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PhD in Genetics & Microbiology

The role of the Bcl-2 Family in Mitochondrial Homeostasis

Thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy

July 2015

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Publications

Carroll, R.G. and Martin, S.J. Autophagy in multiple myeloma: what makes you stronger can also kill you. Cancer Cell *23*, 425-426

Hollville, E., **Carroll, R.G**., Cullen, S.P. and Martin, S.J. Bcl-2 Family Proteins Participate in Mitochondrial Quality Control by Regulating Parkin/PINK1-Dependent Mitophagy. Molecular Cell *55*, 451-466.

Carroll, R.G., Hollville, E. and Martin, S.J. Parkin Sensitizes Towards Apoptosis induced by Mitochondrial Depolarization Through Promoting Degradation of Mcl-1. l Cell Reports 9, 1538-1553.

Summary

Introduction

Mitochondria are required by cells to generate ATP in order to meet their energy requirements. The electron transport chain, within the mitochondria, pumps protons into the intermembrane space generating an electrochemical gradient. This electrochemical gradient is required for production of ATP and is referred to as the mitochondrial potential. During apoptosis, the activation of Bax/Bak results in a loss of mitochondrial potential and a release of cytochrome c into the cytosol. The loss of mitochondrial potential is the critical step that commits a cell to dying. However, release of cytochrome c activates the apoptosis cascade and promotes a non-inflammatory mode of cell death as opposed to the inflammatory mode of cell death, necrosis. The induction of an inflammatory response promotes the recruitment of many different immune cells whose activation can have a damaging effect on the surrounding cells and tissue. As such, induction of apoptosis is important to remove dead cells without eliciting an immune response in order to minimize unwanted tissue damage.

Chapter III

During apoptosis, the dying cell breaks up into smaller fragments known as apoptotic bodies. If not removed quickly, the apoptotic bodies can rupture, release their cytoplasmic constituents and promote an immune response. The externalization of phosphatidylserine is essential for the rapid identification and removal of apoptotic bodies, thus, limiting inflammation. The caspase-activated scramblase responsible for phosphatidylserine externalization during apoptosis has remained elusive and we hypothesized that TMEM16F may be the protein responsible. TMEM16F was characterized as the scramblase responsible for phosphatidylserine externalization in response to calcium fluctuations, however, it was not responsible for phosphatidylserine externalization during apoptosis. During the course of our work, another group published that Xkr8 was the caspase-activated scramblase responsible for phosphatidylserine externalization during apoptosis. Because of this, we ended our investigation at this point.

Chapter IV

The Bcl-2 family members play an important role in mitochondrial homeostasis during apoptosis. The pro-apoptotic members promote mitochondrial dysfunction through the creation of channels resulting in mitochondrial depolarization and cytochrome c release. The anti-apoptotic Bcl-2 proteins maintain mitochondrial function through inhibiting the pro-apoptotic BH3-only proteins and Bax/Bak activation. Here, we have characterized a novel role for the Bcl-2 family in mitochondrial biology. The anti-apoptotic Bcl-2 proteins can inhibit Parkin translocation, therefore, setting a threshold for mitophagy and as a result, maintain a healthy pool of mitochondria within the cell. The pro-apoptotic BH3-only proteins antagonize anti-apoptotic Bcl-2 proteins and their inhibition of Parkin, thereby decreasing the threshold for mitochondrial degradation. This threshold for mitophagy is vital to prevent the removal of healthy mitochondria. This balance in mitophagy allows for the correct abundance of healthy mitochondria that are required to generate ATP requirements of a cell. The importance of mitophagy can be clearly observed in familial Parkinson's disease as loss-of-function mutations in mitophagy genes lead to neurodegeneration.

Chapter V

Here, in addition to its role in mitophagy, we described that Parkin can act as a sensor for mitochondrial damage. Mitochondrial depolarization and cytochrome c release are two distinct events during apoptosis. Mitochondria can be depolarized without the loss of cytochrome by using mitochondrial toxin, such as, CCCP. The loss of mitochondrial potential without cytochrome c release should result in necrosis and inflammation. However, in response to excessive mitochondrial depolarization in the absence of cytochrome c release, Parkin can sense the mitochondrial dysfunction and degrade Mcl-1. Loss of Mcl-1 allows the cell to enter apoptosis and, in doing so, prevents unwanted inflammation. Altogether, we have demonstrated that the Bcl-2 family members play an essential role in mitochondrial homeostasis throughout the lifetime of a cell. The anti-apoptotic Bcl-2 proteins regulate the mitochondrial damage sensor, Parkin, and are involved in regulating the decision between either a mitochondrial repair response or the termination of cells with irreparable mitochondria.

Abbreviations

A1	Bcl-2 related protein-A-1
Apaf-1	Apoptotic protease activating factor 1
Atg	Autophagy related gene
ATP	Adenosine Triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma
Bcl-B	Bcl-2 related gene B
Bcl-W	Bcl-2 related gene W
Bcl-xL	Bcl-2 related gene X long isoform
BH	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting protien Bim
CAD	caspase activated Dnase
caspase	Cysteinyl aspartate -specific protease
DNA	Deoxyribonucleic acid
FACS	Fluorescence -activated cell sorter
FCS	Fetal calf serum
FADD	Fas-associating protein with death domain
GFP	Green Fluorescent Protein
HEK	Human embryonic kidney cells
Hrk	Harakiri
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase activated Dnase
IL	interleukin
IP	Immunoprecipitation
LC3	Microtubule-associated 1A/1B-light chain 3
Mcl-1	Myeloid cell Leukemia
Mfn	Mitofusin

MOMP	Mitochondrial outer membrane permeabilization
NBR1	Neighbor of BRCA1 gene 1
NFκB	Nuclear factor kappa-light-chain enhancer of activated B cells
PAGE	Polyacrylamide gel
PARP	Poly(ADP-ribose) polymerase
PEST	Proline (P), glutamic acid (E), serine (S), threonine (T) rich sequence
PBS	phosphate buffer saline
PINK1	PTEN-induced kinase
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
Puma	p53-upregulated modulator of apoptosis
RNA	Ribonucleic acid
ROCK-1	Rho-associated coiled-coil-containing protein kinase-1
ROS	Reactive Oxygen Species
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
siRNA	small interferring ribonucleic acid
shRNA	short hairpin ribonucleic
Smac	Second mitochondrial activator of caspases
TNFα	tumor necrosis factor alpha
TRADD	TNF receptor associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
YFP	Yellow fluorescent protein
XIAP	X-linked inhibitor of apoptosis protein
zVAD-fmk	Benzylocarbonyl-valine (V) alanine (A) aspartic acid (D)-fluromethylketone

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Chapter I

Introduction

1.1 Apoptosis

Apoptosis is a form of programmed cell suicide, conserved in metazoans, which is crucial for development and maintenance of cellular homeostasis within multicellular organisms (Meier et al., 2000). During development in mammals, apoptosis is required for the elimination of excess cells in the nervous system, the elimination of webbing between digits and the sculpting of numerous other tissues. Throughout the lifetime of an organism, apoptosis also plays a vital role in cellular maintenance by eliminating autoreactive T cells, removing excess lymphocytes after infections and removing potentially cancerous cells carrying deleterious mutations. Recently, apoptosis has also been implicated in tissue homeostasis through compensatory proliferation, where apoptotic cells release mitogenic signals to neighbouring cells to divide in order to compensate for the loss of cells (Huang et al., 2011). The importance of apoptosis is underscored by the observation that deregulation of this process is associated with both neurodegeneration (uncontrolled death of neurons) and cancer (uncontrolled growth of cells).

The phenomenon of apoptosis was first explicitly described by Currie and colleagues in 1972 (Kerr et al., 1972), where they described the morphological characteristics of apoptotic cells. These characteristics include cell shrinkage, nuclear and cytoplasmic condensation, membrane blebbing to form apoptotic bodies, fragmentation of organelles such as the mitochondria or endoplasmic reticulum, loss of plasma membrane asymmetry, DNA fragmentation and cell detachment from neighbouring cells. Apoptosis is an important mode of cell death as it allows for the removal dying cells without activating an immune response (Martin et al., 2012). Apoptotic cells alert the immune system and neighbouring cells to their presence through the release of 'find me' signals which allows for the chemotaxis of phagocytes to remove dying cells before they can illicit an immune response (Cullen et al., 2013; Juncadella et al., 2013). Apoptotic cells are then recognized by phagocytes and are engulfed by phagocytes or neighbouring cells before secondary necrosis of apoptotic bodies can stimulate an immune response (Fadok et al., 1992; Martin et al., 1995).

The plasma membrane encapsulates the cell and is composed of a polar lipid bilayer with one layer facing the cytoplasm and the other facing the extracellular matrix.

There are many different lipids that compose the plasma membrane with their synthesis and insertion into the membrane generating an asymmetric distribution. The lipids phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are exclusively found on the inner leaflet while Phosphatidylcholine and sphingomyelin are found within the outer leaflet of the membrane (Schlegel and Williamson, 1987). During apoptosis, the asymmetry of the plasma membrane is lost, resulting in externalization of phosphatidylserine to the outer leaflet of the membrane (Martin et al., 1995). The externalization of phosphatidylserine acts as a signal to phagocytes and endothelial cell to engulf the dying cell before activation of the immune system can take place. The removal of the dying cell before an immune response is stimulated acts to maintain tissue homeostasis by preventing unnecessary inflammation.

Apoptosis is a tightly regulated biochemical process co-ordinated by many families of proteins and is conserved from nematode to man. The execution of apoptosis relies on a family of cysteine-aspartic proteases called the caspases (Martin et al., 2012; Taylor et al., 2008). While the upstream initiating events of caspase activation can be classified into two distinct pathways (the extrinsic and intrinsic pathways) they both converge downstream to activate a common set of executioner caspases who cleave specific substrates resulting in the morphological characteristics of apoptosis.

1.2 Caspases

Caspases are cysteine aspartic proteases that exist in the cell as inactive zymogens and can be classified into two groups based on their role as positive or negative regulators of inflammation (Martin et al., 2012). Activation of caspase 1, 4, 5 or 11 promotes inflammation through the proteolysis of the pro-inflammatory apical cytokines IL-1β and IL-18 and are also key regulators of the pro-inflammatory form of cell death, pyroptosis (Miao et al., 2010). The remaining members of the caspase family are involved in co-ordinating the controlled destruction of unwanted cells through apoptosis without eliciting an immune response (Taylor et al., 2008). The caspases involved in regulating apoptosis have also been demonstrated to have direct anti-inflammatory properties such as the suppression of IL-33 activity by caspase 3 and 7 cleavage during apoptosis (Luthi et al., 2009).

As mentioned previously, apoptosis can be initiated via two pathways, the extrinsic pathway or the intrinsic pathway (**Figure 1.1**). The extrinsic pathway is initiated following the ligation of death receptors on the cell surface with their respective ligands (Fas, TRAIL or TNF-α). This leads to trimerization of the receptor resulting in a conformational change allowing for the recruitment of the adaptor proteins TRADD and FADD along with the initiator pro-caspase-8/10 to the cytosolic domain of the receptor known as the death inducing signalling complex (DISC). The formation of the DISC brings many molecules of pro-caspase-8/10 into close proximity thereby promoting their autoacatalysis (Muzio et al., 1996; Muzio et al., 1998). The autocatalysis of Caspase-8/10 induces a conformational change which promotes the dimerization of cleaved caspase-8 molecules generating a fully active enzyme. Once active, Caspase-8 cleaves and activates the executioner caspases, Caspase-3 and Caspase-7 leading to apoptosis.

The intrinsic pathway of apoptosis is tightly regulated by proteins of the Bcl-2 family and is activated following the activation and oligomerization of the proteins Bax and Bak (Yin et al., 1994). Once oligomerized, these proteins insert into the outer mitochondrial membrane, creating a channel, and allow the release of the electron transport chain protein, cytochrome c. This process is known as mitochondrial outer membrane permeabilization (MOMP) (Pastorino et al., 1998; Wolter et al., 1997). Once in the cytosol of the cell, Cytochrome c binds to seven Apaf-1 molecules forming a pentameric ring. Oligomerization causes a conformational change within Apaf-1, exposing its CARD domains and allowing the recruitment of pro-caspase-9 to the complex (Li et al., 1997; Liu et al., 1996). The multimeric structure is known as the apoptosome. Recruitment of pro-caspase-9 promotes its autocatalysis and dimerization of caspase-9 molecules for the full activation of the enzyme. Once activated, Caspase-9 cleaves and activates the executioner caspases, Caspase -3 and Caspase-7 and results in apoptosis (Li et al., 1997; Liu et al., 1996; Slee et al., 1999; Zou et al., 1997). While the two pathways are initiated independently there is crosstalk between the two. In cells referred to as type II cells, Caspase-8 can promote apoptosis through the cleavage of the pro-apoptotic BH3-only family member BID, which once cleaved readily translocates to the mitochondria and promotes Bax/Bak oligomerization followed by apoptosis (Desagher et al., 1999).

Caspase-8 and Caspase-9 are referred to as initiator caspases and their activation initiates the proteolytic cascade of apoptosis.

The initiator caspases are activated by oligomeric complexes which promote proximity induced autoactivation through autocatalysis. Caspase-3 and Caspase-7 are known as executioner caspases and they are common to both extrinsic and intrinsic apoptosis. The executioner caspases require initiator caspases for their cleavage and activation. The executioner caspases are associated with the cleavage of over 600 known cellular proteins, however, only a select few contributing to the characteristic morphology of apoptosis (Luthi and Martin, 2007; Taylor et al., 2008). For example, the executioner caspases cleave the inhibitor of Caspase-actived DNase (ICAD). Cleavage of this inhibitor then allows for the Caspase-activated DNase (CAD) to cleave nuclear DNA during apoptosis. Cleavage of cytosolic constituents such as actin, tubulin gelsolin and myosin promote cell rounding and shrinkage. Caspase-3 cleaves the Rho Activated Kinase-1 (ROCK1) to activate this kinase during apoptosis and allows for the formation apoptotic bodies through the enhancement of membrane blebbing (Coleman et al., 2001). While many of the caspase cleavage events which contribute to the morphology of apoptosis have been well characterized, the caspase cleavage event which externalizes phosphatidylserine has remained elusive.

1.3 Phosphatidylserine asymmetry and TMEM16F

Phosphatidylserine is a phospholipid which is exclusively located on the cytoplasmic leaflet of the plasma membrane and contributes to the asymmetry of the plasma membrane. Externalization of phosphatidylserine to the outer leaflet during apoptosis is a key event and it is required for early recognition and removal of apoptotic cells by macrophages (Martin et al., 1995; Schlegel et al., 1996). Phosphatidylserine externalization can be inhibited during apoptosis with the use of the pan-caspase inhibitor zVAD-fmk, strongly suggesting that an activating caspase cleavage event is required for the loss of membrane asymmetry during apoptosis (Martin et al., 1996). A recent study by Suzuki and colleagues (Suzuki et al., 2010) demonstrated that the calcium activated chloride ion channel, TMEM16F, was required for phosphatidylserine exposure in response to cytosolic calcium fluctuations. Phosphatidylserine externalization in platelets following calcium fluctuations is an important activation step for initiation of the clotting cascade (Bevers et al., 1982). Furthermore, they characterized a gain of function mutant of the protein which when overexpressed leads to constitutive exposure of phosphatidylserine.

The mutation was a substitution leading to the conversion of an aspartic acid residue to a glycine. As caspases cleave after aspartic acid residues, the removal of the negative charge from the aspartic acid residue could mimic an activating Caspase cleavage event. The mutation was also located at a Caspase consensus cleavage motif according to predictive software. While TMEM16F is characterized as a calcium-activated chloride channel, it appears also to function in the externalization of phosphatidylserine following calcium activation. Furthermore, it remains possible that TMEM16F could potentially have its activity increased by caspase cleavage and may be the protein responsible for the vital step of phosphatidylserine externalization during apoptosis.

1.4 Regulation of the intrinsic pathway of Apoptosis

As stated previously, the activation of the intrinsic pathway to apoptosis occurs following activation and oligomerization of Bak/Bax, their insertion into the mitochondrial membrane and the release of Cytochrome c (Li et al., 1997; Liu et al., 1996). The oligomerization of Bax/Bak and subsequent mitochondrial outer membrane permeabilization (MOMP) is tightly regulated by the stoichiometric balance of two subsets of proteins in the Bcl-2 families: the anti-apoptotic Bcl-2 proteins and the pro-apoptotic BH3-only proteins (Willis et al., 2007; Yang et al., 1995). These proteins exert their regulatory effects through interaction and neutralization of each other and of Bax or Bak (**Figure 1.2**). In healthy cells, the proteins exist in a stoichiometric balance where the anti-apoptotic Bcl-2 proteins can bind and neutralize the BH3-only proteins, Bax and Bak. Cellular stresses lead to an upregulation or phosphorylation dependent-activation of the pro-apoptotic BH3-only proteins or alternatively a decrease in the anti-apoptotic Bcl-2 proteins. This change in stoichiometry allows the BH3-only proteins to overwhelm the anti-apoptotic Bcl-2 family proteins and promotes Bax/Bak oligomerization and insertion into the mitochondria. Loss of regulation in the intrinsic pathway can be observed in cancer where genetic changes enhance anti-apoptotic protein activity allowing for the cell to survive and grow under stress (Willis and Adams, 2005).

1.5 BH3-only proteins

In contrast to the pro-survival Bcl-2 proteins and Bax and Bak, which have several BH domains, the BH3-only proteins derive their name from their structure as they contain a single conserved BH3 domain; however, the BH3-only proteins are highly divergent allowing for varying functions to regulate apoptosis in response to a variety of cell stresses. BH3-only proteins have two major mechanisms to promote apoptosis. Firstly, they can bind to and inhibit the anti-apoptotic Bcl-2 family proteins that normally sequester Bax and Bak, thereby promoting Bax/Bak insertion into the outer mitochondrial membrane and subsequent release of Cytochrome c. Secondly, they can directly bind Bax or Bak and promote their direct insertion into the membrane. Bim, tBid and PUMA have been shown to interact directly with Bax or Bak, changing their structural reorganization to facilitate apoptosis while the other BH3-only family members (Bad, Bik, Hrk and NOXA) function to neutralize the pro-survival Bcl-2 proteins (Chen et al., 2005b; Kim et al., 2009).

The BH3-only proteins are tightly regulated due to their importance in activating apoptosis and this regulation occurs at both the transcriptional and post-translational level. In unstressed cells the levels of BH3-only proteins are insufficient to overwhelm the anti-apoptotic Bcl-2 proteins. Following specific cellular stresses, the BH3-only proteins NOXA, PUMA, Bim and Bik can be upregulated by several transcription factors, Bad can be liberated from the 14-3-3 proteins or Bid can be activated by caspases, all of which can promote apoptosis. NOXA and PUMA can be upregulated in response to P53 following DNA damage (Jeffers et al., 2003; Oda et al., 2000; Sheridan et al., 2010; Villunger et al., 2003). Bim and Bad are regulated post-translationally through phosphorylation of key residues. The phosphorylation of Bad decreases its pro-apoptotic activity as 14-3-3 proteins bind and neutralize phosphorylated Bad; however, dephosphorylation by phosphatases such as calcineurin, promotes apoptosis through diminishing the interaction of Bad with 14-3-3 proteins allowing for its liberation and pro-apoptotic activity (Wang et al., 1999; Zha et al., 1996). The phosphorylation of Bim by Erk1/2 enhances its degradation and promotes survival, while, phosphorylation of Bim by JNK promotes its interaction with Bax, potentiating apoptosis (Lei and Davis, 2003; Luciano et al., 2003; Putcha et al., 2003).

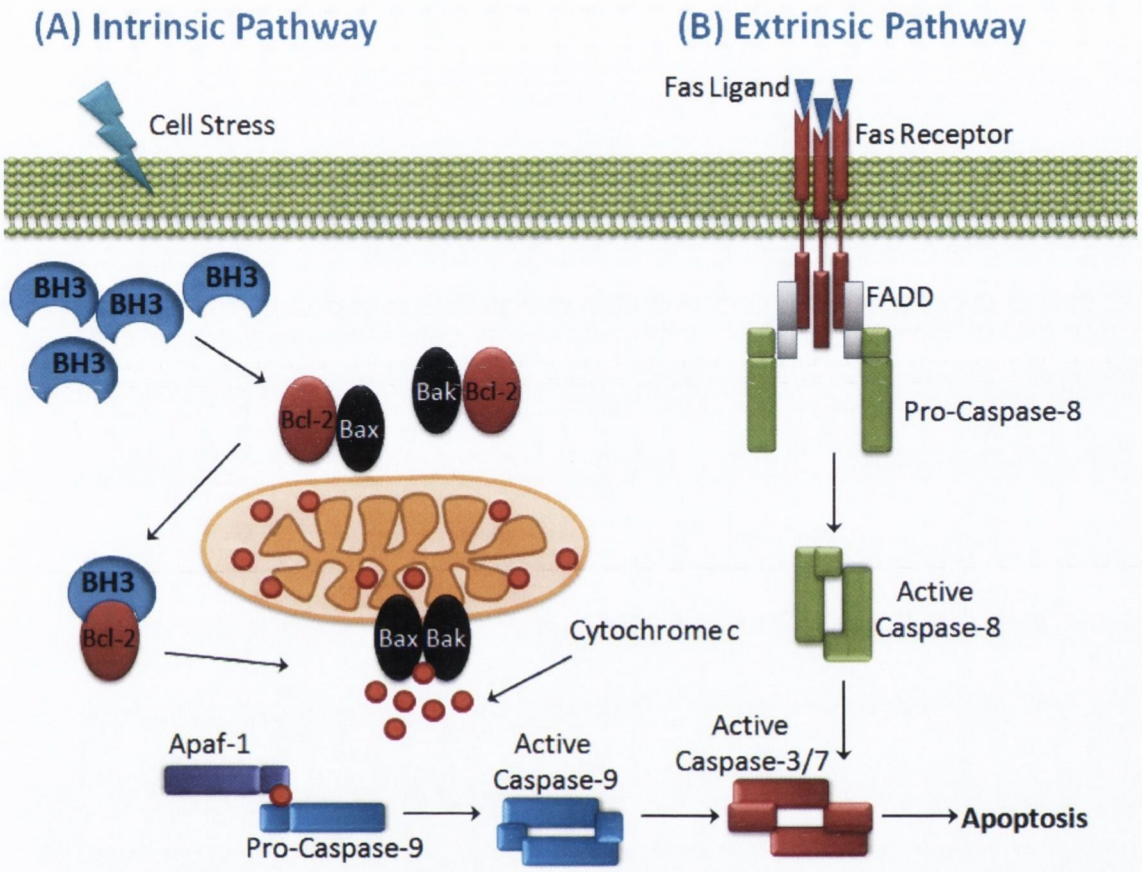

Figure 1.1 Intrinsic and extrinsic pathways of Apoptosis

(A) Cellular stresses can lead to the upregulation of BH3-only family members. This increase overwhelms the Bcl-2 family preventing their inhibitory function and allowing for Bax/Bak oligomerization. This results in Cytochrome c release and caspase-9 activation followed by activation of the executioner caspases. (B) Fas ligand binds to the fas receptor leading to the formation of the DISC. Caspase-8 is activated following DISC formation which then cleaves the executioner caspases to induce apoptosis via the extrinsic pathway.

