transfected with the indicated siRNA along with Parkin cDNA (100 ng) for 48 hr. Cells were treated with CCCP (4 μM) for 45 min before staining for TOM20 and Parkin (I) or for TOM20, Parkin, and Ub (J). Cells presenting Parkin (I) or Ub (J) colocalized with mitochondria were scored.

Results shown are representative of at least three independent experiments. Error bars indicate SD of triplicate counts of 100 cells. Statistical significance was assessed by two-tailed paired Student's t test. Asterisks indicate significance: *$p \leq 0.01$; **$p \leq 0.001$; ***$p \leq 0.0001$. See also Figure S5.

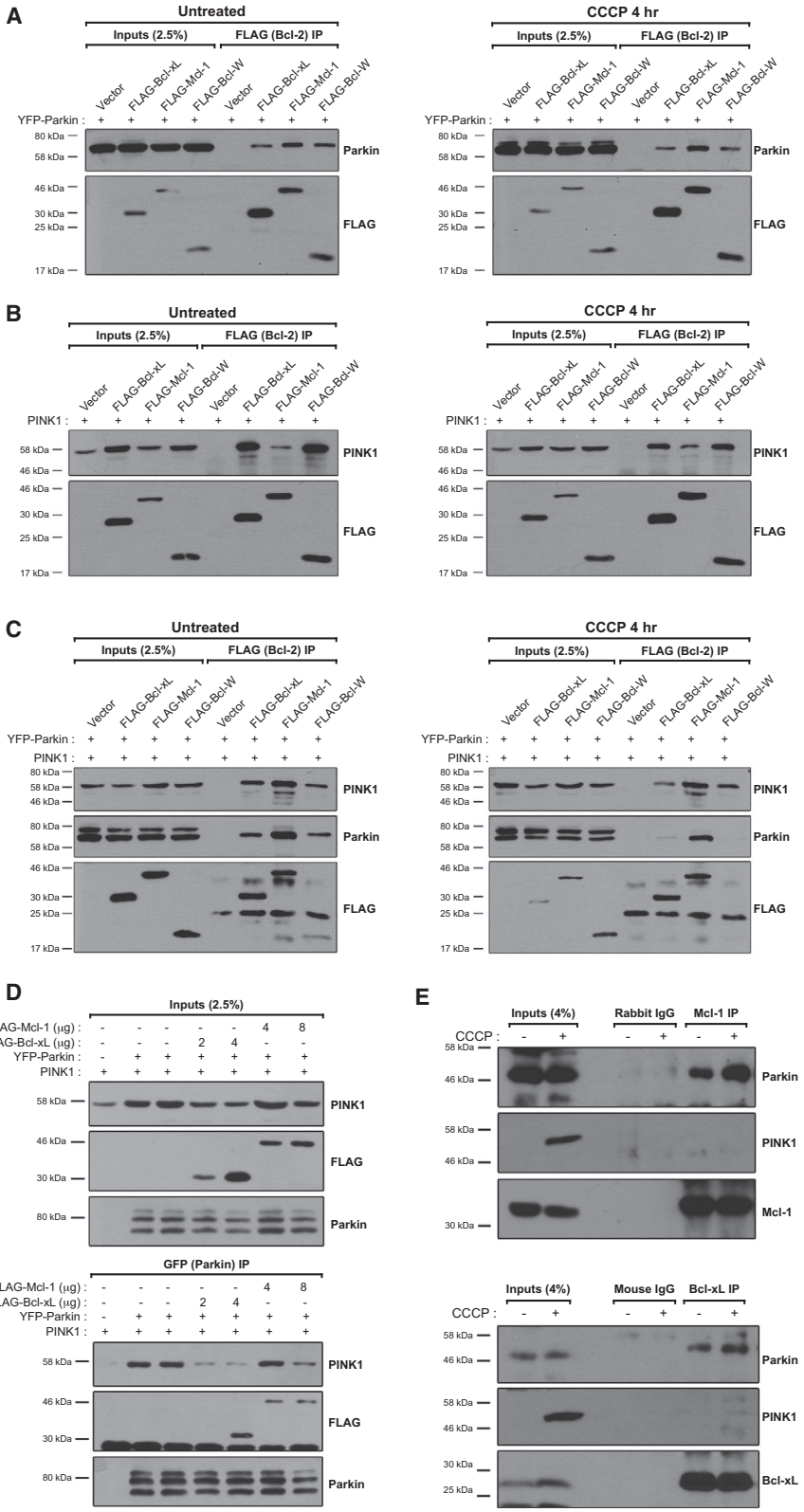


**Figure 7. Bcl-2 Family Proteins Directly Interact with Parkin**

(A) HEK293T cells were transfected with YFP-Parkin cDNA (1 μg) along with FLAG-Bcl-xL (3 μg), FLAG-Mcl-1 (5 μg), or FLAG-Bcl-W (5 μg) plasmids. Cells were left untreated (left) or treated with CCCP (5 μM) for 4 hr (right). Bcl-xL, Mcl-1, and Bcl-W were immunoprecipitated (IP) with an anti-FLAG antibody followed by immunoblotting for Parkin or Bcl-xL, Mcl-1, or Bcl-W (FLAG).

(B) HEK293T cells were transfected with PINK1 cDNA (1 μg) along with FLAG-Bcl-xL (3 μg), FLAG-Mcl-1 (5 μg), or FLAG-Bcl-W (5 μg) plasmids. Immunoprecipitations were performed as in (A).

(C) HEK293T cells were transfected with plasmids encoding YFP-Parkin (1 μg) and PINK1 (1 μg) along with FLAG-Bcl-xL (3 μg), FLAG-Mcl-1 (5 μg), or FLAG-Bcl-W (5 μg) cDNA. Immunoprecipitations were performed as in (A).

(D) HEK293T cells were transfected with plasmids encoding YFP-Parkin (0.5 μg) and PINK1 (0.5 μg) along with the indicated amount of FLAG-Bcl-xL or FLAG-Mcl-1 cDNA. Parkin was immunoprecipitated with an anti-GFP antibody followed by immunoblotting for PINK1, Bcl-xL, or Mcl-1 (FLAG) and Parkin.

(E) HEK293T cells were left untreated or treated with CCCP (50 μM) for 5 hr. Endogenous Mcl-1 (top) and Bcl-xL (bottom) were immunoprecipitated. Immunoprecipitates were analyzed by immunoblotting for Parkin, PINK1 and Bcl-xL, or Mcl-1. IgG, immunoglobulin G.

Results shown are representative of at least three independent experiments.

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