1.6 Anti-apoptotic Bcl-2 proteins

The first protein discovered to inhibit apoptosis was found in a B-Cell Lymphoma and was named Bcl-2. The translocation of the immunoglobulin promoter onto the Bcl-2 gene leads to its enhanced expression and inhibition of cell death (Tsujimoto et al., 1985). This demonstrated the importance of cell death in cellular homeostasis and the role of the anti-apoptotic Bcl-2 proteins in cancer progression.

The primary role of the anti-apoptotic Bcl-2 proteins is to prevent Bax/Bak oligomerization, insertion into the mitochondria and subsequent Cytochrome c release. There are six family members that execute this function: Bcl-2, Bcl-X, Mcl-1, Bcl-B, Bcl-W and A1, however, their ability to interact with pro-apoptotic BH3-only family members, their mode of regulation and their tissue distribution vary greatly (Sorenson, 2004). The anti-apoptotic Bcl-2 family's mode of action is dependent on their abundance within a specific cell. While they bind and neutralize the pro-apoptotic BH3-only family members to prevent Bax/Bak oligomerization, upon an apoptotic stimulus cells upregulate the activity of the BH3-only proteins to overwhelm the pro-survival proteins and initiate apoptosis (Willis et al., 2007). Post-translational modification of the anti-apoptotic Bcl-2 family also plays a role in the regulation of apoptosis, cleavage or phosphorylation of the proteins can alter the threshold required for the initiation of this mode of cell death (Haldar et al., 1995; Kirsch et al., 1999).

The importance of the anti-apoptotic Bcl-2 family in cell death can be further demonstrated by the phenotypes observed in mice deleted of one or more of these genes. Removal of the *mcl-1* gene is embryonically lethal as the embryos do not implant in utero, while removal of the *bcl-x* gene is embryonically lethal due to massive hematopoietic and neuronal cell death (Motoyama et al., 1995; Rinkenberger et al., 2000). Mice lacking the *bcl-2* gene survive until birth, however, they display fulminant lymphoid apoptosis, polycystic kidneys, hypopigmented hair and 50% die within 1-6 weeks after birth (Veis et al., 1993).

While the importance of the Bcl-2 family in regulating apoptosis has been extensively researched, recent studies place the Bcl-2 family as regulators cellular health through their regulation of key processes such as autophagy, mitochondrial dynamics and mitochondrial function (Berman et al., 2009; Karbowski et al., 2006; Pattingre et al., 2005; Sheridan et al., 2008; Wang et al., 2013b).

1.7 Mcl-1

Mcl-1 is a member of the anti-apoptotic Bcl-2 family and, in contrast to the stable expression of the other family members, such as, Bcl-2 or Bcl-X, Mcl-1 is rapidly turned over. Mcl-1 expression can be increased in response to mitogen activated protein kinase (MAPK) or phosphatidylinositol-3 (PI3-K) signalling to promote survival. Mcl-1 is regulated by the ERK pathway, through both transcriptional upregulation and phosphorylation which increases protein stability (Domina et al., 2004; Townsend et al., 1999). Mcl-1 also contains a PEST sequence, a region of the protein which is rich in proline (P), glutamic acid (E), serine (S) and threonine (T). This sequence in Mcl-1 targets the protein for constitutive ubiquitination and proteosomal degradation (Rogers et al., 1986). Further work on Mcl-1 also found that it was also a substrate for the E3 ligase, Mule. The importance of Mcl-1 regulation in apoptosis is demonstrated by the knockdown of Mule to diminish Mcl-1 ubiquitination. Following Mule knockdown, cells are much more resistant to apoptotic stimuli due to an accumulation of Mcl-1 (Zhong et al., 2005). Three more E3 ligases responsible for Mcl-1 ubiquitination have also been described: $SCF^{\beta\text{-}TrCP}$, SCF^{Fbw7} and APC/C^{Cdc20} (Ding et al., 2007; Harley et al., 2010; Inuzuka et al., 2011). Furthermore, Mcl-1 is also cleaved by caspases at a site within its PEST sequence to enhance the degradation of Mcl-1 and allow apoptosis to proceed (Herrant et al., 2004). There are also reports which suggest that this clevage event not only inhibits the anti-apoptotic function but also converts it to an active pro-apoptotic molecule (Michels et al., 2004). It is clear that Mcl-1 plays a major role in the survival of many cell types due to the extensive processes available to degrade it once a cell commits to apoptosis. Due to the potent anti-apoptotic ability of Mcl-1, the cell must protect itself against excessive Mcl-1 degradation. Under normal conditions, Mcl-1 is primarily degraded in a proteosome-dependent manner. Ubiquitination is a reversible event and the deubiquitinase (DUB) ubiquitin specific peptidase 9 X (USP9X) was identified as a Mcl-1 DUB (Schwickart et al., 2010). The ability of USP9X to specifically remove polyubiquitin chains from Mcl-1 allows for maintenance of a steady state level of the protein to allow for its anti-apoptotic activity. Indeed, ablation of USP9X increased the half-life of Mcl-1 and provided protection against a number of apoptotic stimuli (Schwickart et al., 2010). Schwickart and colleagues also demonstrated

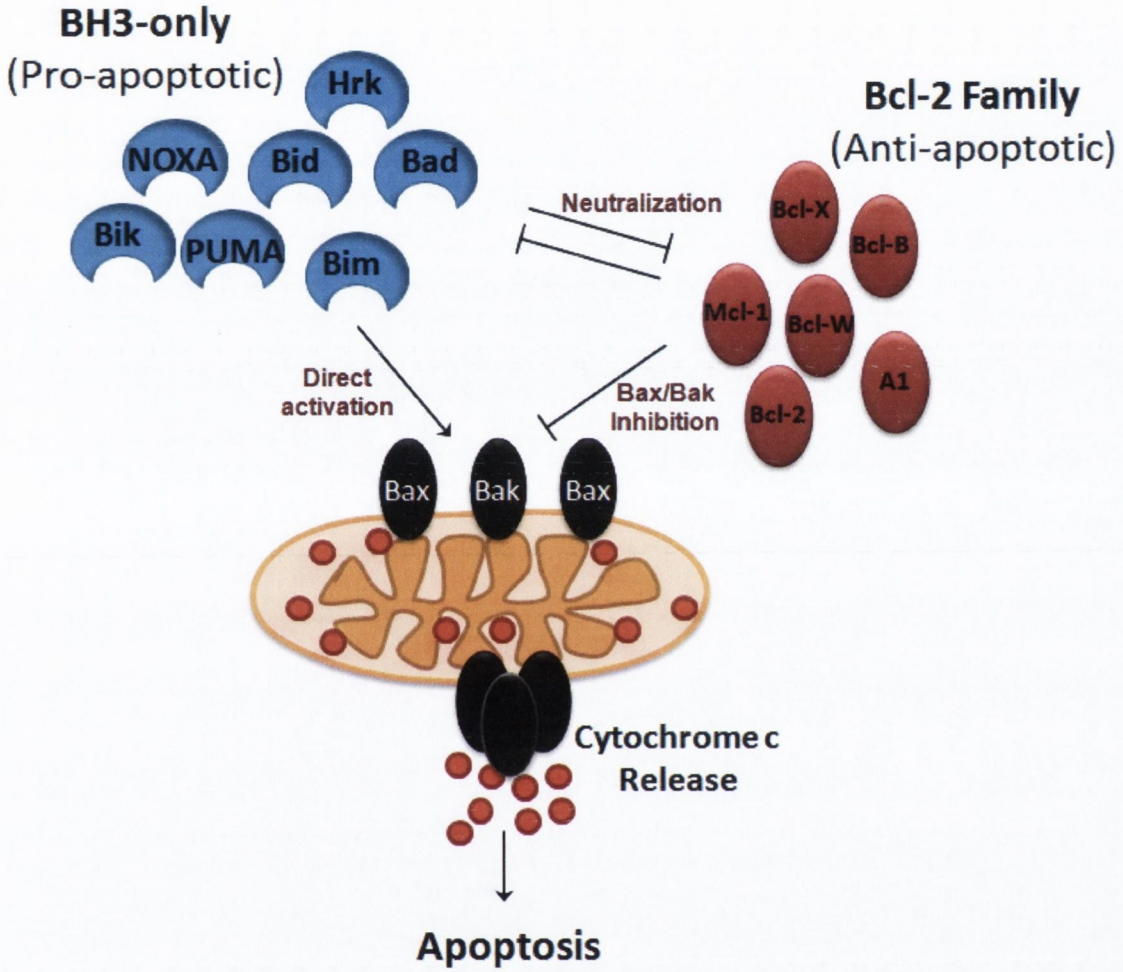

Figure 1.2 Regulation of Cytochrome c release

The anti-apoptotic Bcl-2 family interact with Bax and Bak to prevent their oligomerization and subsequent Cytochrome c release. The pro-apooptotic BH3-only proteins inhibit this function of the Bcl-2 family, however, they exist in a tight balance whereby there is no non-specific activation of Bax or Bak. In order for cytochrome c to be release from mitochondria, modulation of the abundance or activity of either the anti-apoptotic Bcl-2 family or the pro-apoptotic BH3-only family needs to occur.

that Mcl-1 phosphomimetics show reduced interaction with USP9X which may provide a mechanism as to how phosphorylation of Mcl-1 can contribute to its degradation.

While removal of the *mcl-1* gene in mice is embryonically lethal, conditional knockout of *mcl-1* in various tissues has also highlighted its importance as an anti-apoptotic protein as it was demonstrated that Mcl-1 is vital for the survival of several different cell types. Lymphocytes, hematapoietic stem cells, neurons, cardiomyoctes and hepatocytes spontaneously die following the conditional knockout of the Mcl-1 gene in specific tissues (Arbour et al., 2008; Opferman et al., 2005; Opferman et al., 2003; Vick et al., 2009; Wang et al., 2013b). The ability of a cell to change the levels of Mcl-1 within a short space of time (within minutes) can be used to influence both pro-survival and pro-apoptotic signals when needed.

1.8 Non-apoptotic roles of the Bcl-2 family

Historically, the Bcl-2 family of proteins have been associated with the protection of cells against apoptosis. However, recent studies have highlighted the role of the Bcl-2 family in other non-apoptotic functions within the cell. It has been reported that Bcl-2 and Bcl-X_L regulate autophagy through an interaction with Beclin-1, a protein vital for autophagy (Elgendy et al., 2011; Pattingre et al., 2005). Bax and Bak have also been implicated in regulating the dynamic fission and fusion which occurs between mitochondria to maintain their health (Autret and Martin, 2009; Karbowski et al., 2006; Sheridan et al., 2008). In addition a truncated form of Mcl-1 has been reported to be imported into mitochondria where it modulates the respiratory output of the mitochondria (Perciavalle et al., 2012). Autophagy, mitochondrial morphogenesis and mitochondrial respiration are all vital for the maintenance of the health of individual cells. The fact that the Bcl-2 family play a role in all of the above cellular functions suggests that the Bcl-2 family play a more global role in monitoring and maintaining the health of a cell rather than just having a role in determining the fate of a cell in response to a stress (Autret and Martin, 2009).

1.9 The Mitochondrion

Mitochondria are double membrane dynamic organelles which are vital to maintaining the health of a cell. Mitochondria play a central role in initiation of apoptosis, however, in

healthy cells they are the primary source of ATP generation. While over ninety percent of the proteins located within mitochondria are encoded by the nuclear genome, mitochondria contain their own genomes that encode for several proteins of the electron transport chain. The electron transport chain is located on large invaginations on the inner mitochondrial membrane known as cristae. The electron transport chain functions by transferring electrons in a stepwise manner from complex I (NADH dehydrogenase) to complex II (succinate dehydrogenase) to complex III (cytochrome bc_1 complex) and finally to complex IV (cytochome c- oxidoreductase). The transfer of electrons from complex III to complex IV is mediated by cytochrome c and when this protein moves to the cytosol it functions in the formation of the apoptosome and initiation of apoptosis. The transport of electrons along the electron transport chain generates protons which are pumped into the intermembrane space of the mitochondria, creating a proton gradient referred to as the mitochondrial membrane potential (Nicholls, 2008). This proton gradient is used by the ATP synthase to generate molecules of ATP through oxidative phosphorylation (Elston et al., 1998; Wang and Oster, 1998).

In addition to producing energy for the cell, mitochondria also play a role in maintaining cellular homeostasis through calcium and reactive oxygen species (ROS) signalling. Mitochondria also control fluctuations in cytosolic calcium which contributes to calcium dependent enzymes and scaffold proteins (Rizzuto et al., 2012). Alternatively, mitochondria can be induced to produce reactive oxygen species in response to immune activation, under hypoxia or starvation or in response to oncogenic transformation (Matsuzawa et al., 2005; Scherz-Shouval et al., 2007; Weinberg et al., 2010; West et al., 2011). While the production of reactive oxygen species can promote NFκB signalling, HIF1-α signalling or calcium signalling, excessive reactive oxygen production can be very damaging for a cell (Hamanaka and Chandel, 2010).

The mitochondrial genome encodes for proteins which are essential for the electron transport chain and correct functioning of mitochondria. The mitochondrial genome is particularly sensitive to mutations due to its proximity to reactive oxygen species generated through mitochondrial respiration.

Mitochondria have multiple mechanisms to protect against deleterious mutations within their genome. These organelles contain a number of proteins which can repair mutations directly (Sickmann et al., 2003), they can maintain mitochondrial heterogeneity through

mitochondrial fission and fusion events (Chen et al., 2005a) or deleterious mitochondria can be completely removed by mitophagy before they negatively impact the remainder of the network (Kurihara et al., 2012). The failure of these protective mechanisms has been associated with mitochondrial myopathies and also neurodegenerative diseases such as Parkinson's disease demonstrating the importance of mitochondria in the health of a cell (Holt et al., 1988; Schapira et al., 1990).

1.10 Autophagy

Normal cell function requires a balance between protein synthesis and protein degradation. A major degradation pathway involved in the recycling of proteins and organelles is macroautophagy (hereafter referred to as autophagy). Autophagy, which literally means 'self eating', is conserved in eukaryotes from yeast to mammals suggesting an important role in the maintenance of cellular health (Meijer et al., 2007). Autophagy is constitutively active at basal levels in all cells and contributes to the turnover of proteins and organelles to maintain cellular health (Degenhardt et al., 2006; Hara et al., 2006; Singh et al., 2009). Autophagy involves the dynamic rearrangement of membranes, forming an organelle known as the autophagolysosome, to sequester and target cytosolic constituents for lysosomal degradation and recycling (Dunn, 1990; Klionsky and Emr, 2000; Yorimitsu and Klionsky, 2005).

Autophagy is actively inhibited by mammalian target of rapamycin (mTOR), a serine/threonine kinase which constitutively phosporylates and inhibits UKL1 from initiating autophagy. When a cell becomes stressed, through starvation or infection, mTOR activity is down-regulated through the increase in activity of the GTPase Rheb. Once mTOR activity is diminished, it can no longer phosphorylate and inhibit ULK1 (Kim et al., 2011). Consequently, this leads to autoactivation of ULK1 and the formation of the initiation complex, the first step in autophagy activation. Once activated ULK1 phosphorylates Beclin-1, a post translational modification occurs resulting in the recruitment of Atg14L and VPS34. This complex is known as the initiation complex and is required for the nucleation of the membrane to initiate the formation of an autophagosome (Di Bartolomeo et al., 2010). For autophagy to proceed there must be a system for generating membranes to form the autophagosome required for protein and organelle sequestration. Two ubiquitin-like conjugation systems, involving the Atg

proteins, promote the assembly of the Phagophore that is required for the sequestration. Firstly Atg7 forms a thioester bond with Atg12 between the C-terminal glycine of Atg12 and the active cysteine of Atg7 at the expense of an ATP molecule (Mizushima et al., 1998a; Mizushima et al., 1998b). The primed Atg12 molecule is now transferred to Atg12 through a thioesterification reaction. Finally, Atg12 is bound to Atg5 through an isopeptide bond between the c-terminal glycine of Atg12 and the active lysine of Atg5. The Atg5-12 complex bind to Atg16L forming a multmeric complex that is required for the addition of phosphatidylethanolamine to LC3-I (Ichimura et al., 2000; Mizushima et al., 1998a). Before the Atg 5-12-16L complex can conjugate phosphatidylethanolamine to LC3-I, LC3 must be primed for conjugation. Firstly, Atg4 cleaves the c-terminal Arginine on LC3-I to reveal a glycine residue. Atg7 can then form a thioester bond with LC3-I before transfer of LC3-I to Atg3 through a transthioester bond. The Atg3-LC3-I complex interacts with the Atg 5-12-16L complex to remove LC-3-I from Atg3 and attach phosphatidylethanolamine to LC3-I through an isopeptide linkage (Geng and Klionsky, 2008). Lipidated LC3 is recruited to the Beclin-1 initiation complex where it elongates the autophagosomal membrane during the formation of an autophagosome (Mizushima et al., 1998a).

Once the autophagosome is completely sealed, it is transported for fusion with a lysosome with the resulting vacuole named the autophagolysosome. This fusion event results in the lytic contents of the lysosome and the contents of the autophagosome being combined allowing for the degradation and recycling of amino acids and lipids. Autophagy is regulated by the Bcl-2 family proteins through a direct interaction with and inhibition of Beclin-1 (Maiuri et al., 2007a; Pattingre et al., 2005; Wei et al., 2008). This regulation by the Bcl-2 family proteins places them within a cellular process that has a direct effect on overall cellular homeostasis (Pattingre et al., 2005).

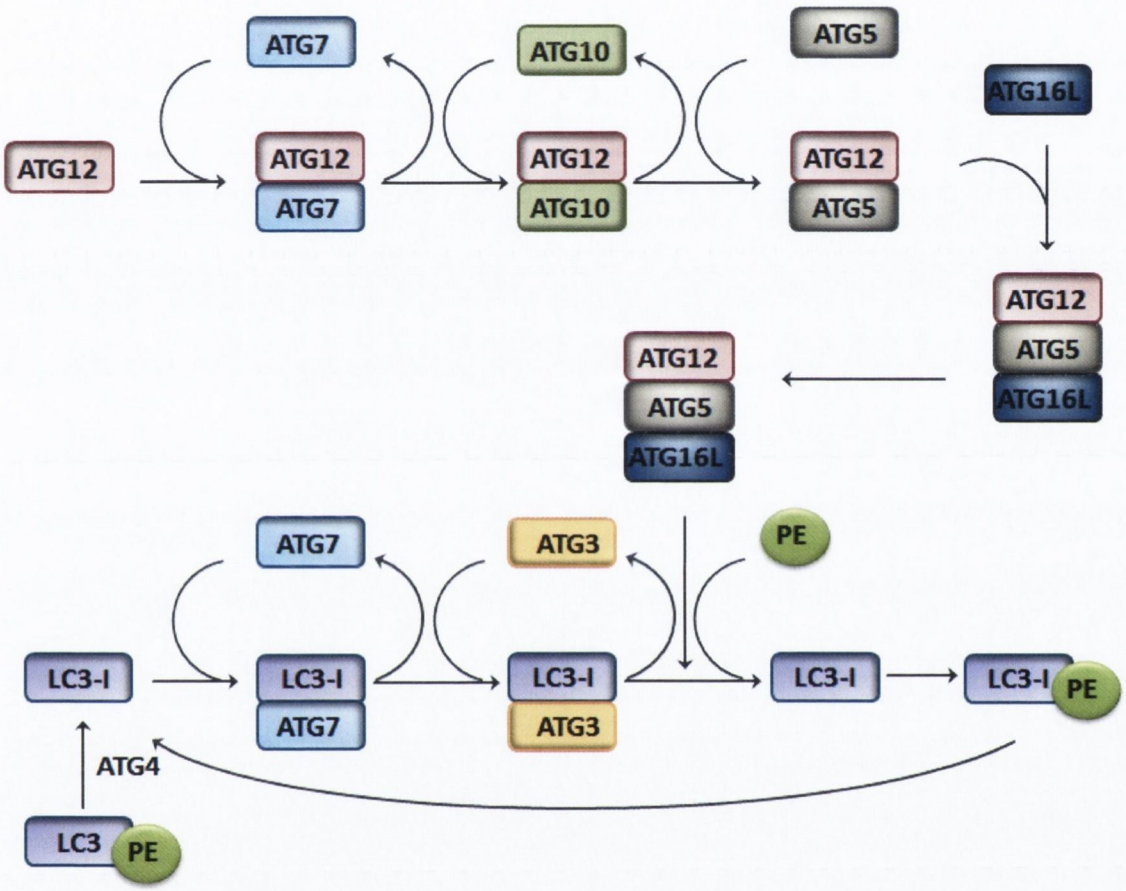

Figure 1.3 Atg conjugation pathways

The c-terminal glycine of Atg12 and the active cysteine of Atg7 form a thioester bond to initiate the pathway. Atg12 is then transferred to Atg10 which allows the interaction with Atg5 and isopeptide bond formation between the c-terminal glycine of Atg12 and the active lysine in Atg5. The Atg5-12 binds to Atg16L forming an active complex responsible for the conjugation of phosphatidylethanolamine to LC3-I.

1.11 Mitophagy

Mitochondria are dynamic organelles, which evolved from primitive bacteria, and are the major energy generators for most cellular functions; however, damaged mitochondria are a source of reactive oxygen species which can have destructive effects on cellular health (Cabiscol et al., 2000; Kujoth et al., 2005). In order to prevent excessive damage and possibly the death of the cell, a cell can undergo a mitophagy to remove damaged/depolarized mitochondria. Mitophagy is the removal of damaged mitochondria through autophagy (Dunn, 1990; Matsuda et al., 2010; Narendra et al., 2008; Narendra et al., 2010b). In healthy mitochondria, the electron transport chain pumps H^+ ions into the intermembrane space of the mitochondria which can be used to produce ATP. The high concentration of H^+ ions in the intermembrane space is referred to as mitochondrial polarization or mitochondrial potential and loss of this potential, through any means, signals the mitochondrial as damaged and it will be removed by mitophagy.

Following mitochondrial damage, PINK1 accumulates on the outer mitochondrial membrane of damaged mitochondria due to a failure of mitochondrial import. PINK1 then phosphorylates both Parkin and ubiquitin allowing for full activation of Parkin (**Figure 1.3**). Once active, Parkin ubiquitinates many proteins on the outer mitochondrial membrane and decorates damaged mitochondria with ubiquitin. The adaptor proteins, P62 and NBR-1, interact with both ubiquitinated proteins on the mitochondria as well as lipidated LC3 (Geisler et al., 2010). The presence of LC3 concentrated at the surface of the mitochondria promotes the formation of a phagophore engulfing damaged mitochondria within an autophagosome (**Figure 1.4**). Following engulfment, the autophagosome containing the mitochondria fuse with a lysosome and lysosomal proteases degrade and recycle the amino acids and lipids of damaged mitochondria (Youle and Narendra, 2011).

Dysfunction of mitophagy and the failure to remove damaged mitochondria has been strongly associated with neurodegenerative disorders with many of the proteins involved being elucidated from genetics studies of recessive familial Parkinson's disease (Bentivoglio et al., 2001; Kitada et al., 1998).

The Bcl-2 family have been shown to be key regulators of mitochondrial dynamics, mitochondrial fission/fusion, mitochondrial biogenesis and autophagy in maintaining the health of a cell, therefore, Bcl-2 proteins may also play a key regulatory role in the removal

of damaged mitochondria (Berman et al., 2009; Karbowski et al., 2006; Pattingre et al., 2005; Sheridan et al., 2008; Wang et al., 2013b).

1.12 PINK1/Parkin independent Mitophagy

While PINK1/Parkin mediated mitophagy is by far the most studied, there are alternative ways to induce mitophagy. In most mammals, mature erythrocytes lack mitochondria as the production of ROS in the presence of the iron in haemoglobin would be toxic to red blood cells. It has been reported that the protein responsible for the removal of mitochondria in immature erythrocytes before terminal differentiation is the outer mitochondrial membrane protein, Nix (Schweers et al., 2007). Mice lacking a functional Nix gene presented with anaemia and their erythrocytes had mitochondria which were not removed during differentiation (Mortensen et al., ; Schweers et al., 2007). This demonstrates that Nix-mediated mitophagy is absolutely required during development. Interestingly, addition of the mitochondrial depolarizor, CCCP, could induce the removal of mitochondria from Nix deficient erythrocytes. However, it remains possible that CCCP could just activate PINK1/Parkin-mediated mitophagy to circumvent Nix-dependent mitophagy but it does demonstrate the importance of Nix-dependent mitophagy in the health of an organism (Sandoval et al., 2008).

Mitophagy can also be induced, independently of PINK1/Parkin, in cells under hypoxic conditions as a pro-survival mechanism. BNIP3, a homologue of Nix, is a Hif-1α-dependent gene whose expression is induced under hypoxic conditions (Zhang et al., 2008). BNIP3 can then directly interact with LC3B promoting the engulfment of mitochondria into phagosomes. A second mechanism for mitophagy under hypoxic conditions has also been characterized involving the outer mitochondrial membrane protein FUNDC1. FUNDC1 is normally phosphorylated by src kinase and this phosphorylation maintains the protein in an inactive conformation. Under hypoxic stress, src kinase becomes dephosphorylated and loses its ability to phosphorylate and therefore fails to inhibit FUNDC1. FUNDC1 is an LC3 interacting protein which can recruit LC3 directly to the mitochondria and target the organelle for phagosomal engulfment (Liu et al., 2012). It is likely that both BNIP3 and FUNDC-1 function in parallel to ensure the removal of mitochondria during hypoxia. It is also interesting to consider what may occur

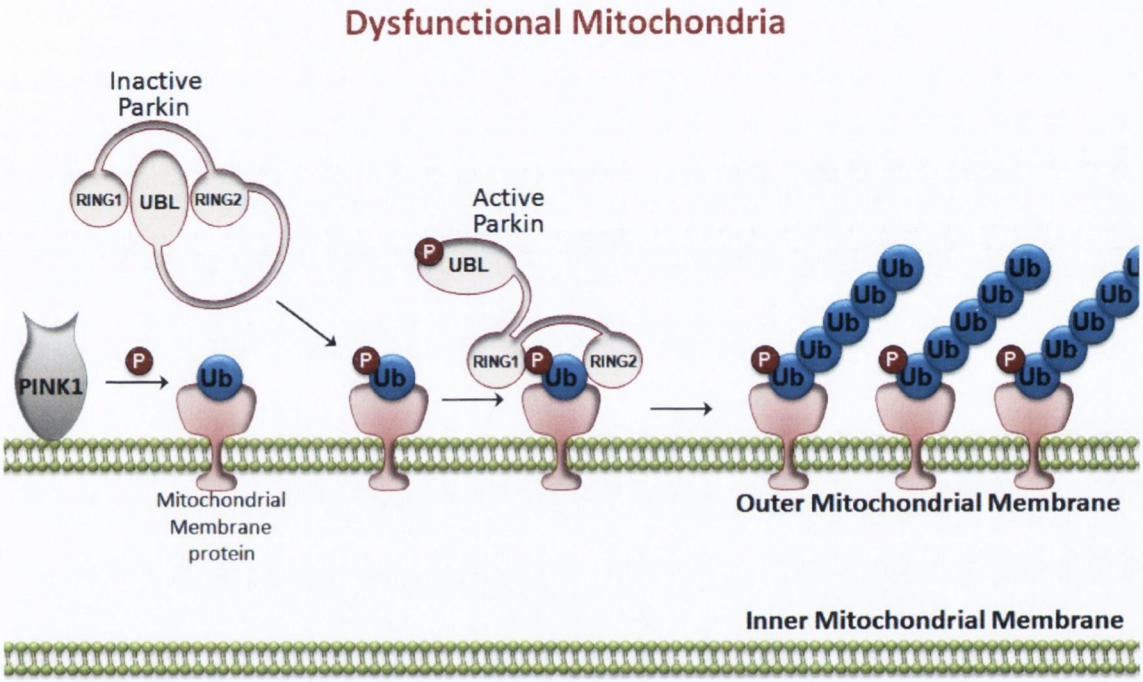

Figure 1.4 PINK1 stabilization and Parkin activation

In healthy mitochondria, PINK1 is translated in the cytosol before being trafficked to mitochondria where it is imported by the TOM complex and degraded by the mitochondrial protease, PARL. Following mitochondrial damage or depolarization, the TOM complex fails to import PINK1 leading to its accumulation on the outer mitochondrial membrane. PINK1 then phosphorylates ubiquitin attached to mitochondrial outer membrane proteins. Phosphorylated ubiquitin then acts to recruit inactive Parkin to damaged mitochondria allowing for full activation of the latter. Following activation, Parkin polyubiquitinates outer mitochondrial membrane proteins and targets dysfunctional mitochondria for degradation via mitophagy.

under pseudohypoxia which is observed during an immune response, considering the importance of the mitochondria in controlling the immune response.

1.13 PINK1 regulation

PINK1 is a serine threonine kinase and is ubiquitously expressed in all tissues (Berthier et al., 2011). It was first described in a screen where PTEN-null cancer cells had exogenous PTEN re-introduced and gene analysis identified PINK1 as a PTEN regulated gene (Unoki and Nakamura, 2001). PINK1 was subsequently identified as a gene associated with autosomal recessive Parkinson's disease (Valente et al., 2004). PINK1 is a nuclear transcribed protein which contains a mitochondrial targeting motif for trafficking to mitochondria. PINK1 mRNA levels are high within cells, however, overall proteins levels are quite low. In cells with unperturbed mitochondria, PINK1 is trafficked to mitochondria where it is imported into the inner mitochondrial membrane by the TOM20 complex (Lazarou et al., 2012). PINK1 is then degraded by the protease PARL in the inter membrane space of mitochondria, reducing the protein levels of PINK1 and consequently its kinase activity (Jin et al., 2010). In the case of damaged or depolarized mitochondria, PINK1 is translated in the cytosol and trafficked to mitochondria as normal, however, the TOM complex becomes dysfunctional and fails to import PINK1 into the inner mitochondrial membrane. As a result, PINK1 accumulates on the outer membrane of damaged mitochondria and this allows for an increase in its kinase activity specifically on damaged mitochondria (Matsuda et al., 2010; Narendra et al., 2010b; Vives-Bauza et al., 2010).

PINK1 then activates Parkin in a two step process. PINK1 must phosphorylate both Parkin and ubiquitin to promote full activation of the E3 ligase activity of Parkin and interestingly it phosphorylates both proteins at a conserved serine at position 65. The importance of both phosphorylation events was clearly demonstrated as phosphomimetic Parkin still required PINK1 activity for full activation (Kane et al., 2014; Koyano et al., 2014). Furthermore, overexpression of both phosphomimetic Parkin and phosphomimetic Ubiquitin did bypass the requirement of PINK1 in Parkin activation (Koyano et al., 2014). As Parkin also requires autoubiquitination for full activation, phosphorylated ubiquitin may have a higher affinity for Parkin that promotes its autoubiquitination and activation. The source of ubiquitin for phosphorylation is likely to be mitochondrial outer membrane

proteins that are ubiquitinated at steady state levels in cells as this is where PINK1 is located. PINK1-mediated activation of Parkin initiates the autophagic cascade at the site of damaged mitochondria and ultimately leads to their degradation and removal (Matsuda et al., 2010; Narendra et al., 2008; Narendra et al., 2010b; Vives-Bauza et al., 2010). The importance of PINK1 is underpinned by its association with Parkinson's disease. Mutations within the PARK6 gene (PINK1) result in a more severe form of Parkinson's disease which develops at a much earlier stage than idiopathic Parkinson's disease (Bentivoglio et al., 2001). Furthermore, while mice lacking PINK1 do not display a phenotype, rats which have had PINK1 removed do develop Parkinson's-like symptoms further underpinning the importance of PINK1 in this neurodegenerative disease (Baptista et al., 2013). Understanding why knockout mice do not develop Parkinson-like symptoms while knockout rats do could be key in understanding the causes of Parkinson's disease.

1.14 Mitophagy-independent roles of PINK1

Parkin activation by PINK1 phosphorylation has been extensively studied; however, several other PINK1 substrates have also been characterized. PINK1 promotes mitophagy not only through phosphorylation and activation of Parkin but also through phosphorylation of Ubiquitin and of the mitochondrial motility protein, Miro (Kane et al., 2014; Koyano et al., 2014; Wang et al., 2011). Phosphorylation of Ubiquitin is essential for Parkin auto-ubiquitination and activation of its E3 ligase ability while phosphorylation of Miro inhibits mitochondria motility and maintains the damaged mitochondria in a stationary state for mitophagy to occur. These phosphorylation events co-ordinate mitophagy for the efficient removal of damaged mitochondria.

PINK1 also phosphorylates substrates which have no role in mitophagy. PINK1 was shown to phosphorylate Bcl-X_L and in doing so impairs its pro-apoptotic cleavage. Other substrates include the phosphorylation of TNF-receptor associated protein 1 (TRAP1) to protect against oxidative damage (Pridgeon et al., 2007), phosphorylation of rictor to promote cell survival through increased mTORC2 activity (Murata et al., 2010) and finally phosphorylation of HtrA2 to increase its proteolytic activity and limit mitochondrial stress (Plun-Favreau et al., 2007).

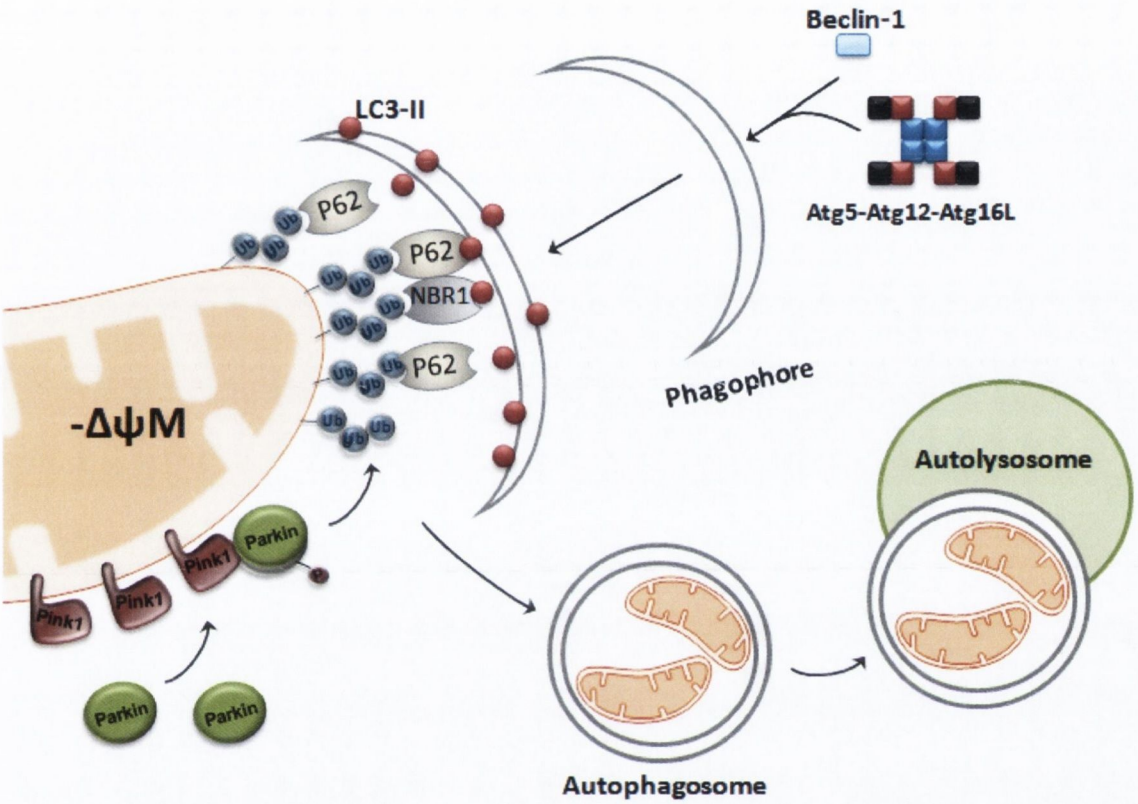

Figure 1.5 Mechanism of Mitophagy

Following mitochondrial damage or depolarization, PINK1 accumulates on the outer mitochondrial membrane where it phosphorylates ubiquitin and also possibly Parkin. Following activation, Parkin decorates damaged mitochondria with ubiquitin and targets these organelles for destruction. The adaptor proteins, P62 and NBR-1, interact with both LC3 and ubiquitin therefore recruiting LC3 to damaged mitochondria. The phagophore is expanded around mitochondria through the Atg conjugation system and damaged mitochondria are engulfed in an autophagosome. This autophagosome then fuses with an Autolysosome forming an autophagolysosome. Lysosomal proteases then degrade mitochondria and recycle the constituents.

1.15 Parkin and its neuroprotective role

Parkin was discovered in 1998 as one of the genes linked to autosomal recessive early onset Parkinson's disease (Kitada et al., 1998). The Parkin gene spans 1.5 Mb containing 12 exons and encodes a 46.5 KDa protein. Parkin is comprised of an amino terminal ubiquitin-like domain (UBL) and a carboxyl-terminal RING Box domain which is two RING domains separated by a RING-between-RING domain. Parkin expression is regulated by the transcription factor N-myc and Parkin expression inversely correlates with N-myc expression and this may explain the tissue expression patterns of Parkin (West et al., 2004).

Parkin exists in an inactive form within the cytosol and requires a phosphorylation at serine 65 in the presence of phosphorylated ubiquitin for its full activation (Kane et al., 2014; Koyano et al., 2014; Shiba-Fukushima et al., 2012). Following full activation, Parkin translocates to damaged mitochondria, through binding to phosphorylated ubiquitin, where it functions as an E3 ligase ubiquitinating outer mitochondrial membrane proteins of damaged mitochondria only and targets these damaged mitochondria for degradation and removal by mitophagy.

Recently, Parkin has also been implicated as a tumor suppressor as loss of function of Parkin in cancer lead to an increase in cyclin D1 and E1 implicating Parkin in a role in cell cycle regulation (Gong et al., 2014). This suggests that Parkin may act as a tumor suppressor, however, whether this function is independent of mitophagy remains to be elucidated.

1.16 Proteosome-Ubiquitin pathway for protein degradation

Protein modification is an important aspect of cell biology for regulating both intrinsic and extrinsic signalling processes. Ubiquitination is the addition of one or multiple ubiquitin molecules to proteins resulting in either proteolytic degradation of the protein or altering the signalling capabilities of proteins within the cell. Ubiquitin is a small (8.5 kDa), well conserved protein expressed in all tissue which can be covalently attached to proteins, targeting them for multiple different cell fates such as degradation via the proteosome, cellular trafficking, modulation of target protein activity or enhancing or decreasing target

protein interactions (Huang et al., 2006; Li et al., 2006; Pankiv et al., 2007; Zhong et al., 2005).

Ubiquitination requires an enzymatic cascade in order to attach ubiquitin molecules to target proteins. Three classes of enzymes are required for the process: an E1 ubiquitin activating enzyme, of which there are only two known in humans, forms a thioester bond with the ubiquitin molecule at the expense of an ATP molecule (Lee and Schindelin, 2008). The ubiquitin molecule is now primed for transfer to the active site of the second enzyme in the cascade known as the E2 ubiquitin-conjugating ezyme via a transthioesterification bond. The E2-ubiquitin complex then interacts with an E3 ligase and this complex provides the substrate specificity for targeting of the ubiquitin molecule.

An E3 ligase interacts with E2-ubiquitin complex and recognizes the target protein resulting in the addition of a ubiquitin molecule to a lysine residue on the target protein via an isopeptide bond with the C-terminal glycine on ubiquitin (Ciechanover et al., 1980; Nandi et al., 2006; Wilkinson et al., 1980). There are several hundred known E3 ligases allowing for highly specific ubiquitination of proteins to regulate a plethora of cellular processes (Pickart, 2001).

Proteins can be modified by monoubiquitination (addition of a single ubiquitin), multi-monoubiquitination (addition of a single ubiquitin to many sites of a protein) or polyubiquitination (addition of several ubiquitin molecules). There are several different forms of polyubiquitination and they have a variety of modifying effects on the target protein. The different types of polyubiquitination can be identified by the lysine residue number in which subsequent ubiquitin molecules are attached to and leads to the formation of specific polyubiquitin chains (Kulathu and Komander, 2012). Chains that are linked at lysine 48 within the ubiquitin molecule act as a trafficking signal to target the protein to the proteosome for destruction. Chains which have been linked at lysine 63 within ubiquitin molecules are associated with the assembly of signalling complexes which can have diverse functions within the cell such as endocytic trafficking and inflammation (Chen et al., 1996; Tanowitz and Von Zastrow, 2002). Other ubiquitin chains can be formed with the lysines at positions 6, 11, 27 and 29, however, functions of these polyubiquitin chains are still in the process of elucidation. Recently, linear ubiquitin chains have been characterized where the N-terminal methionine is conjugated to the C-terminal

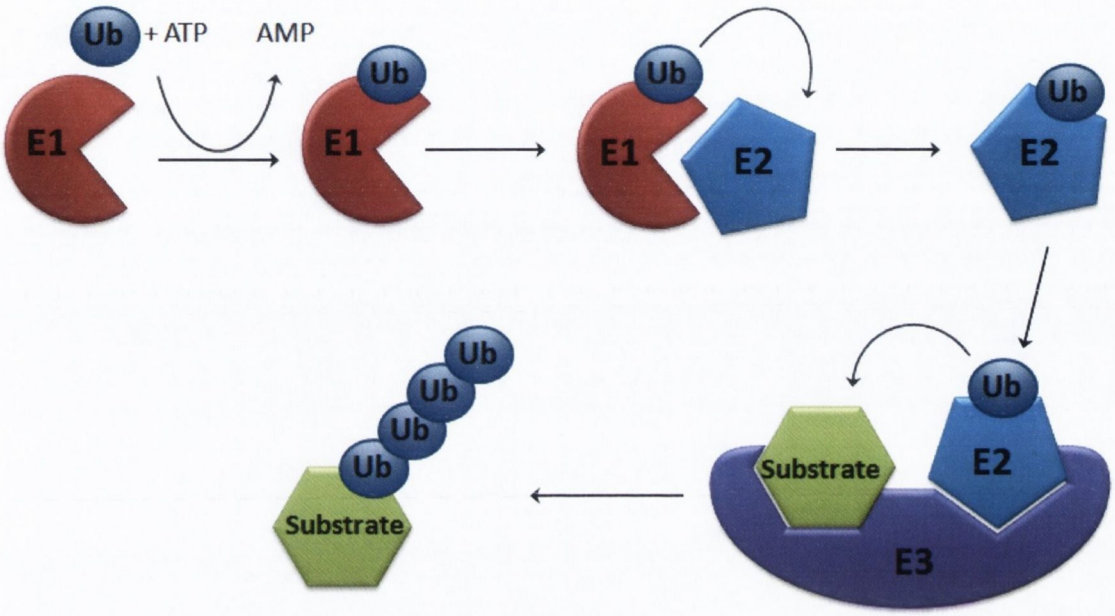

Figure 1.5 Ubiquitination cascade

The E1 ligase forms a thioester bond with the ubiquitin molecule at the expense of an ATP molecule. The E1 ligase primed with ubiquitin then interacts with the E2 ligase and the transfer of ubiquitin to the E2 occurs via a thioesterification bond. An E3 ligase then interacts with both the primed E2 ligase and the substrate for ubiquitination. This complex catalyzes the isopeptide bond formation between a lysine residue on the substrate and the ubiquitin molecule on the E2.

glycine on the previous ubiquitin molecule and this modification appears vital for correct immune signalling (Chen et al., 1996; Keusekotten et al., 2013).

As protein degradation is rapid and irreversible, it is important for the ubiquitination system to be tightly regulated to prevent uncontrolled protein destruction, as a consequence, ubiquitination pathways are regulated by a set of enzyme known as de-ubiquitinases (DUBs). DUBs are isopeptidases that catalyze the removal of ubiquitin or ubiquitin-chains from proteins and act in concert with the ubiquitination system to regulate the cellular effects of ubiquitination.

Modulation in ubiquitination-associated processes following a cellular stress can therefore arise from an increase/decrease in activity from the ubiquitination pathway itself or an increase/decrease in DUB activity allowing for a rapid response to environmental and cellular signals.

The ubiquitination system is very important for the overall health of an organism which is illustrated by from diseases associated with ubiquitination defects. 3-M syndrome is a growth retardation disorder associated with mutations in the E3 ligase Cullin-7 (Huber et al., 2005), Angelman syndrome is a neurological development disorder associated with truncations in the E3 ligase UBE3A (Matsuura et al., 1997) while failure of the ubiquitination pathway has long been associated with the neurodegenerative diseases Alzheimer's and Parkinson's (Kitada et al., 1998; Leroy et al., 1998; van Leeuwen et al., 1998).

1.17 Parkinson's Disease

Parkinson's disease is a neurodegenerative disease first described in 1886 by James Parkinson's and he described it as the "shaking palsey". There was little progress in determining the aetiology of Parkinson's disease until the discovery of dopamine and the nigrostriatal pathway in 1957 (Carlsson et al., 1957). It was then subsequently discovered that patients with Parkinson's disease had a dramatic decrease in the number of dopaminergic neurons (dopamine-producing neurons) in the Substantia Nigra within the brain resulting in a diminished nigrostriatal pathway (Hirsch et al., 1988; Lloyd, 1977; Riederer and Wuketich, 1976). The nigrostriatal pathway is involved in the subconscious co-ordination of muscle movements to allow for conscious actions and the loss of this

pathway results in the symptoms associated with Parkinson's disease such as resting tremors, rigidity, bradykinesia and loss of balance. While 90% of cases of Parkinson's disease are idiopathic with no known cause, there are 10% of cases with familial heritability and at least two of the causative genes implicated in familial Parkinson's disease are genes associated with mitophagy (Kitada et al., 1998; Valente et al., 2004) . The association of mitophagy with Parkinson's has provided valuable insight into possible causes of the disease as Parkinson's has long been considered a mitochondrial disease and is also associated with diminished activity in the ubiquitination pathway (Leroy et al., 1998).

Idiopathic Parkinson's disease patients generally suffer with a deficiency in mitochondrial Complex I activity and their mitochondria have an increase in reactive oxygen production (Swerdlow et al., 1996; Zhang et al., 1999). Autopsies of Parkinson's disease brains show an accumulation of aggregated protein complexes termed Lewy bodies, though it is not known if Lewy bodies are causative or a protective mechanism (Maries et al., 2003).

While the cause of idiopathic Parkinson's disease remains unclear, recent studies of recessive early onset Parkinson's disease have provided valuable insight into possible causes of this disorder. Mutations within alpha-synuclein and the Leucine-rich repeat kinase 2 (LRRK2) were identified in early studies, however, genes involved in mitophagy, such as PINK1, Parkin and DJ-1, have been found in autosomal recessive Parkinson's disease (Healy et al., 2004; Kitada et al., 1998; Li et al., 2009; Polymeropoulos et al., 1997; Valente et al., 2004). Disease-associated mutations in Parkin and PINK1 are loss of function mutations which are now known to decrease mitophagy (Seibler et al., 2011). This strongly suggests that clearance of damaged mitochondria is critical for maintaining mitochondrial and cellular health particularly in neurons. The specific loss of dopaminergic neurons within the Substantia Nigra of the brain indicates that these cells are particularly sensitive to mitochondrial stress. One reason for the sensitivity of dopaminergic cells to mitophagic deficiency could arise from the fact that dopamine is capable of auto-oxidation and results in the production of free radicals (Kang et al., 1998). Mitochondria may act as a buffer for free radicals and any mitochondria damaged as a result need to be removed. Failure to remove damaged mitochondria would result in the further production of reactive oxygen species and damage to neurons possibly culminating in the death of the cell. This is supported from studies showing an increase in oxidative

damage in the brains of patients with Parkinson's disease (Sherer et al., 2002; Zhang et al., 1999; Zhang et al., 2000).

1.18 Thesis Aims

The regulation of apoptosis by the Bcl-2 family has been extensively studied over the last 30 years and, as a result, many of the pathways involved have been elucidated in detail. The Bcl-2 family regulate the release of cytochrome c and the subsequent activation of caspases during apoptosis. The activation of caspases leads to a proteolytic cascade and the cleavage of hundreds of proteins culminating in the demise of the cell and the hallmark morphology of apoptosis.

An important event in apoptosis is the caspase-dependent externalization of the phospholipid, phosphatidylserine, on the outer leaflet of the plasma membrane. An important event for the recognition and engulfment of apoptotic cells, the protein responsible for the scrambling of the lipid bilayer and externalization of phosphatidylserine has remained elusive. We hypothesized that TMEM16F, a protein which acts as a scramblase to externalize phosphatidylserine during calcium fluctuations may also be activated in a caspase-dependent manner and lead to the externalization of phosphatidylserine during apoptosis. Here, we investigated the role of TMEM16F in the externalization of phosphatidylserine during apoptosis.

While the role of the Bcl-2 family in apoptosis has been clearly elucidated, the Bcl-2 family have also been implicated in roles other than cell death. Studies have demonstrated a role for the Bcl-2 family in regulating mitochondrial dynamics, mitochondrial function, mitochondrial biogenesis and autophagy. This suggested a broader role for the Bcl-2 family in mitochondrial homeostasis throughout the life of the cell in addition to the role of these proteins in apoptosis. As damaged mitochondria can affect cellular homeostasis through increased production of reactive oxygen species, these organelles could also affect the remainder of the mitochondrial network through fusion and distribution of damaged organelles into healthy mitochondria. The removal of damaged mitochondria would appear to be necessary for maintaining overall cellular health. Thus, we hypothesized that the Bcl-2 family may have a role in the regulation of mitophagy to

maintain both cellular and mitochondrial health. This hypothesis was also explored herein.

Parkinson's disease has long been described as a mitochondrial impairment disease and genetic association studies in familial Parkinson's disease have implicated proteins which promote mitophagy, PINK1 and Parkin, as causative agents. As mutations within PINK1 and Parkin are loss-of-function mutations and lead to a decrease in mitophagic function, it has widely been assumed that PINK1 and Parkin are cytoprotective though this has not been explicably tested and a clear anti-apoptotic mechanism has not been described. Parkin activation has only been characterized in the last five years and many of these cytoprotective studies pre-date our current understanding of Parkin activation. Here, we set out to investigate the role of activated Parkin in the regulation of apoptosis.

Chapter II

Materials and Methods

2.1 Materials

2.1.1 General Reagents

Cisplatin, Actinomycin D, Hoechst and CCCP were purchased from Sigma. Super-killer TRAIL was purchased from ENZO. ABT-737 (Bcl-2 inhibitor) and α-Fas (clone CH11) were purchased from Merck Millipore. Genejuice was purchased from Merck Millipore. Annexin V was expressed, purified and conjugated to FITC in the lab. All DNA related reagents were purchased from NEB Biolabs. Cell culture related materials were purchased from Gibco. Unless otherwise stated all reagents were purchased from Sigma. MitoTracker Red CMXRos was obtained from Molecular Probes (Life Technologies). CCCP, Valinomycin, Oligomycin, Antimycin A, Actinomycin D and Cisplatin were obtained from Sigma-Aldrich. The poly-caspase inhibitor Z-VAD-fmk was obtained from Bachem. The poly-caspase inhibitor Q-VD-OPh was obtained from ApexBio. The proteasome inhibitor MG-132, Daunorubicin hydroxychloride, Etoposide and SuperKillerTRAIL were obtained from Enzo Life Sciences.

2.1.2 Antibodies

Antibodies for western-blot, immunostaining and immunoprecipitation were obtained from the following suppliers: α-Parkin (PRK8), α-TOM20 (F-10), α-TOM20 (FL-145), α-Beclin-1 (D-18), α-Mcl-1 (S-19), anti-Bax (N-20) and α-Bid (FL-195) were purchased from Santa Cruz Biotechnology, α-TMEM16F (NBP1-84207), α-PINK1 (BC100-494) was purchased from Novus Biologicals, α-Bim (22-40) was purchased from Calbiochem (Millipore), α-Actin (clone C4) was purchased from MP biomedicals, α-FLAG (clone M2), α-FLAG (F7425), anti-Bax (clone 6A7) and anti-Vimentin (clone V9) were purchased from Sigma Aldrich, α-HSP60 (clone LK-2) was purchased from Stressgen (Enzo Life Sciences), α-Ubiquitin (clone FK2) was purchased from Enzo Life Sciences, anti-ICAD was from MBL, anti-Cytochrome *c* (clone 7H8.2C12) for immunoblotting, anti-Cytochrome *c* (clone 6H2.B4) for immunostaining, anti-PARP (566494) and anti-Apaf-1 (559683) were from BD Pharmingen, α-p62/SQSTM1 (clone 3/p62), α-Bcl-2 (clone 7/Bcl-2), α-Bcl-XL (#610211), α-Bad (clone 48/Bad), anti-Caspase-3 (#610323), anti-Caspase-7 (#610813) and anti-XIAP (#610763) were purchased from BD Transduction Laboratories, α-Bcl-B (#3869, α-Bcl-W (clone 31H4), anti-cleaved-Caspase-3 (#9961s), anti-cleaved-Caspase-7 (#9491s), anti-

cleaved-Caspase-9 (#9501s) and anti-cleaved-PARP (#9541s) were purchased from Cell Signalling Technology, α-PUMA (#3041) was purchased from ProSci, α-GFP (JL-8) was purchased from Clontech, anti-caspase-9 (Ab-2) was from Oncogene (Merck Millipore), α-Mouse Alexa Fluor488, α-Rabbit Alexa Fluor488, α-Mouse alexaFluor350, α-Rabbit Alexa Fluor350, α-Mouse Alexa Fluor Rhodamine and α-Rabbit Alex Fluor Rhodamine secondary antibodies for immunofluorescene were purchased from Molecular Probes (Invitrogen). α-Mouse-HRP and α-Rabbit-HRP secondary antibodies for western-blotting were purchased from Jackson Laboratories.

2.1.3 siRNA sequences

TMEM16F siRNA #1 - CGAUGUAUCAAAACGCACA

TMEM16F siRNA #2 - GGAUCAACAGACUUGUAAA

TMEM16F siRNA #3 - UGUGCGUUUUGAUACAUCG

TMEM16F siRNA #4 - UUUUACAAGUCUGUUGAUCC

TMEM16F siRNA #5 - UGCGUUUUGAUACAUCGGG

PINK1 siRNA #1 - GAAAUCCGACAACAUCCUUUU

PINK1 siRNA #2 - CCAUCAAGAUGAUGUGGAA

Parkin siRNA #1 - CUUGGCUACUCCCUGCCUU

Parkin siRNA #2 - CAGCCAAAUUGCAGAAGAA

Parkin siRNA #3 - UGUAAAGAAGCGUACCAU

Beclin 1 siRNA #1 - GGAUGACAGUGAACAGUUA

Beclin1 siRNA #2 - UAAGAUGGGUCUGAAAUUU

Bcl-2 siRNA #1 - GGGAGAUAGUGAUGAAGUA

Bcl-2 siRNA #2 - GGAUCAUGCUGUACUUAAA

Bcl-X_L siRNA #1 - GGAGACUAGAUUGCCUUUG

Bcl-X_L siRNA #1 - GGAGACUAGAUUGCCUUUG

Mcl-1 siRNA #1 - GGACUUUUAUACCUGUUAU

Mcl-1 siRNA #2 -	GGACACAAAGCCAAUGGGC
Bcl-B siRNA #1 -	GGCUUUUCUGUCAUGCUUG
Bcl-B siRNA #2 -	GGCAGAUUCACCGGUCCUU
Bcl-W siRNA #1 -	GGGACAAAUAGGGACUUUG
Bcl-W siRNA #2 -	CUCGGUCCUGCGAUUAUUAAU
BID siRNA #1 -	GGCAGAUUCUGAAAGUCAA
BID siRNA #2 -	GGAGAAGACCAUGCUGG
Bad siRNA #1 -	GUACUUCCCUCAGGCCUAU
Bad siRNA #2 -	AAGAAGGGACUUCCUCG
Bim siRNA #1 -	GCACCCAUGAGUUGUGACA
Bim siRNA #2 -	GACCGAGAAGGUAGACA
PUMA siRNA #1 -	CCGAGAUGGAGCCCAAUUA
PUMA siRNA #2 -	UCUCAUCAUGGGACUCC
IAP1 siRNA #1 -	GGCAAAUGCUGCGGCCAACA
IAP1 siRNA #2 -	UUCGUACAUUUCUCUCUUUA
IAP2 siRNA #1 -	GUUCAAGCCAGUUACCCUC
IAP2 siRNA #2 -	AAGUGGUAGGGACUUGUGC
XIAP siRNA #1 -	GUGGUAGUCCUGUUUCAGC
XIAP siRNA #2 -	GCAGUUGACAAGUGUCCCA

2.2 DNA Purification

2.2.1 Preparation of competent bacteria

To prepare E. Coli DH5α competent bacteria, a colony from a LB agar plate previously streaked with DH5α cells was used to inoculate a 3 ml starter culture and grown overnight at 37°C at 280 rpm. Starter culture was added to 50 ml of Super optimal Broth (SOB) medium (2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ pH 7) until an OD_{600} of approx 0.1 was reached. The bacterial culture was incubated at 25°C at 280 rpm until an OD_{600} of approx 0.4 was achieved. Bacterial cells were chilled on ice for 10 min before centrifugation at 2,500 g for 10 min at 4°C. The bacterial pellet was gently resuspended in 20 ml of sterile Inoue Transformation Buffer (10 mM PIPES pH 6.7, 55 mM $MnCl_2$, 15 mM $CaCal_2$, 250 mM KCl) and incubated on ice for 10 min. The bacterial cells were centrifuged at 2,500 g for 10 min at 4°C before resuspension in 4 ml Inoue Transformation Buffer. Finally, 280 µl of DMSO was added and bacteria were aliquoted in cryovials. Competent bacteria were stored at -70°C.

2.2.2 Transformation of competent bacteria

50 µl of competent bacteria were typically used for the transformation of about 1 µg of plasmidic DNA. After 30 minutes on ice, time required for the plasmid to coat the bacteria, the cells were heat-shocked at 42ºC for 40 seconds and allowed to recover on ice for 2 minutes. 450 µl of LB were added and the bacteria were incubated at 37ºC at 220 rpm for one hour to allow the expression of the antibiotic resistance gene. 2.5 µl to 100 µl of each transformation in a total volume of 200 µl of LB were then plated on LB Agar plates containing 100 µg/ml Ampicillin. A negative control, using H_2O in place of plasmid was also set up to ensure that competent bacteria were no contaminated with alternate plasmids.

2.2.3 Plasmid DNA preparation

Following transformation, 3 ml starter culture from a colony from a transformed plate was grown overnight at 37°C at 280 rpm. 1 ml of bacterial starter culture was then centrifuged at top speed for 1 min. Supernatant was removed and bacterial pellet was resuspended in

100 µl of P1 Buffer (50 mM Tris-HCl pH 8, 10 mM EDTA), followed by addition of 200 µl of P2 Buffer (0.2M NaOH, 1% SDS) to lyse the cells. The suspension was incubated on ice for 1 min prior to addition of 200 µl of P3 Buffer (3M KAc pH 5.5) to precipitate proteins and genomic DNA. Unwanted proteins and genomic DNA was then removed from the suspension by centrifugation at top speed for 15 min. 0.7 Volumes of isopropanol was then added to the suspension and incubated under constant agitation for 20 min to precipitate plasmid DNA. Plasmid DNA was then retrieved by centrifugation at top speed for 15 min. Plasmid pellet was washed using ice cold 70% alcohol to removed contaminating salts and residual isopropanol. Plasmid DNA was then resuspended in TE solution stored at 4°C.

Plasmid midipreps were prepared using the QIAGEN Tip-100 columns as per manufacturer's instructions. DNA was quantified on a spectrophotometer by obtaining the absorbance at 260 nm and the Beer-Lambert equation. Plasmid DNA was sterilized prior to mammalian transfection using Spin-X tubes (Sigma).

2.3 Protein Analysis

2.3.1 Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated using standard SDS PAGE conditions. Protein samples were denatured by resuspension in SDS loading buffer (2% SDS, 50 mM Tris-HCL, pH 6.8, 10% glycerol, 2.5% β-mercaptoethanol) and boiled at 94ºC for 7 minutes. Typically, samples were loaded on 8% to 12% polyacrylamide gels and the gels were resolved at 55 V (stacking gel) / 75 V (resolving gel) in SDS-PAGE Running Buffer (25 mM Tris-HCl pH 8.3, 250 mM Glycine, 0.1% SDS)

2.3.2 Western Blotting

Proteins separated by SDS PAGE were transferred for 12 to 16 hours at 35 mA onto 0.2 µm nitrocellulose membranes (Schleider and Schull) in transfer buffer (48 mM Tris-HCL, pH 8.3, 39 mM Glycine, 0.037% SDS, 20% Methanol). Membranes were then blocked for 30 minutes in TBST (10 mM Tris-HCL, pH 8.0, 150 mM NaCL, 0.05% Tween-20) with 5%

(w/v) non-fat dried milk (Marvel) and then probed with the primary antibody diluted at 1:500 to 1:5000 for two hours in blocking buffer. The membranes were then washed for three times for 10 minutes in TBST and re-incubated with the appropriate horse-radish peroxidase coupled secondary antibody at 1:1000 in TBST with 5% (w/v) non-fat dried milk for one hour. The membranes were washed again for 3 times for 10 minutes in TBST and the signal was detected with enhanced chemiluminescence Supersignal West Dura / Pico (Pierce) by exposing the blots to sensible film (HyperfilmMP, Amersham).

2.3.3 Immunoprecipitation

HEK293T cells were plated at 2 x 106 and were calcium phosphate transfected 24 hours later with indicated amounts of plasmid DNA. Cells were lysed in 500µL of IP lysis buffer (50 mM Tris-HCl pH 8, 150mM NaCl and 1% Nonident P-40 containing 100µM phenylmethylsulfonyl fluoride, 10µg/ml leupeptin, 2µg/ml aprotinin and 10µM MG132). Lysates were incubated on ice for 15 min to allow for lysis to occur. Lysates were then clarified by centrifugation at 15,000 x g for 30 min at 4 °C. Lysates were then subjected to immunoprecipitation with 2µg of appropriate antibody and 25µL of protein A/G agarose beads (Santa Cruz Biotechnology) at 4 °C overnight. Complexes were then washed three times in IP wash buffer (50 mM Tris-Hcl pH 8, 150mM NaCl and 0.1% Nonident P-40 containing 100µM phenylmethylsulfonyl fluoride, 10µg/ml leupeptin, 2µg/ml aprotinin and 10µM MG132). Proteins were eluted from the protein A/G agarose beads by boiling for 7 min in SDS sample buffer. Immunoprecipitates were then analyzed by western blotting.

2.3.4 Capture of ubiquitinated proteins with His-tagged ubiquitin

HeLa-YFP-Parkin cells (1 x 10^6) or HEK293T (2 x 10^6) were transfected with His-tagged ubiquitin and incubated for 24 hours. Cells were lysed in 500 µL of 1% NP40 lysis buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 20 mM imidazole) containing 100 µM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 10 µM MG132 and 20 mM imidazole. Lysates were incubated for 15 min under rotation at 4°C followed by centrifugation at 15,000 g for 15 min at 4°C. Precleared lysates were subjected to pulldown using 40 µL of Ni-NTA agarose beads (Amintra) for 4 h at 4°C. Complexes were

washed three times in NP40 wash buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40, 20 mM imidazole) containing 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin and 10 μM MG132. Ubiquitinated proteins were then eluted off Ni-NTA agarose beads by addition of 50 μL SDS loading buffer (2% SDS, 50 mM Tris-HCL, pH 6.8, 10% glycerol, 2.5% β-mercaptoethanol) and boiled for 7 min at 94ºC. Presence of ubiquitinated proteins was then determined by western blotting using antibody targeted against protein of interest.

2.4 Mammalian cell culture

2.4.1 Routine cell culture

HeLa-YFP-Parkin cells were a kind gift from Dr. Stephen Tait. HeLa and HeLa-YFP-Parkin cells were cultured in RPMI supplemented with 5 % Fetal Calf Serum (FCS) and 2mM L-glutamine. HEK293T cells were cultured in DMEM supplemented with 10 % FCS and 4 mM L-glutamine. SH-SY5Y cells were cultured in DMEM supplemented with 15 % FCS, 4 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids. Cells were incubated at 37ºC with 5 % CO_2 and were passaged every 2-3 days. Cells were passaged through a process of trypsinzation (0.05 % trypsin-EDTA), dilution and replating. HeLa, HeLa-YFP-Parkin and HEK293T were diluted 1:8 every 2-3 days. SH-SY5Y cells were diluted 1:3 every 2-3 days.

2.4.2 Transfection of mammalian cells using Genejuice®

HeLa and HeLa-YFP-Parkin cells were transfected by the Genejuice® method. Cells were plated at 1 x 10^5 cells/well on 6-well plates or 1 x 10^6 cells/plate on 10 cm plates. Cells were transfected using the Genejuice reagent (Merk-Germany) according to manufacturer's protocol, briefly Genejuice was added to RPMI in a 1:100 respectively. 100 μL Genejuice®-RPMI mixture was added dropwise to the plasmid combination in 96 well plates and was allowed 15 min for the DNA-Genejuice® complexes to form. Genejuice-DNA complexes were then added to cells. Cells were incubated with Genejuice-DNA complexes for 24 hours before cell-based assays were performed. Reporter plasmid (eGFP) was used to indicate transfected cells.

2.4.3 Transfection of mammalian cells using Calcium Phosphate

HEK293T cells were plated at 2 x 10^6 cells/plate on 10 cm plates and allowed 24 hours to adhere to plates. Desired amount of DNA was added to a well within a 24-well plate and brought to a final volume of 200 µL with dH_2O. 50 µL of $CaCl_2$ (2.5M) was added to the DNA and mixed, followed by dropwise addition to 250 µL 2X HBS buffer (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Dextrose , 50 mM HEPES pH 7.1). Mixture was incubated for 30 min to allow for the calcium phosphate and the DNA to form precipitates. Precipitates were then added to HEK293T cells and cells were incubated for 24 hours before any cell-based assays were performed.

2.4.4 Transfection of mammalian cells using nucleofection

Nucleofection was used for introduction of siRNA (200 nM) in HeLa or HeLa-YFP-Parkin. Cells (5 x 10^5 – 1 x 10^6) were centrifuged and resuspended in 100 µL of nucleofection buffer (5mM KCl, 15mM $MgCl_2$, 20mM HEPES, 150mM Na_2HPO_4, pH 7.2). Cells were then added to nucleofection cuvette (0.2 cm, Sigma) and cells were nucleofected using the Amaxa Nucleofector (program I-013). Cells were added to warm R5F and subsequently plated at 1 x 10^5 cells/well on 6-well plates. Cells were incubated for 24-48 hours before performing cell based assays.

2.4.5 Transfection of SH-SY5Y with siRNA using Lipofectamine-2000

SH-SY5Y cells were plated at 5 x 10^4 cells/well on 6 well plates and allowed 24 hours to adhere to plate. Cells were transfected using the Lipofectamine-2000 reagent (Invitrogen) according to manufacturer's protocol, briefly Lipofectamine-2000 was added to RPMI in a 1:100 respectively. 100 µL Lipofectamine-2000-RPMI mixture was added dropwise to siRNA (200 nM) in 96 well plates and was allowed 15 min for the DNA-Lipofectamine-2000 complexes to form. DNA-Lipofectamine-2000 complexes were then added to SH-SY5Y cells. Cells were incubated with DNA- Lipofectamine-2000 complexes for 48 hours before cell-based assays were performed.

2.5 Cell-Based Assays

2.5.1 Apoptosis Assays

Cells were treated with indicated pro-apoptotic drugs for 18-24 hours before cell death was counted using phase contrast microscopy. Apoptosis of cells was determined by morphological criteria such as membrane blebbing and detachment from the plate. Modulation of apoptosis by proteins of interest was performed by either transient transfection or knockdown of desired proteins, by methods described above, and analysis of alteration in apoptosis counts in response to the same stimuli. In each apoptosis assay, a minimum of triplicate counts of 100 cells were counted and each assay was performed a minimum of three times.

2.5.2 Phosphatidylserine exposure assay

To quantitate phosphatidylserine exposure in treated cells, cells were trypsinized and combined with detached cells in medium before centrifugations at 800 g for 10 min. Cells were then resuspended in Annexin V staining buffer (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2) containing 1-2 μg/ml of annexin V-FITC. Cell fluorescence was measured on a flow cytometer (FACSCalibur, Becton Dickinson, CA) and analysed using CellQuest software.

2.5.3 Immunocytochemistry

Cells were plated on coverslips and allowed 24 hours to attach before cells were then prepared as indicated by methods above. After treatments, cells were washed in PBS pH 7.2, fixed with 3.7% paraformaldehyde in PBS for 10 min followed by a further three washes with PBS pH 7.2. Cells were then permeabilised using 0.15% triton X for 15min followed by three washes with PBS pH 7.2. Cells were blocked with 2% BSA for 30 min to reduce non-specific binding before incubation with primary antibody (1:100-1:500 dilution) for 45 min. Cells were subsequently washed three times with 0.2%BSA/0.15% triton X to remove any non-specific primary antibody. Cells were then incubated with 1:1000 dilution of desired fluorophore-conjugated secondary antibody for 45min followed by washing with 0.2%BSA/0.15% triton X to remove any non-specific secondary antibody.

Where indicated cell nuclei were stained using 500nM Hoechst incubation for 10 min. Cells were mounted onto slides along with Slow Fade (Invitrogen) and observed on a laser scanning microscope (Olympus FV1000) using a 488 nm Argon laser (green fluorescence), a 543 nm HeNe laser (red fluorescence) and a 405 nm LD laser (blue fluoressence). Quantification of Parkin translocation, mitochondrial ubiquitination, p62 decoration or mitophagy was estimated by counting a minimum of 3 x 100 cells for each treatment and each experiment was performed in triplicate.

2.5.4 Sub-G1 analysis

HEK293T cells were plated at 1 x 10^5 cells/well in 6-well plates 24 hours prior to transfection with PINK1 and Parkin plasmids and incubated for a further 48 hours post transfection. 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 staining with 50 µg/mL Propidium Iodide before Cell fluorescence was measured on a flow cytometer (FACSCalibur, Becton Dickinson, CA) and analysed using CellQuest software.

Chapter III

The Role of TMEM16F in Phosphatidylserine Exposure during Apoptosis

3.1 Introduction

Bcl-2 family members are key regulators of apoptosis (Kluck et al., 1997; Yang et al., 1997). Triggering apoptosis results in the activation of caspases and subsequent cleavage of hundreds of proteins (Luthi and Martin, 2007; Taylor et al., 2008). While many of these cleavage events have no effect on the fate of the cell, cleavage of a subset of caspase substrates initiates the morphological hallmarks of apoptosis such as DNA fragmentation and membrane blebbing (Coleman et al., 2001; Enari et al., 1998). Another important caspase-dependent event during apoptosis is the externalization of phosphatidylserine to the outer leaflet of the cell membrane (Fadok et al., 1992; Martin et al., 1995). This acts as a signal for neighbouring cells and macrophages to engulf the dying cell rapidly before the occurrence of secondary necrosis and the release of danger-associated molecular patterns (DAMPs) followed by inflammation (Cullen et al., 2013; Juncadella et al., 2013). Externalization of phosphatidylserine is a key event for identification of apoptotic cells by macrophages and is a key feature of apoptosis which currently has no elucidated mechanism.

It has been recently demonstrated that the calcium-activated chloride channel, TMEM16F, mediates phosphatidylserine externalization in response to calcium fluctuations within a cell (Suzuki et al., 2010). Thus, we postulated that TMEM16F may also be the protein that mediates phosphatidylserine externalization during apoptosis. Suzuki and colleagues characterized an activating point mutation within TMEM16F where aspartic acid 409 was replaced with a glycine. This is interesting, as caspases preferentially cleave after aspartic acid residues and predictive software suggest that this aspartic acid may lie within a consensus motif for caspase cleavage (Wee et al., 2007). It is possible that caspase cleavage at aspartic acid 409 in TMEM16F results in a conformational change within the protein to promote its scramblase activity. Hence, we wished to investigate the potential role for TMEM16F in phosphatidylserine externalization during apoptosis.

3.2 Results

3.2.1 Phosphatidylserine exposure during Apoptosis

The exposure of phosphatidylserine during apoptosis is well established and plays a vital role in the recognition and removal of apoptotic cells (Schlegel et al., 1996). The loss of a functional phosphatidylserine pathway has been associated with the mild bleeding disorder, Scott Syndrome, due to its role in coagulation. We first wished to characterize the role of the Bcl-2 family in caspase activation and the externalization of phosphatidylserine during apoptosis. As previously reported by several studies, induction of apoptosis by chemotherapeutic drugs led to the characteristic externalization of phosphatidylserine which was effectively blocked by transfection of exogenous anti-apoptotic Bcl-2 family members (**Figure 3.1 A-C**). Furthermore, blocking the downstream executioners of apoptosis, caspases, using the pan-caspase inhibitor, zVAD-fmk, also efficiently blocked the exposure of phosphatidylserine (**Figure 3.1 D**). Thus, phosphatidylserine externalization is tightly regulated by the Bcl-2 family of proteins, as expected.

3.2.2 TMEM16F is a Caspase substrate

While Suzuki et al. (Suzuki et al., 2010) demonstrated that TMEM16F modulated phosphatidylserine exposure in response to intracellular calcium fluctuations; we wished to investigate whether TMEM16F had the ability to modulate phosphatidylserine exposure during apoptosis. Caspases exert their effects through the cleavage of proteins after an aspartic acid residue. Suzuki and colleagues (Suzuki et al., 2010) characterized a gain-of-function mutation within TMEM16F at a key aspartic acid residue and we wished to investigate if cleavage after this residue during apoptosis mimics the gain-of-function mutation reported in the latter study.

In order to investigate if TMEM16F is cleaved during apoptosis, we first overexpressed exogenous FLAG-tagged TMEM16F in Hela cells which were then treated with the pro-apoptotic stimuli TRAIL and Fas (**Figure 3.2 A**). Overexpression of TMEM16F did not modulate the degree apoptosis observed (**Figure 3.2 A**). Cells were subsequently lysed and analyzed by western blot, using anti-FLAG, to ask whether exogenous TMEM16F was

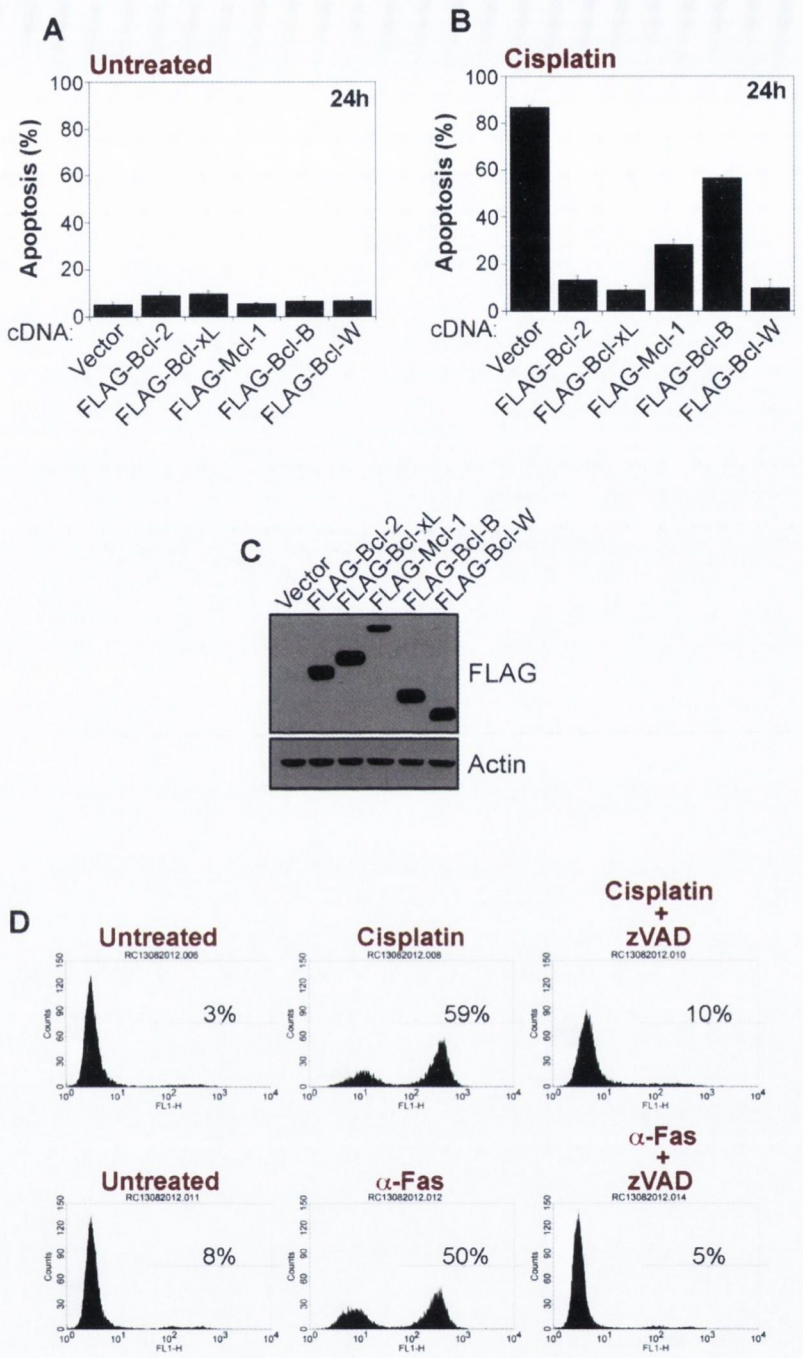

Figure 3.1 Phosphatidylserine exposure during apoptosis

(A) HeLa cells were transfected with 500ng of cDNA encoding for indicated Bcl-2 family member. Cells were then either left untreated or (B) treated with Cisplatin (50 μM) for 24 hr. Cell death was scored based on morphology. (C) Western blot analysis demonstrating expression of exogenous Bcl-2 family members. (D) Cells were treated with Cisplatin (50 μM) or anti-Fas (100 ng/ml) in the presence or absence of zVAD-fmk (50μM) for 24 hr. Phosphatidylserine exposure was analyzed by FITC-Annexin V and flow cytometry. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

cleaved during apoptosis (**Figure 3.2 B**). Results show that under conditions where caspases were active, there was a reduction in the full length form of TMEM16F suggesting cleavage of the protein. We next investigated if endogenous TMEM16F was cleaved during apoptosis. To this end, Hela cells were treated with death receptor ligands and subsequently cells were lysed for analysis by western blotting (**Figure 3.2 C and D**). Activation of caspases was confirmed through blotting as there was a reduction in the amount pro-caspase-8 and also the appearance of the caspase-8 active fragment. Strikingly, under the same conditions there was a clear reduction of full length TMEM16F suggesting that it could be a potential caspase substrate. These results suggest that TMEM16F may be cleaved during apoptosis and it was possible that this cleavage could act as an activation event for TMEM16F activation and phosphatidylserine externalization.

3.2.3 TMEM16F mutants fail to modulate phosphatidylserine exposure

As reported by Suzuki and colleagues, mutation of an aspartic acid at residue 409 to a glycine in TMEM16F resulted in a gain-of-function and constitutive externalization of phosphatidylserine (Suzuki et al., 2010). Caspases predominantly cleave after an aspartic acid residue and this indicated that TMEM16F function may be positively regulated by caspases. Many cleavage events result in a conformational change within the cleaved protein. The mutation of an aspartic acid to a glycine residue results in an acidic polar residue replaced with a neutral non-polar residue.

This change in charge within the protein could lead to a permanent conformational change and increased activity of TMEM16F. Using site directed mutagenesis, we generated a plasmid encoding TMEM16F with Asp409 mutated to a glycine. We also used site-directed mutagenesis to replace Asp409 with a glutamic acid residue. We postulated that the glutamic acid would maintain the charge and conformation within TMEM16F, however, would be resistant to cleavage by caspases and therefore would act as a loss-of-function mutation, reducing phosphatidylserine externalization during apoptosis. To further investigate the role of TMEM16F in phosphatidylserine exposure, we transiently transfected these TMEM16F mutants into Hela cells and measured phosphatidylserine exposure for both constitutive exposure and exposure in response to apoptotic triggers (**Figure 3.3 A**). Phosphatidylserine exposure can be measured using the Annexin V assay (Martin et al., 1995). Annexin V is a protein which preferentially binds phosphatidylserine

in the presence of calcium and can be used to detect phosphatidylserine exposure on the outer leaflet of the cell membrane. The constitutively active mutant, TMEM16F D409G, and the putative dominant-negative mutant, TMEM16F D409E, both failed to modulate phosphatidylserine exposure either constitutively or in response to apoptotic triggers (**Figure 3.3 B**). Together these results suggest that Asp409 in TMEM16F is dispensable for phosphatidylserine externalization during apoptosis. However, the presence of endogenous TMEM16F could have affected the potential enhancement or inhibitory effects from the exogenous mutants.

3.2.4 Ablation of TMEM16F does not modulate phosphatidylserine exposure during apoptosis

As a consequence of the TMEM16F mutations having no modulating effects on phosphatidylserine exposure, we next investigated the requirement for the endogenous protein for phosphatidylserine exposure during apoptosis.

To this end, we ablated endogenous TMEM16F expression in Hela cells using 5 individual siRNAs targeted against TMEM16F (**Figure 3.4 A**) and analyzed phosphatidylserine externalization during apoptosis. TMEM16F was effectively knocked down and apoptosis was induced through death receptor ligation. Ablation of TMEM16F by RNA interference had no effect on apoptosis in response to Fas activation (**Figure 3.4 B**). Phosphatidylserine exposure is a downstream event of caspase activation, so modulating its expression would not have been expected to alter the fate of the cell. However, we also failed to observe modulation of phosphatidylserine externalization following TMEM16F knockdown (**Figure 3.4 C and D**).

Because the previous experiments had all been endpoint experiments, the possibility remained that externalization of phosphatidylserine may have been saturated at these timepoints, while decreases at earlier timepoints may have been missed. To address this, we again ablated TMEM16F expression in Hela cells using RNA interference and measured phosphatidylserine externalization over a timecourse in response to the pro-apoptotic agent, Cisplatin (**Figure 3.5 A**). However, once again, there was no difference in phosphatidylserine exposure, upon TMEM16F ablation, at any of the timepoints measured (**Figure 3.5 B**). This suggested that while TMEM16F was found to be important for

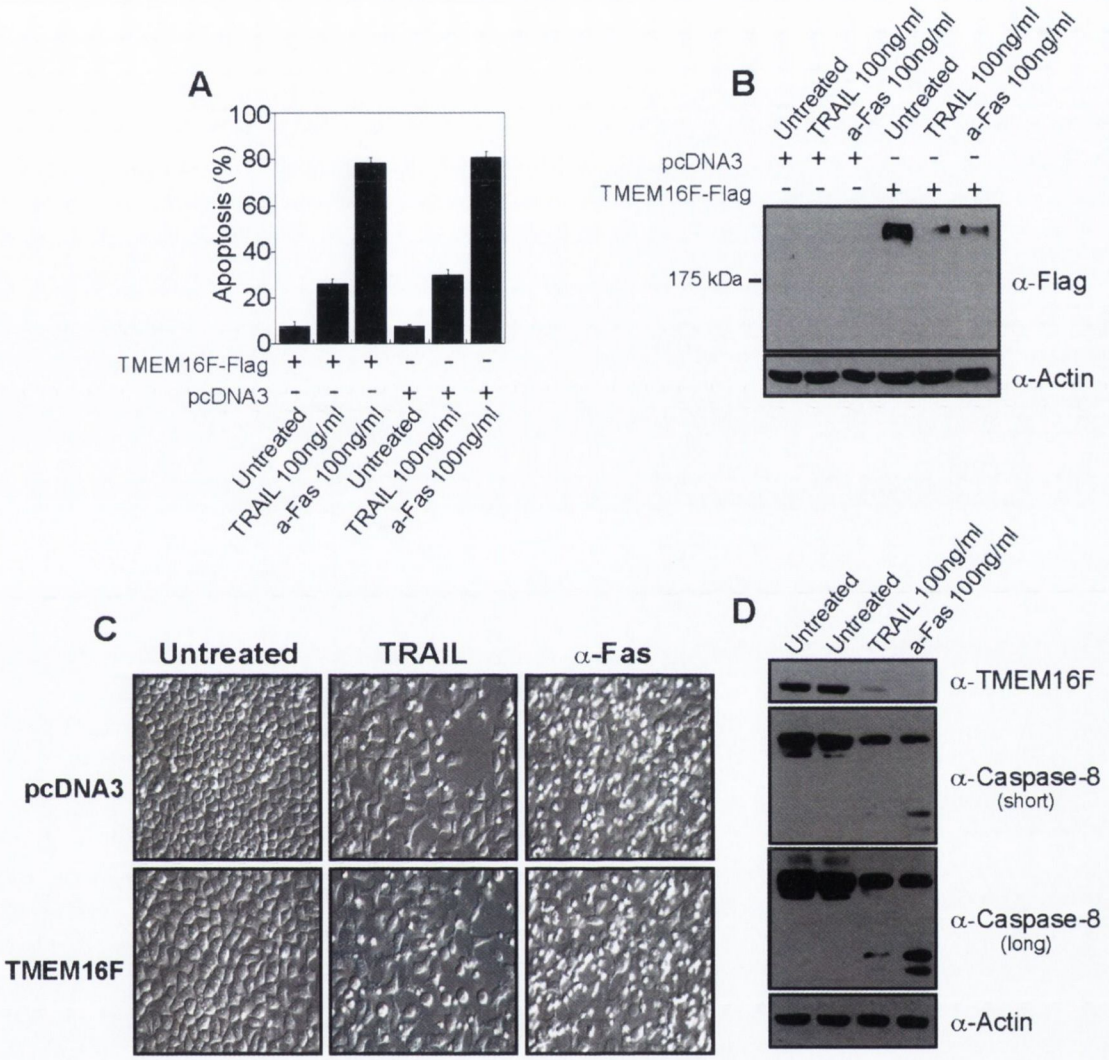

Figure 3.2 TMEM16F is degraded during apoptosis

(A) HeLa cells were transfected with plasmid encoding TMEM16F for 24 h. Cells were then treated with TRAIL (100 ng/ml) or anti-Fas (100 ng/ml) for 24 h and cell death was scored based on morphology. (B) HeLa cells wre transfected with plasmid encoding for TMEM16F for 24 h. Cells were then treated with TRAIL (100 ng/ml) or anti-Fas (100 ng/ml) for 24 h and TMEM16F cleavage was analyzed by western blot. (C) Phase contrast images of HeLa treated with TRAIL (100 ng/ml) or anti-Fas (100 ng/ml) for 24 h. (D) HeLa cells were treated with TRAIL (100 ng/ml) or anti-Fas (100 ng/ml) for 24 h and endogenous TMEM16F cleavage was analyzed by western blotting. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

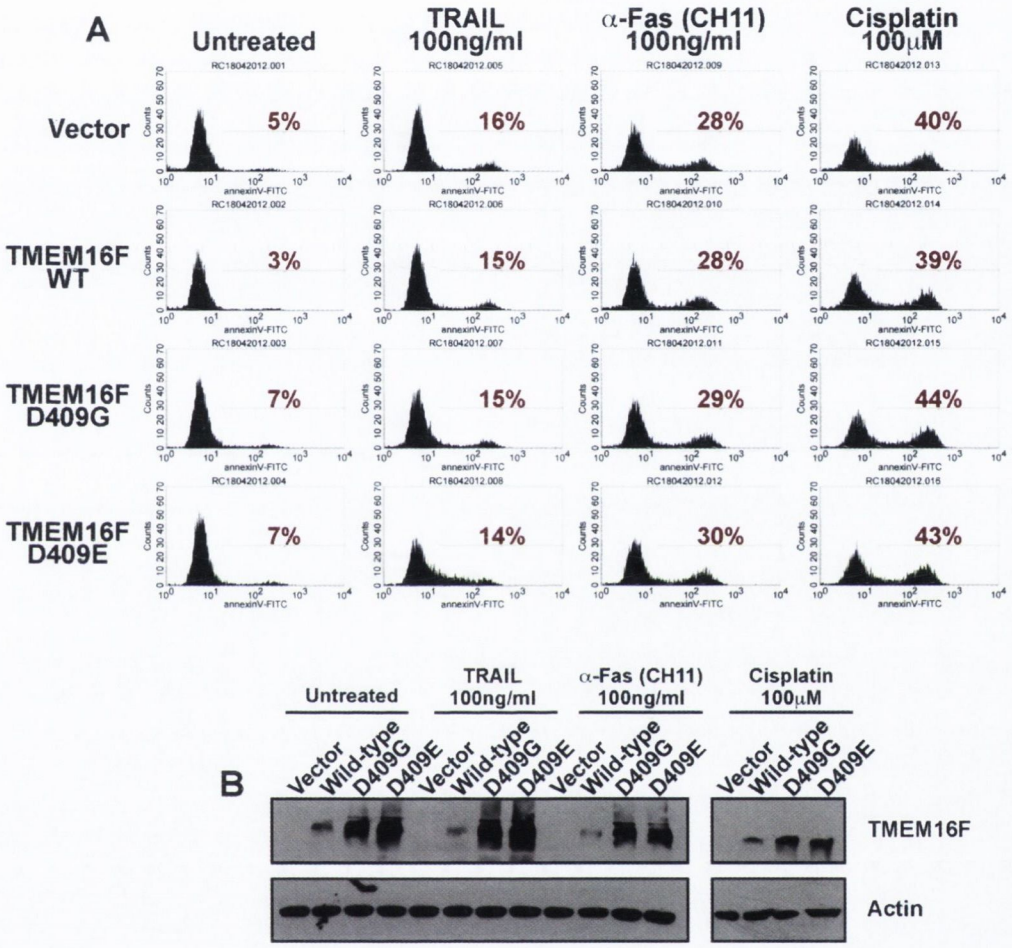

Figure 3.3 TMEM16F mutants fail to modulate phosphatidylserine exposure

(A) HeLa cells were transfected with plasmid encoding TMEM16F WT and indicated mutants (1 μg). Cells were then either left untreated or treated with TRAIL (100 ng/ml), anti-Fas (100 ng/ml) or Cisplatin (100 μM) for 10 hr. Phosphatidylserine exposure was analyzed with FITC-Annexin V and flow cytometry. (B) HeLa cells were treated as in (A) and cell lysates were analyzed by western blot to verify the expression of TMEM16F mutants.

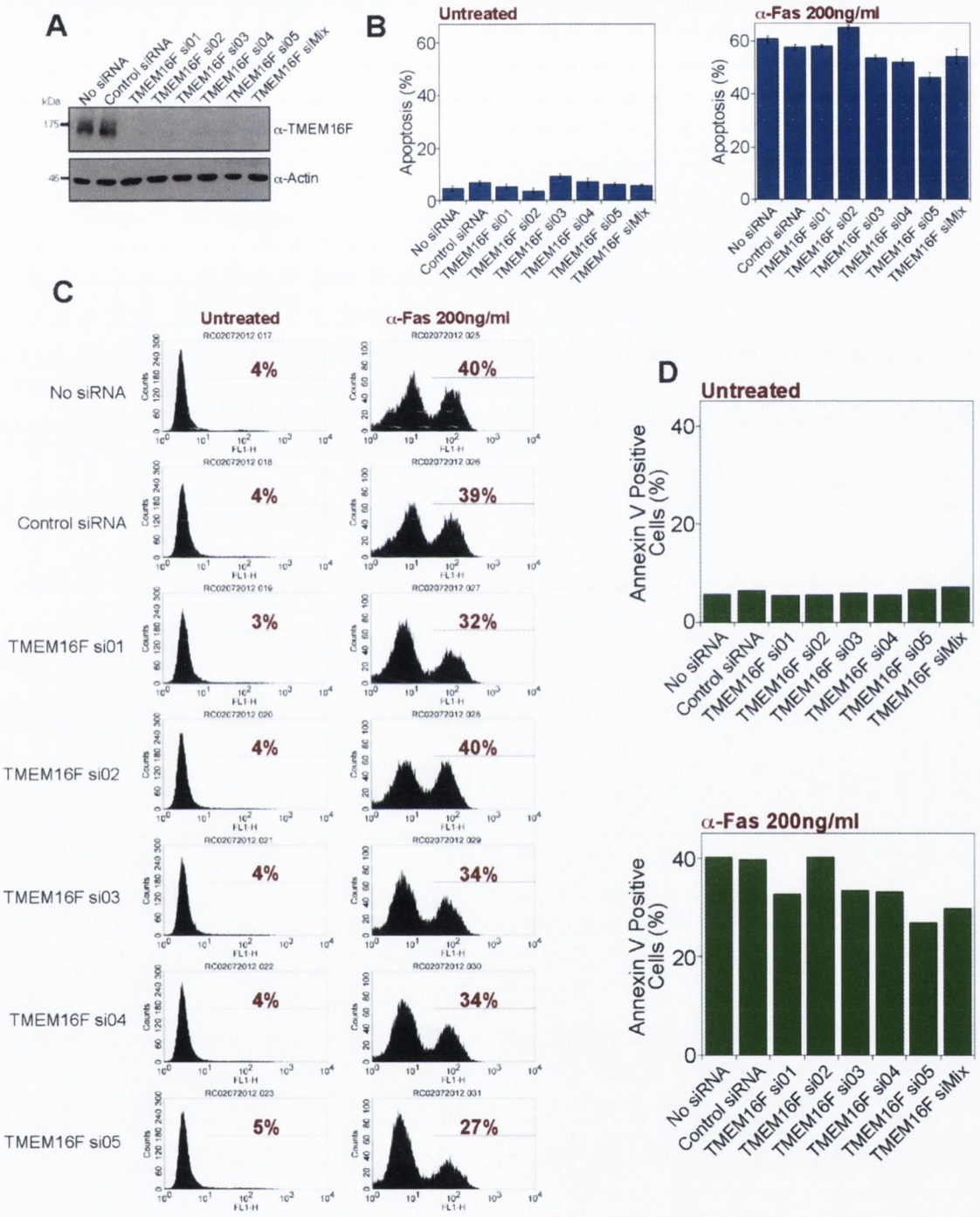

Figure 3.4 Knockdown of TMEM16F does not modulate phosphatidylserine exposure during apoptosis

(A) HeLa cells were transfected with 5 unique siRNAs(200 nM) targeted against TMEM16F and incubated for 48 h. Knockdown was then verified by western blot. (B) HeLa cells were transfected with siRNA as in (A) and treated with anti-Fas (100 ng/ml) for 24 h. Cell death was scored based on morphology. (C and D) HeLa cells were transfected and treated as in (B) and phosphatidylserine exposure was measured with FITC-Annexin V and flow cytometry.

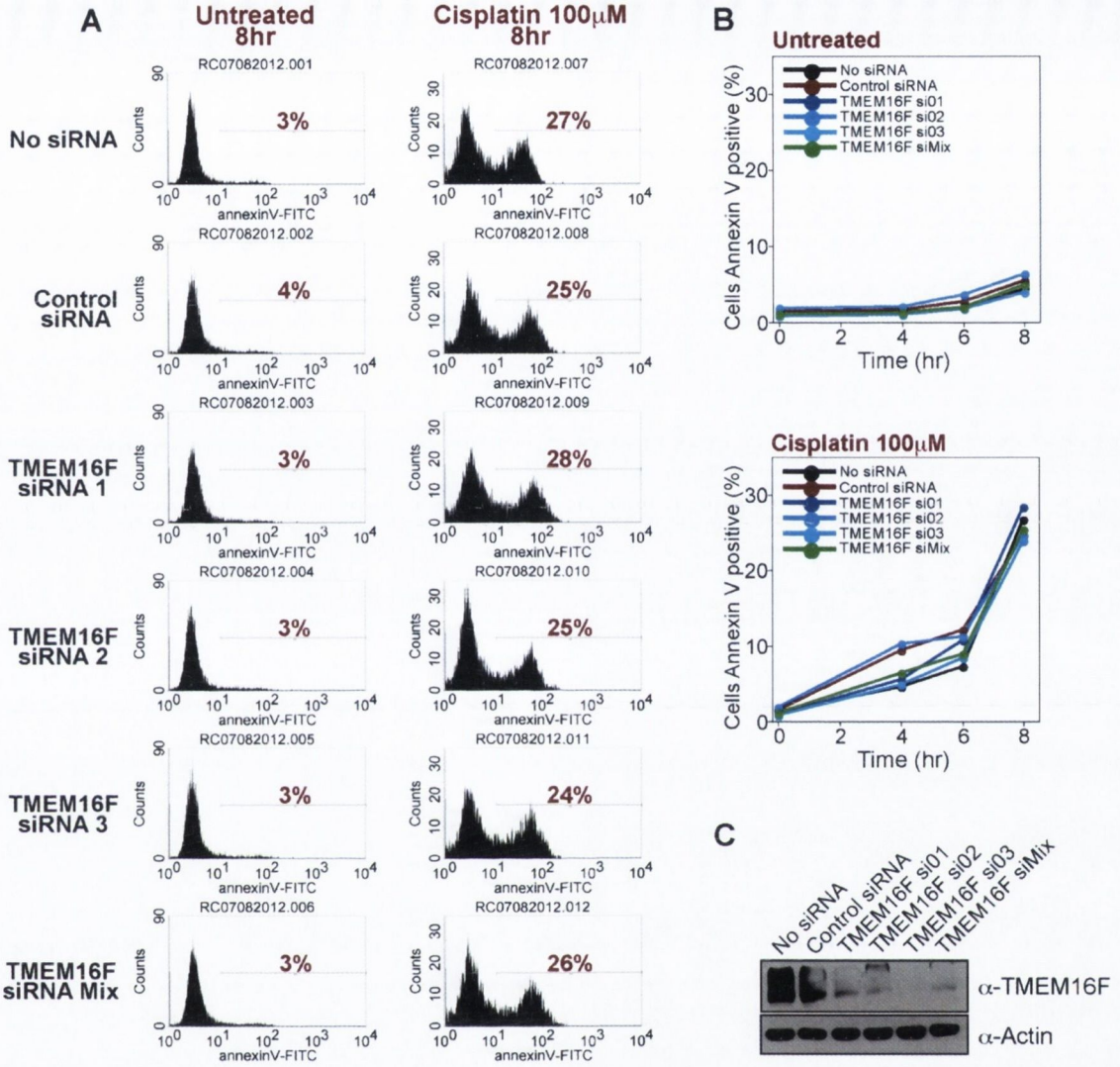

Figure 3.5 Timecourse of phosphatidylserine exposure in cells with TMEM16F ablated

(A) HeLa cells were transfected with three unique siRNAs (200 nM) targeted against TMEM16F and incubated for 48 h. Cells were then treated with Cisplatin (100 μM) for 8 h and phosphatidylserine exposure was analyzed with FITC-Annexin V and flow cytometry. (B) HeLa cells were transfected and treated as in (A) and phosphatidylserine exposure was measured at indicated time points. (C) HeLa cells were transfected as in (A) and TMEM16F knockdown was verified by western blot.

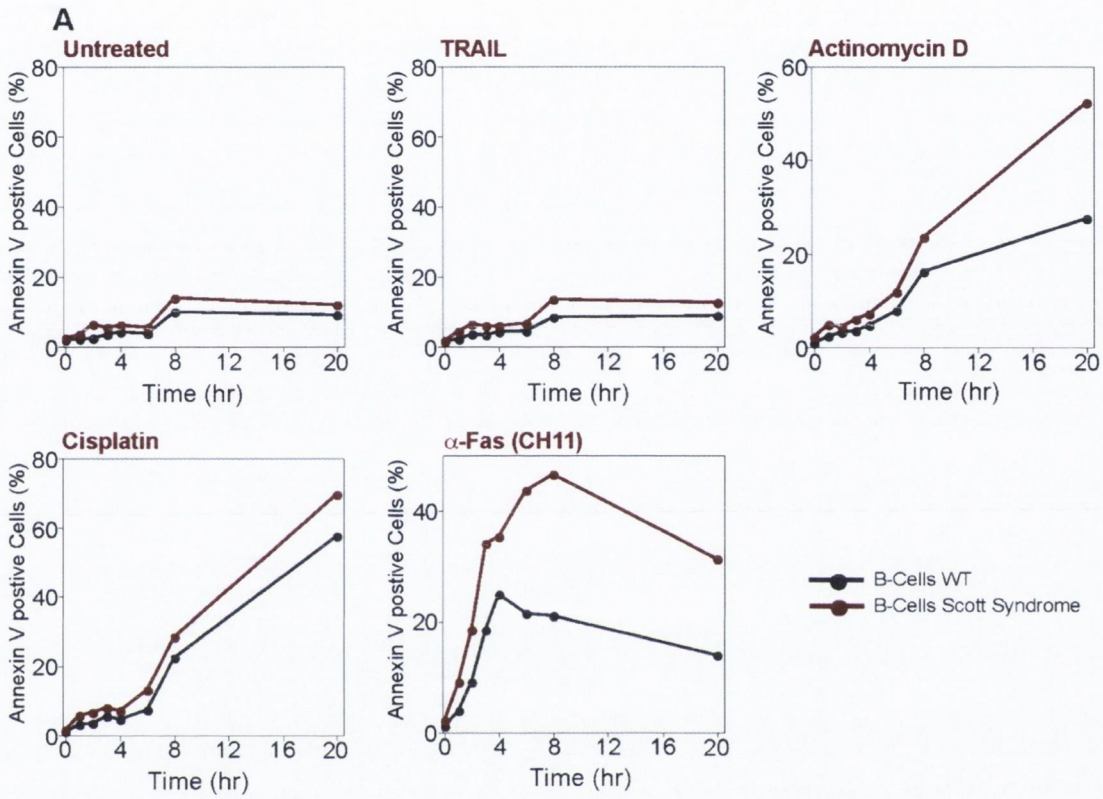

Figure 3.6 Phosphatidylserine exposure in TMEM16F null cells

(A) Immortalized B-lymphocytes from a patient who is a natural knockout for TMEM16F (Scott Syndrome) were treated with TRAIL (100 ng/ml), anti-Fas (100 ng/ml), Cisplatin (100 μM) and Actinomycin D (5 μM). Phosphatidylserine exposure was measured with FITC-Annexin V at indicated timepoints.

phosphatidylserine exposure in response to calcium fluctuations, it appeared dispensable for phosphatidylserine exposure during apoptosis.

3.2.5 Phosphatidylserine exposure is not modulated in TMEM16F-null cells

In a final effort to investigate a role for TMEM16F in apoptosis, we used a cell line which contains no TMEM16F. Scott syndrome was first described by Weiss et al and was characterised by a mild bleeding disorder as a result of reduced platelet procoagulant activity (Weiss et al., 1979).

Subsequent research found that a frameshift mutation within the TMEM16F gene resulted in a premature stop codon and a loss of TMEM16F protein expression (Castoldi et al., 2011; Suzuki et al., 2010).

We thus investigated apoptosis-induced phosphatidylserine externalization in immortalized B-cells isolated from a Scott Syndrome patient. We obtained both wild-type B-cells and Scott syndrome B-cells and cells were treated with activators of both intrinsic and extrinsic apoptosis (Castoldi et al., 2011). The externalization of phosphatidylserine was monitored over a timecourse and there was no reduction in its externalization despite the cells completely lacking TMEM16F (**Figure 3.6 A**). In fact, wild-type B-cells exposed less phosphatidylserine in response to apoptotic triggers than their Scott Syndrome counterparts; however, we can only speculate that this is a result of patient to patient variation. Thus, that while TMEM16F appears to be important for phosphatidylserine exposure in response to calcium fluctuations within cells, it is dispensable for caspase-dependent phosphatidylserine exposure during apoptosis (Suzuki et al., 2010).

3.3 Discussion

3.3.1 TMEM16F is degraded during apoptosis

The TMEM16 family are a family of proteins that are regulated by calcium ions and have been reported to function as ion channels. Studies showed that TMEM16F has phospholipid scramblase activity in response to fluctuation of cytosolic calcium levels (Suzuki et al., 2010). Further experimentation from the same group also characterized a gain of function mutation within TMEM16F where an Aspartic acid residue was mutated to a glycine residue. Expression of TMEM16F containing this residue lead to constitutive exposure of phosphatidylserine on the outer membrane of the cell. The sequence which contained the aspartic acid residue was suggested by predictive software to lie within a caspase cleavage consensus motif (Wee et al., 2007). As a result we investigated whether caspase cleavage at this residue could result in the activation of TMEM16F's scramblase activity irrespective of calcium levels and whether it was the protein responsible for caspase-dependent phosphatidylserine exposure during apoptosis.

Analysis of both endogenous and exogenous TMEM16F strongly suggested that it was cleaved during apoptosis further suggesting that the protein may play a role in phosphatidylserine exposure during cell death. We postulated that TMEM16F may exist in both active and inactive forms requiring a modification for activation. The constitutively active mutation characterized by Suzuki et al may result in conformational changes as the glutamic acid and glycine differ in both charge and polarity and we postulated that this conformational change results in the activation of TMEM16F. As with the cleavage of executioner caspases by initiator caspases results in a conformational change required for protease activity, it was possible that a similar caspase cleavage of TMEM16F promoted its scramblase activity also through a conformational change.

We analyzed this through overexpression of the constitutively active mutant characterized by Suzuki et al and also generated a mutant with the aspartic acid residue replaced with a glutamic acid residue, a residue with a similar charge and polarity; which however, would not be cleaved by caspases. As TMEM16F requires oligomerization for full function, the mutation of the aspartic acid to glutamic acid should also act as a dominant-negative to reduce phosphatidylserine externalization; however, this was not the case. In all cases, overexpression of wild-type or either mutant did not alter phosphatidylserine externalization in response to apoptotic triggers.

The constitutively-active mutant also failed to increase phosphatidylserine externalization, an observation which is in direct contrast to reported observations (Suzuki et al., 2010). This suggests to us that the aspartic acid residue at position 409 is not vital for TMEM16F function. Other groups also failed to re-produce data that TMEM16F D409G failed to constitutively externalize phosphatidylserine (Yang et al., 2012). These results suggest that TMEM16F does not play a role in phosphatidylserine exposure during apoptosis.

Further results casting doubt on TMEM16F's role in phosphatidylserine exposure during apoptosis came from studies using either siRNA to deplete cells of TMEM16F or using cells lacking TMEM16F. Both approaches indicated that TMEM16F had no role to play in the externalization of phosphatidylserine during apoptosis.

To date there are approximately 600 Caspase substrates characterized with very few having a direct effect on apoptosis or the morphology associated with apoptosis. However, as phosphatidylserine externalization acts as a signal for an apoptotic cell to be engulfed and removed before secondary necrosis, TMEM16F was a potentially exciting caspase substrate. However, our results argue that TMEM16F is not the caspase-activated scramblase responsible for phosphatidylserine externalization during apoptosis.

Our results only allowed us to conclude that, while TMEM16F has been shown to be involved in the redistribution of phospholipids in response to fluctuations in cytosolic calcium levels, it was not involved in the redistribution of phospholipids during apoptosis. Worryingly, we were not the only group that failed to reproduce the reported effects of the constitutively active TMEM16F mutant. Yang and colleagues generated a TMEM16F knockout mouse and during their investigation and they generated the TMEM16F constitutively-active mutant as a control and they also failed to observe any enhancement of phosphatidylserine externalization (Yang et al., 2012).

Moreover, during the course of this work the scramblase responsible for the caspase dependent phosphatidylserine exposure, XKR8, was identified (Suzuki et al., 2013) therefore we ended our investigation at this point.

Chapter IV

Bcl-2 family Proteins Participate in Mitochondrial Quality Control by Regulating Parkin/PINK1-dependent Mitophagy

4.1 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 (Chen and Klionsky, 2011; Mizushima, 2007; Xie and Klionsky, 2007). 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 (Ashrafi and Schwarz, 2013; Youle and Narendra, 2011). However, the molecular events that orchestrate selective recognition and removal of damage mitochondria are only now emerging.

Parkin is a Ub E3 ligase which 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). PTEN-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 (Clark et al., 2006; Park et al., 2006; Valente et al., 2004; 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 (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2008; Narendra et al., 2010b; 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 (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2008; Narendra et al., 2010b; Shiba et al., 2009; Um et al., 2009; Vives-Bauza et al., 2010).

Parkin is activated upon association with PINK1, and recent studies suggest that PINK1 phosphorylates Parkin at Ser65 (Kondapalli et al., 2012; Shiba-Fukushima 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 ubiquitin and LC3 binding adaptor protein p62 to these organelles (Geisler et al., 2010; Lee et al., 2010; Narendra et al., 2010a; 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., 2010a; Okatsu et al., 2010), possibly due to redundancy with the related Ub and Atg8/LC3II-binding protein NBR1.

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; Goldstein et al., 2000). 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 (Slee et al., 1999). Apart from this key role, studies have also shown that pro-survival 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 (Erlich et al., 2007; Pattingre et al., 2005). 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 (Autret and Martin, 2009; Youle and van der Bliek, 2012), processes that have been implicated in the quarantining of impaired mitochondria during mitophagy (Gomes et al., 2011; Rambold et al., 2011; Twig et al., 2008). Thus, several members of the Bcl-2 family are localized to mitochondria, impact upon 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 mitochondria can be inhibited by pro-survival members of the Bcl-2 family such as Bcl-xL and Mcl-1. However, we found no evidence for involvement of Beclin-1 in Parkin-induced 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, mitochondrial fission/fusion dynamics, as well as the removal of impaired mitochondria via mitophagy.

4.2 Results

4.2.1 Parkin rapidly translocates to and ubiquitinates depolarized mitochondria

Parkin and PINK-1 are proteins involved in the elimination of damaged mitochondria through a specific mode of autophagy termed mitophagy (Narendra et al., 2008). To investigate the role of in mitophagy, HeLa cells which contain a deletion mutation within the Parkin gene (Denison et al., 2003), were stably transfected with YFP-Parkin (HeLa-YFP-Parkin hereafter). Upon mitochondrial depolarization, induced by the uncoupling agent CCCP, YFP-Parkin is activated by PINK1 and rapidly translocates from the cytosol to mitochondria within 3 hr (**Figure 4.1 A**). Activated Parkin functions as a broad E3 ligase ubiquitinating many proteins on the outer surface of mitochondria, thereby tagging damaged mitochondria for destruction (**Figure 4.1 B**). The latter event allows for recruitment of the adaptor molecule p62 to mitochondria (**Figure 4.1 C**) which interacts with LC3 to initiate autophagosome formation and engulfment of damaged mitochondria.

To investigate the degradation of mitochondria by mitophagy, HeLa-YFP-Parkin cells were treated with CCCP for 24 hours and the presence or absence of mitochondrial markers was analyzed. TOM20 and VDAC are both channels located in the outer mitochondrial membrane and serve as convenient mitochondrial markers which are completely lost 24 hr after treatment with CCCP (**Figure 4.2 A and B**). As both TOM20 and VDAC are located on the outer mitochondrial membrane, it is feasible that Parkin can target these proteins for degradation in a proteosome-dependent manner rather than via mitophagy (Tanaka et al., 2010; Yoshii et al., 2011). To further demonstrate that mitophagy was responsible for removal of TOM20 and VDAC, the inner mitochondrial membrane protein, cytochrome c, was analyzed after CCCP treatment. Even though Parkin could not directly interact with cytochrome c it was also rapidly degraded in response to CCCP treatment (**Figure 4.2 C**).

Thus, depolarization of mitochondria leads to activation of Parkin and decoration of mitochondria with ubiquitin allowing p62 to target these damaged mitochondria for degradation.

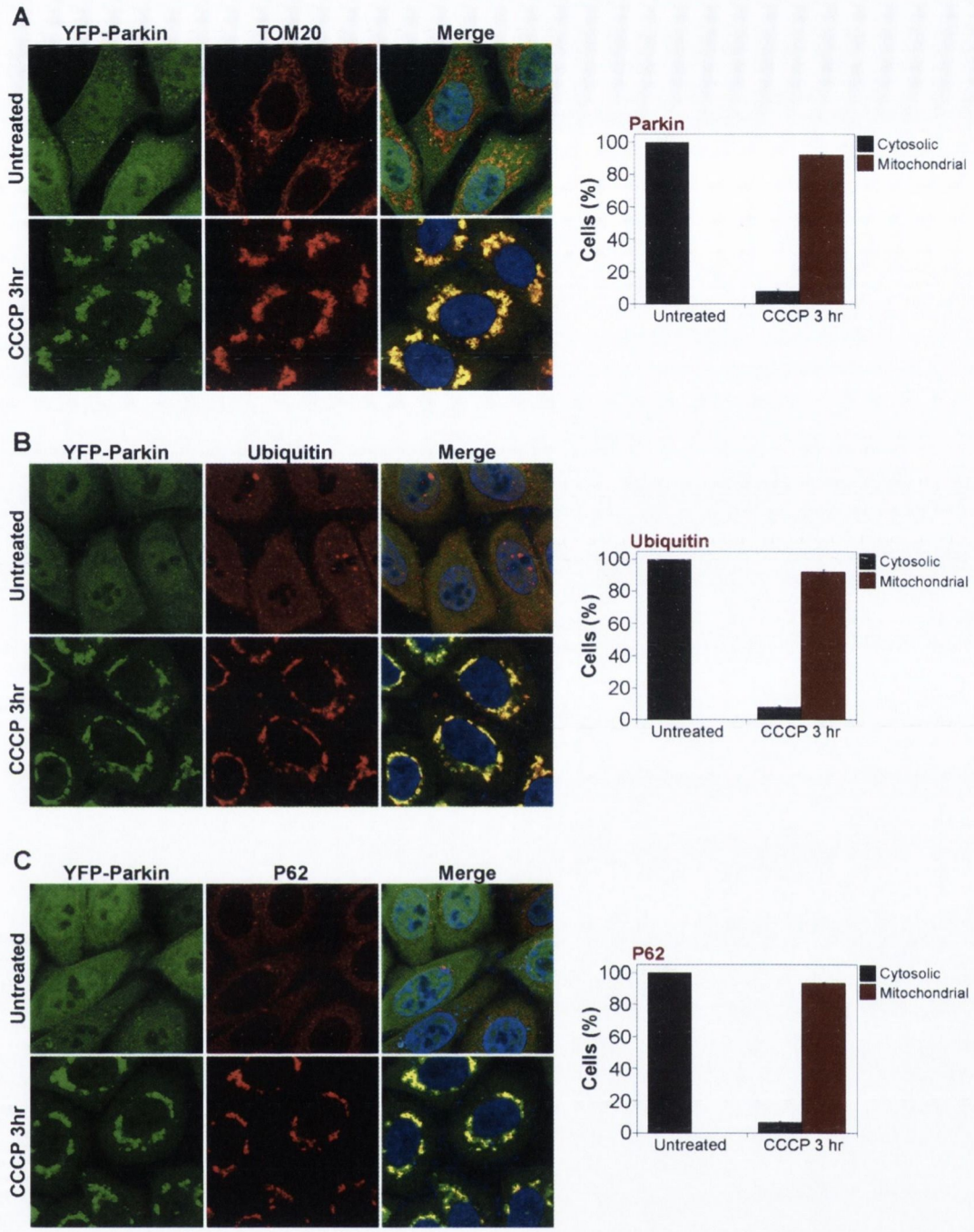

Figure 4.1 Parkin promotes Ubiquitination and p62 recruitment to damaged mitochondria

(A) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for 3 hr. Mitochondria were immunostained for TOM20 (Red) and nuclei were stained with Hoechst (Blue). Co-localization between YFP-Parkin and mitochondria was analyzed by Confocal microscopy. (B) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for 3 hr. Cells were immunostained for P62 (Red) and nuclei were stained with Hoechst (Blue). Co-localization between P62 and mitochondria was analyzed by confocal microscopy. (C) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for 3 hr. Cells were immunostained for ubiquitin (Red) and nuclei were stained with Hoechst (Blue). Co-localization between ubiquitin and mitochondria was analyzed by Confocal microscopy. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

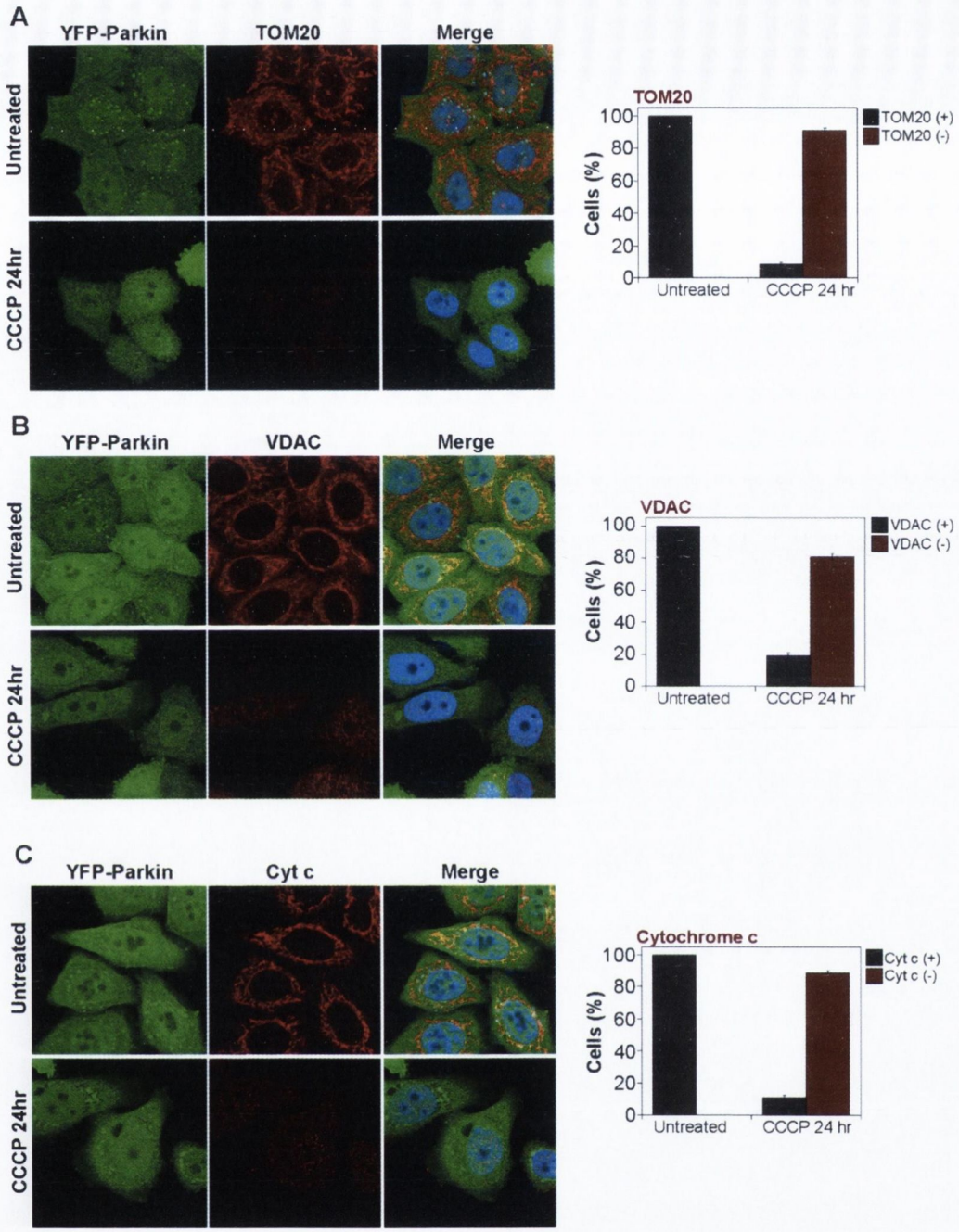

Figure 4.2 Parkin promotes the removal of damaged mitochondria

(A) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for 24 hr. Cells were immunostained for mitochondria (TOM20 – Red) and nuclei were stained with Hoechst (Blue). TOM20-negative cells were counted as a read-out for mitophagy. (B) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for 24 hr. Cells were immunostained for mitochondria (VDAC – Red) and nuclei were stained with Hoechst (Blue). VDAC-negative cells were counted as a read-out for mitophagy. (A) HeLa-YFP-Parkin cells were treated with CCCP (10 μM) for 24 hr. Cells were immunostained for mitochondria (Cyt c – Red) and nuclei were stained with Hoechst (Blue). Cyt c-negative cells were counted as a read-out for mitophagy. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

4.2.2 Parkin is activated by multiple mitochondrial depolarizors

Activation of Parkin also occurs in response to multiple mitochondrial toxins that depolarize the mitochondria similar to CCCP. HeLa-YFP-Parkin cells treated with the *Streptomyces* toxins, Valinomycin or a combination of Antimycin A and Oligomycin, all showed clear mitochondrial depolarization as indicated by Mitotracker staining (**Figure 4.3 A and C**). Furthermore, Valinomycin or the combination of Antimycin A and Oligomycin also led to rapid Parkin activation and translocation to mitochondria followed by decoration of the damaged mitochondria with Ubiquitin (**Figure 4.3 B, D and E**). As YFP is a substrate for Parkin, activation of Parkin E3 ligase activity can be measured by its autoubiquitination (**Figure 4.3 F**). It has been reported that the E3 ligase activity of Parkin can ubiquitinate intracellular pathogens and that Parkin knockout mice are more susceptible to infection. As Parkin requires an activation event, this may be provided by mitochondrial toxins released by pathogens (Manzanillo et al., 2013).

4.2.3 Bcl-2 family proteins antagonize Parkin translocation

Efficient removal of damaged mitochondria is vital to maintain the health of a cell. Damaged mitochondria release reactive oxygen species leading to oxidation of proteins or lipids and further cell damage. Uncontrolled release of reactive oxygen from depolarized mitochondria could ultimately lead to the untimely death of the cell.

To avoid this, depolarized or impaired mitochondria are removed from the healthy network through either increased fission or decreased fusion followed by autophagosome engulfment and removal (Gomes et al., 2011; Twig et al., 2008). As there have been many studies implicating the Bcl-2 family as regulators of mitochondrial fission/fusion as well as autophagy (Autret and Martin, 2009; Delivani et al., 2006; Karbowski et al., 2006; Pattingre et al., 2005; Sheridan et al., 2008), we wondered whether the Bcl-2 family could also play a role in regulating the removal of damaged mitochondria via PINK1/Parkin-dependent mitophagy. To explore this, we asked whether Bcl-2 family proteins could regulate Parkin translocation to depolarized mitochondria. HeLa-YFP-Parkin cells were transiently transfected with anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-X_L, Mcl-1 and Bcl-W) followed by CCCP treatment. As can be seen in **Figure 4.4**, overexpression of the Bcl-2 family efficiently blocked translocation of Parkin from the cytosol to the

mitochondria in response to CCCP. Furthermore, overexpression of the Bcl-2 family not only prevented Parkin translocation but also blocked mitophagy, even after 24 hr CCCP treatment (**Figure 4.5 A and B**). Together these data suggest that the anti-apoptotic members of the Bcl-2 family negatively regulate PINK1/Parkin-mediated mitophagy.

4.2.4 Endogenous Bcl-2 proteins are involved in regulating PINK1/Parkin-mediated mitophagy

To rule out the possibility that the inhibitory effect on mitophagy seen with Bcl-2 family proteins was as a result of overexpression artefacts, we then explored the role of endogenous Bcl-2 family members in mitophagy. As anti-apoptotic Bcl-2 family members can be neutralized by overexpression of the pro-apoptotic BH3-only family members, we initially used this approach to explore whether overexpression of BH3-only proteins could modulate Parkin translocation.

To this end, several BH3-only proteins (BAD, NOXA, BIM and PUMA) were co-expressed with mCherry-Parkin in the presence of zVAD-fmk to block apoptosis. Cells were then treated with CCCP and translocation of Parkin to mitochondria was analyzed by confocal microscopy. As shown in **Figure 4.6**, neutralization of endogenous anti-apoptotic Bcl-2 family members, through overexpression of BH3-only proteins, led to an increase in Parkin activation as measured through translocation of the latter from the cytosol to mitochondria.

As a second approach to investigate the role of endogenous Bcl-2 family members in mitophagy, we used the BH3-only mimetic, ABT-737. To this end, HeLa cells were transfected with cDNA encoding for mCherry-Parkin and treated with a titration of CCCP and a fixed dose of ABT-737 followed by confocal analysis (**Figure 4.7 A**). As demonstrated in **Figure 4.7 B**, neutralization of the anti-apoptotic Bcl-2 proteins using ABT-737 significantly increased the number of cells with mitochondrial Parkin and this observation was titratable (**Figure 4.7 C**). Similar results were obtained in HeLa-YFP-Parkin cells (**Figure 4.7 D – F**).

The atypical BH3-only proteins, BNIP3 and BNIP3L/Nix, have been implicated in PINK1/Parkin-independent mitophagy or in the regulation of autophagy (Beclin-1). Thus,

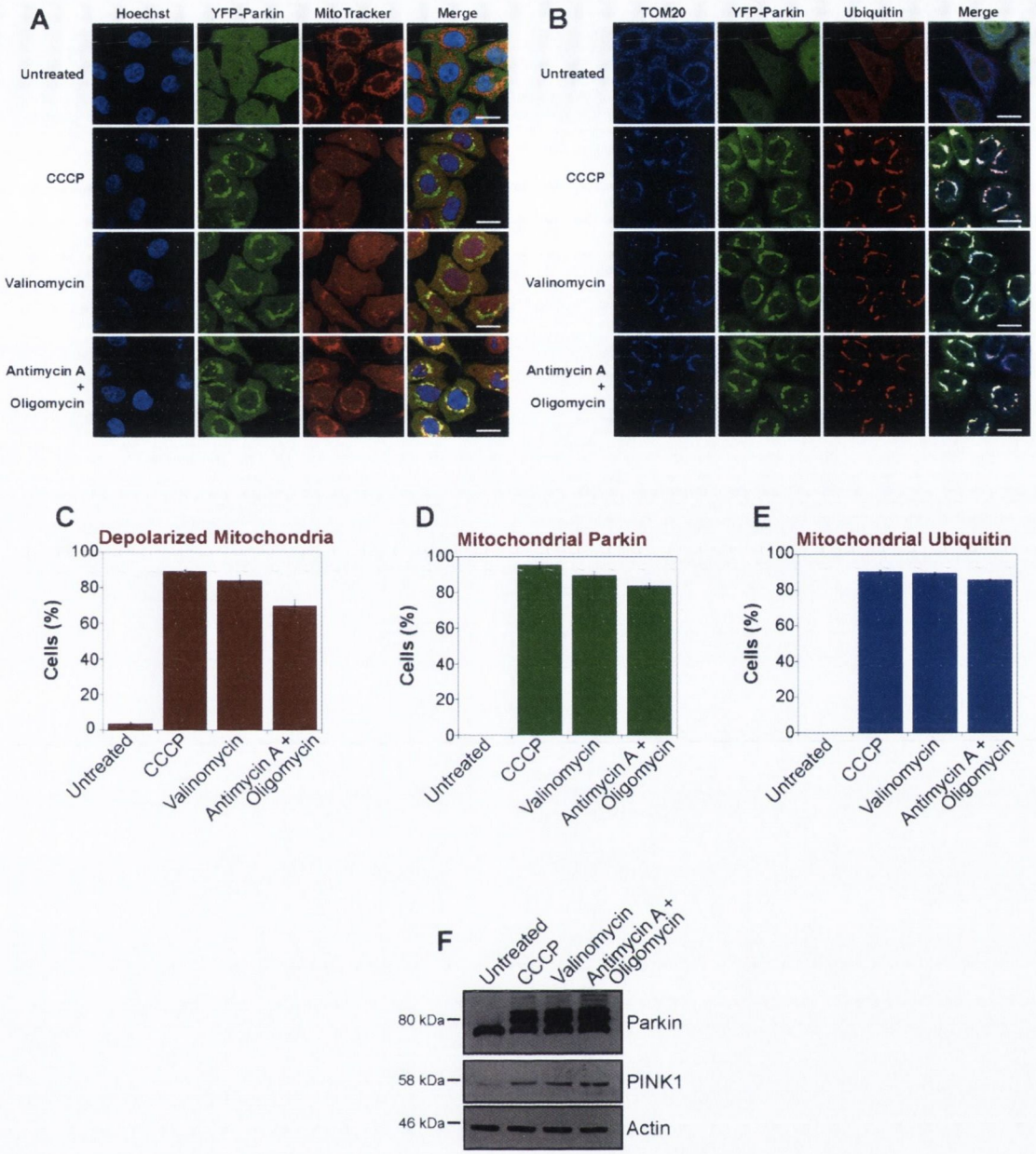

Figure 4.3 Parkin can be activated by multiple mitochondrial depolarizors

(A and C) HeLa-YFP-Parkin cells were treated CCCP (10 μM), Valinomycin (50 nM) or a combination of Antimycin A (500 nM) and Oligomycin (250 nM) for 3 hr. Cells were then stained for polarized mitochondria (Mitotracker – Red) and nuclei were stained with Hoechst (Blue). Cells were analyzed by confocal microscopy and (A) images were acquired or (C) polarized mitochondria were quantified. (B, D and E) HeLa-YFP-Parkin cells were treated CCCP (10 μM), Valinomycin (50 nM) or a combination of Antimycin A (500 nM) and Oligomycin (250 nM) for 3 hr. Cells were then stained for mitochondria (TOM20 - Blue) and ubiquitin (Red). Cells were analyzed by confocal microscopy and (B) images were acquired, (D) Parkin translocation to mitochondria was scored or (E) ubiquitination of mitochondria were quantified. (F) HeLa-YFP-Parkin cells were treated CCCP (10 μM), Valinomycin (50 nM) or a combination of Antimycin A (500 nM) and Oligomycin (250 nM) for 3 hr. Lysates were prepared and Parkin activation was analyzed by western blotting. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

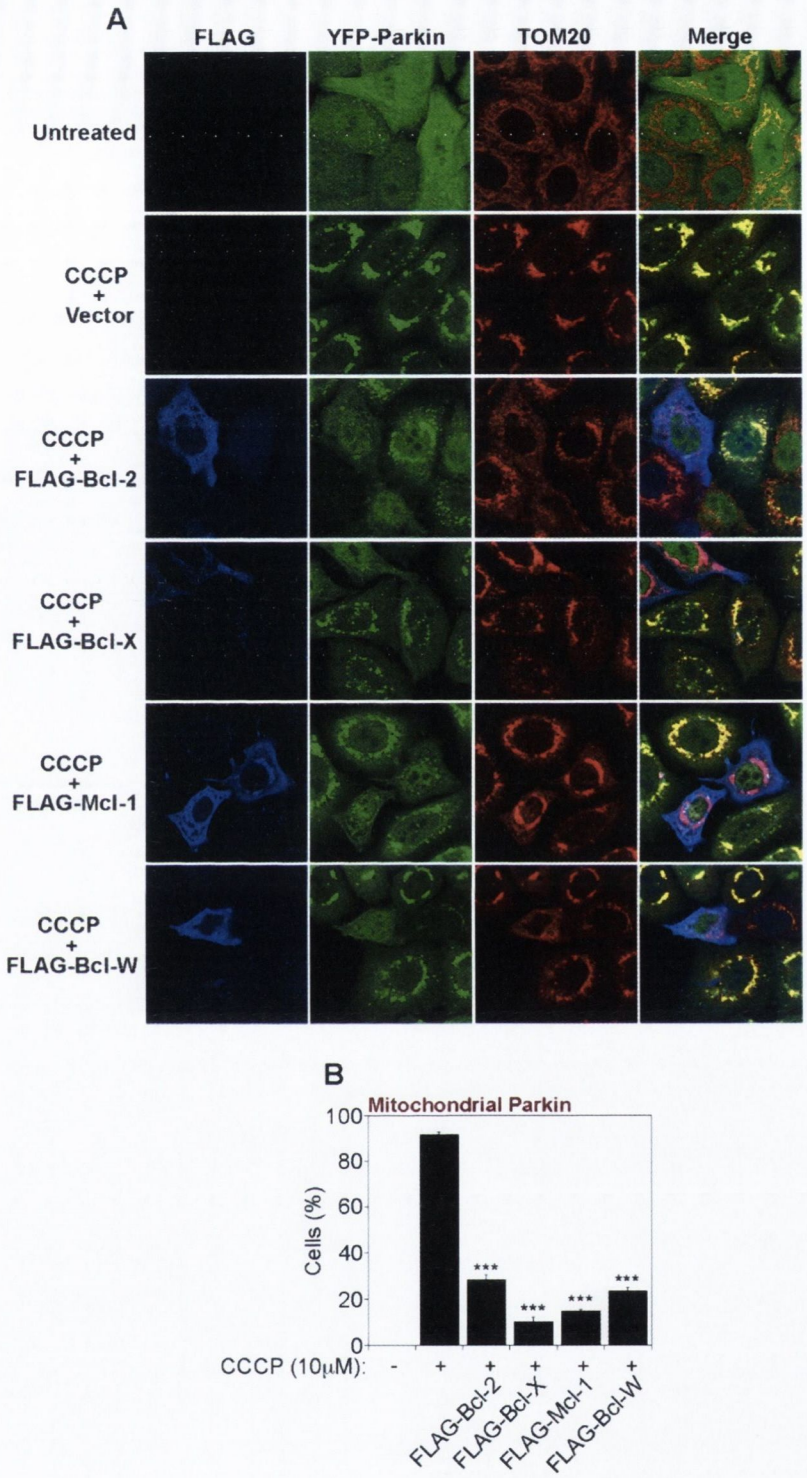

Figure 4.4 Bcl-2 family proteins antagonize Parkin translocation

(A and B) HeLa-YFP-Parkin cells were transfected with cDNA encoding for indicated Bcl-2 family member (500 ng) and incubated for 24 hr. Cells were then treated with CCCP (10 μM) for 3 hr. Cells were immunostained for mitochondria (TOM20 – Red) and Bcl-2 proteins (FLAG – Blue). Parkin translocation to mitochondria was then analyzed by confocal microscopy where (A) images were acquired and (B) translocation was quantified in Bcl-2-positive cells. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

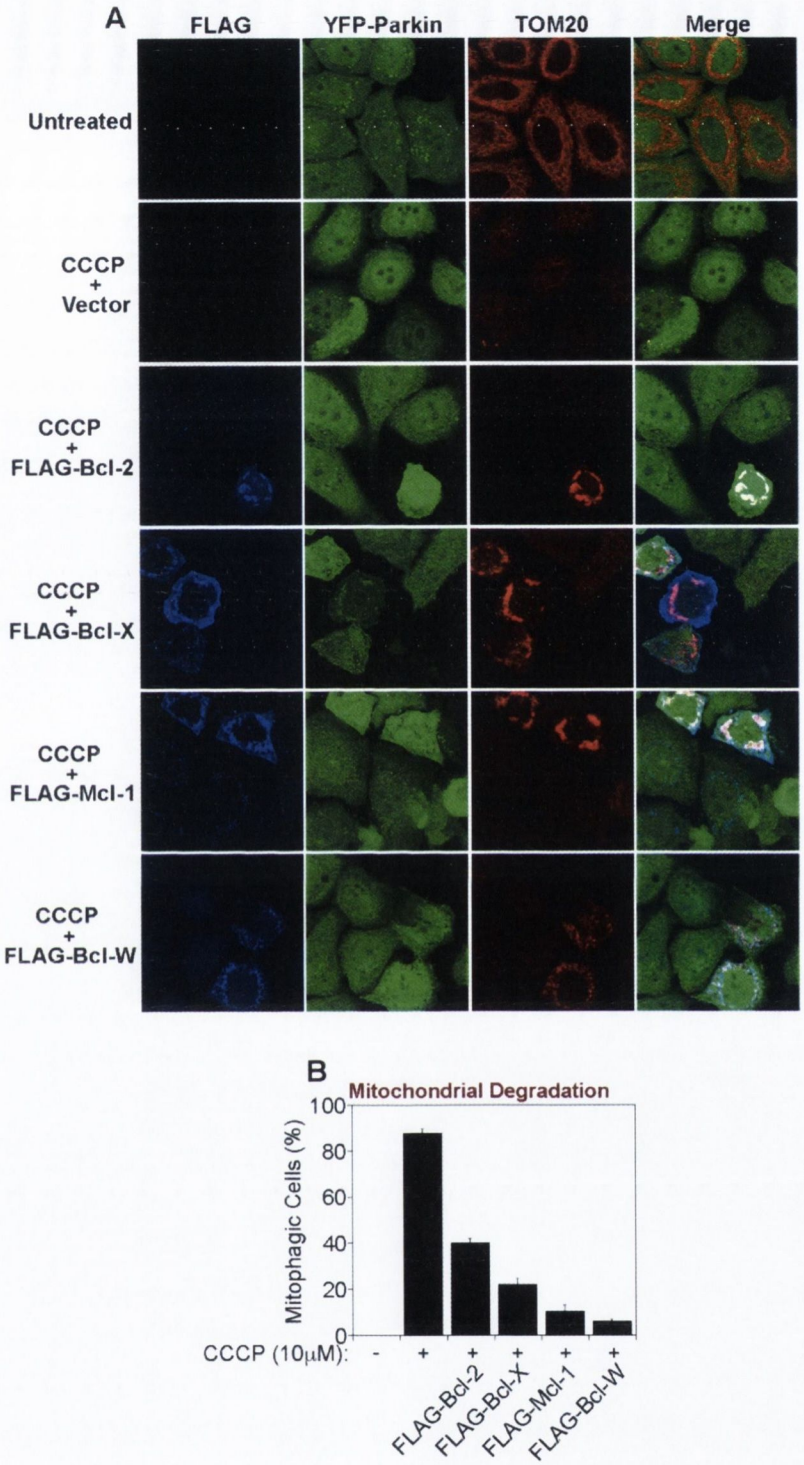

Figure 4.5 Bcl-2 family proteins antagonize Parkin-mediated mitophagy

(A and B) HeLa-YFP-Parkin cells were transfected with cDNA encoding for indicated Bcl-2 family member (500 ng) and incubated for 24 hr. Cells were then treated with CCCP (10 μM) for 24 hr. Cells were immunostained for mitochondria (TOM20 – Red) and Bcl-2 proteins (FLAG – Blue). Cells were analyzed by confocal microscopy and (A) images were acquired. (B) TOM20-negative cells were quantified among Bcl-2-postive cells. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

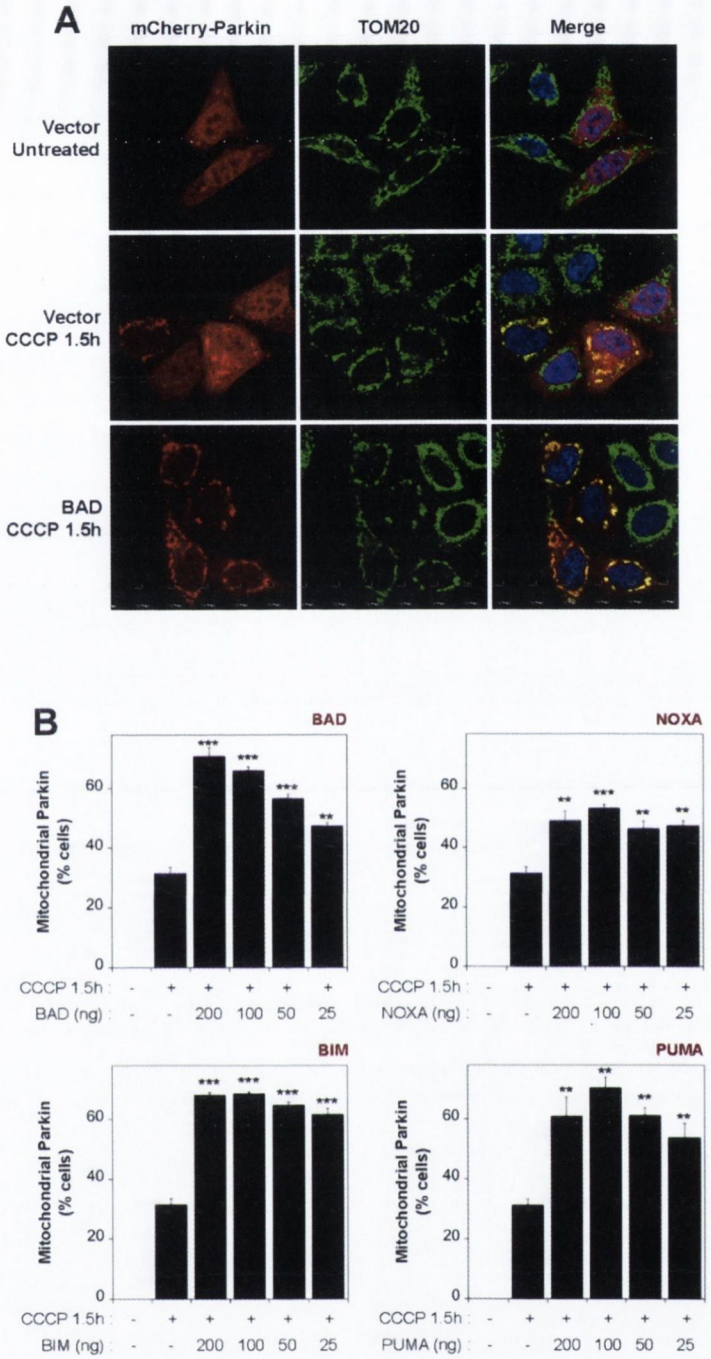

Figure 4.6 Parkin translocation to mitochondria is accelerated by BH3-only proteins

(A) Confocal images of HeLa cells transfected with 200 ng of mCherry-Parkin and 100 ng of Bad cDNA in the presence of zVAD-fmk (20 μM) for 8 h. Cells were then treated with CCCP (5 μM) for 1.5 h, followed by immunostaining for mitochondria (TOM20). (B) HeLa cells were transfected with 200 ng of plasmid encoding mCherry-Parkin and the indicated amount of BH3-only expression plasmid for 8 h in the presence of zVAD-fmk (20 μM). Cells were treated and immunostained as in (A) and colocalization between Parkin and mitochondria was scored in mCherry positive cells. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

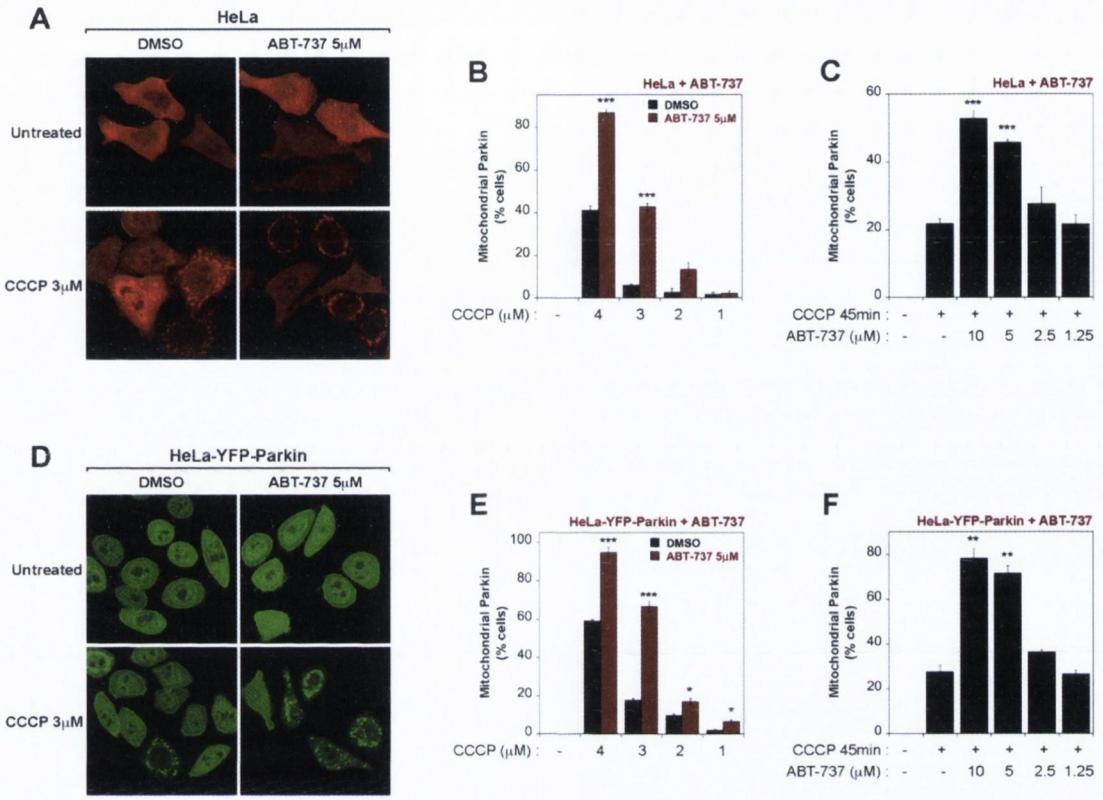

Figure 4.7 Parkin translocation to mitochondria is accelerated by the BH3-only mimetic ABT-737

(A) Confocal images of HeLa cells with 400 ng of mCherry-Pakin cDNA, pretreated with ABT-737 (5 μM) for 1 hr and then treated with CCCP (3 μM) for 45 mins. (B) HeLa cells were treated as in (A) and Parkin translocation to mitochondria was quantified. (C) HeLa cells were transfected with 400 ng of mCherry-Parkin cDNA, pretreatred with indicated dose of ABT-737 for 1 hr and then treated with CCCP (3 μM) for 45 min. Cells were scored for Parkin translocation to mitochondria. (D) Confocal images of HeLa cells transfected with 400 ng of YFP-Pakin, pretreated with ABT-737 (5 μM) for 1 hr and then treated with CCCP (3 μM) for 45 mins. (E) HeLa cells were treated as in (D) and Parkin translocation to mitochondria was scored. (F) HeLa cells were transfected with 400 ng of plasmid encoding for YFP-Parkin, pretreatred with indicated dose of ABT-737 for 1 hr and then treated with CCCP (3 μM) for 45 min. Cells were scored for Parkin translocation to mitochondria in YFP-positive cells. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

we wished to investigate a possible role for these proteins in PINK1/Parkin-dependent mitophagy. To explore this, HeLa cells were transfected with cDNAs encoding the atypical BH3-only family members along with YFP-Parkin (BAD cDNA was used as a positive control). As can be seen in **Figure 4.8 A-C**, none of the atypical BH3-only family members had a significant effect on Parkin translocation, in contrast with overexpression of BAD. These results demonstrate a specific role for endogenous Bcl-2 family proteins in regulating PINK1/Parkin-mediated mitophagy.

4.2.5 Ablation of anti-apoptotic Bcl-2 family members enhances Parkin translocation while ablation of BH3-only proteins has no effect

We also wished to explore whether knockdown of endogenous Bcl-2 family proteins could modulate Parkin translocation to mitochondria. As shown in **Figure 4.9 A**, anti-apoptotic Bcl-2 family members were efficiently ablated. As can be seen in **Figure 4.9 B**, knockdown of anti-apoptotic Bcl-2 proteins were significantly reduced by specific siRNAs and a functional effect was observed as knockdown of the latter sensitized HeLa cells to the pro-apoptotic agents, Fas and Daunorubicin. While knockdown of individual members of the anti-apoptotic Bcl-2 family significantly sensitized to apoptosis, they did not enhance Parkin translocation to mitochondria in response to CCCP. However, when several members of the family were knocked down in combination (with Mcl-1 excluded) there was a significant enhancement of Parkin translocation to mitochondria in response to CCCP (**Figure 4.9 C**). This suggests that there is redundancy among the anti-apoptotic Bcl-2 family in their ability to regulate Parkin translocation to mitochondria and that multiple members of the family play a role in regulating PINK1/Parkin-mediated mitophagy.

We also wished to explore the possibility that BH3-only proteins might also regulate mitophagy as our approach of overexpressing anti-apoptotic Bcl-2 family proteins could also act to neutralize endogenous levels of BH3-only proteins. In contrast, knockdown of BH3-only proteins either singly or in combination failed to modulate Parkin translocation too mitochondria to any significant degree (**Figure 4.10 A and B**). This clearly demonstrates that it is the anti-apoptotic Bcl-2 family proteins which regulate Parkin activity and its translocation to damaged mitochondria during PINK1/Parkin-mediated mitophagy.

4.2.6 Bcl-2 inhibits mitophagy downstream of PINK1 stabilization and is independent of mitochondrial depolarization

The Bcl-2 family regulate autophagy, mitochondrial fission/fusion and mitochondrial dynamics to maintain the fitness of mitochondrial networks (Autret and Martin, 2009), so it is conceivable that overexpression of Bcl-2 family members prevent Parkin activation due to blocking depolarization induced by mitochondrial toxins.

However, this was not found to be the case as HeLa cells transfected with cDNA encoding Bcl-2 family proteins and Parkin failed to prevent mitochondrial depolarization in response to CCCP, as indicated by Mitotracker, a mitochondrial potential sensitive dye (**Figure 4.11**).

In healthy mitochondria, PINK1 stability is regulated by its import into the inter-mitochondrial membrane via the TOM complex and degradation by the protease, PARL. To rule out the possibility of the Bcl-2 family operating upstream of PINK1, we explored the effects of Bcl-2 proteins on CCCP-induced PINK1 stabilization. We also wished to determine whether Beclin-1, a known Bcl-2 interacting protein (Pattingre et al., 2005) was required for activation of Parkin and its translocation to mitochondria. To investigate this, HeLa-YFP-Parkin cells were transfected with siRNAs targeted against either PINK1 or Beclin-1 (**Figure 4.12 A**). Cells were then treated with CCCP for 2 hours and Parkin translocation to the mitochondria was analyzed by confocal microscopy. As shown in **Figure 4.12 B and C**, while knockdown of PINK1 effectively blocked Parkin activation and translocation, knockdown of Beclin-1 had no effect. This allows us to deduce that the Bcl-2 family proteins functioned downstream of PINK1 as their overexpression does not prevent accumulation of PINK1 on damaged mitochondria (Hollville et al., 2014). **Figure 4.12 B and C** also demonstrates that regulation of mitophagy by the Bcl-2 family is independent of their role in regulating autophagy via Beclin-1. This confirms that PINK1/Parkin-dependent mitophagy is Beclin-1-independent.

4.2.7 Bcl-2 Family Members interact with the PINK1/Parkin Complex

The preceding experiments have shown that Bcl-2 family members suppress translocation of Parkin to depolarized mitochondria and block their subsequent degradation via mitophagy. We also found that Bcl-2 family proteins do not prevent PINK1 accumulation on damaged mitochondria.

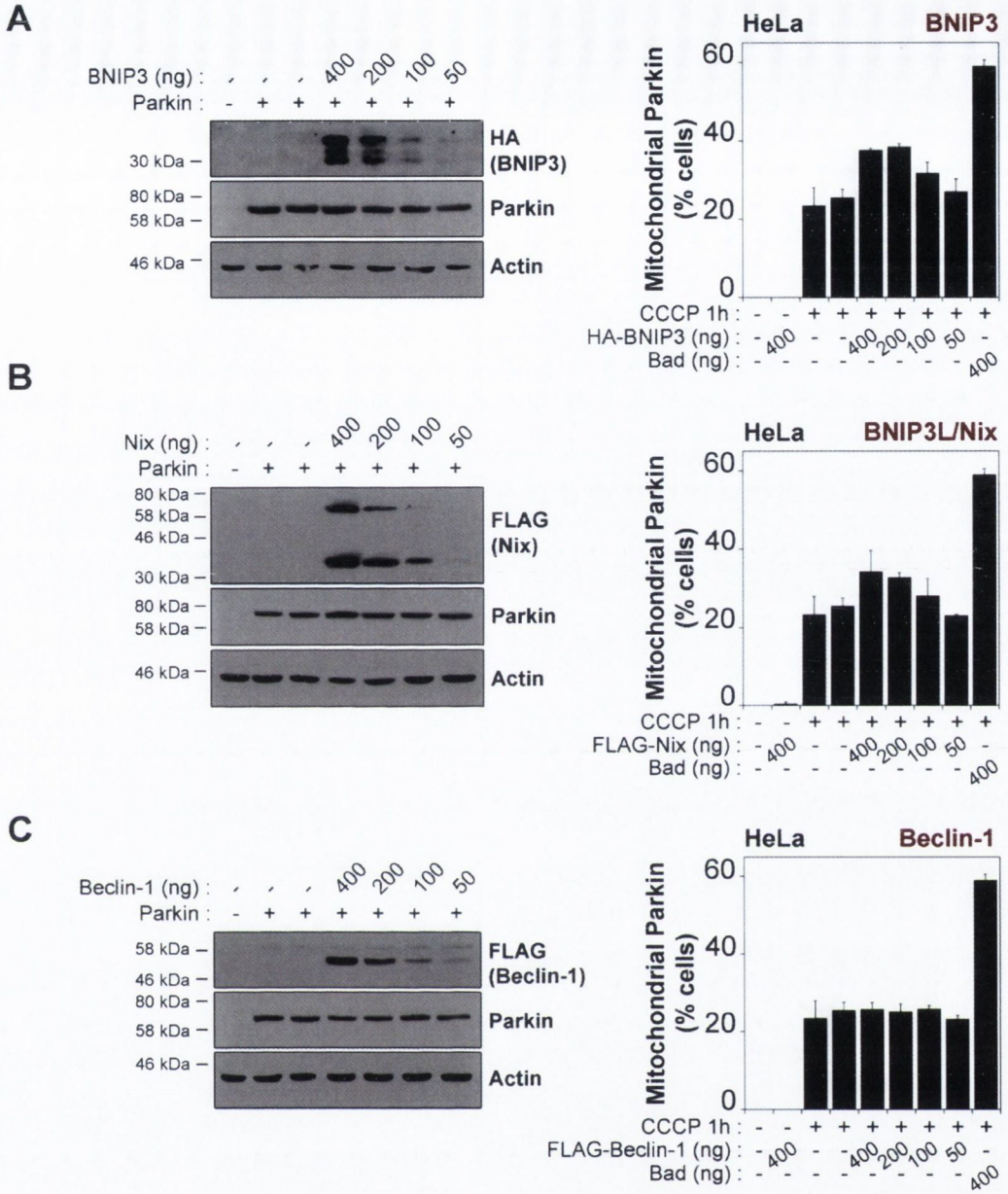

Figure 4.8 Atypical BH3-only proteins BNIP3, BNIP3L/Nix and Beclin-1 are not involved in Parkin translocation to mitochondria

(A) HeLa cells were transfected with plasmid encoding for YFP-Parkin (200 ng) and indicated amounts of HA-BNIP3. Cells were then treated with CCCP (4 μM) for 1 hr and translocation of Parkin to mitochondria was scored in YFP-positive cells. (B) HeLa cells were transfected with plasmid encoding for YFP-Parkin (200 ng) and indicated amounts of FLAG-BNIP3L/Nix. Cells were then treated with CCCP (4 μM) for 1 hr and translocation of Parkin to mitochondria was scored in YFP-positive cells. (C) HeLa cells were transfected with plasmid encoding for YFP-Parkin (200 ng) and indicated amounts of FLAG-Beclin-1. Cells were then treated with CCCP (4 μM) for 1 hr and translocation of Parkin to mitochondria was scored in YFP-positive cells. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

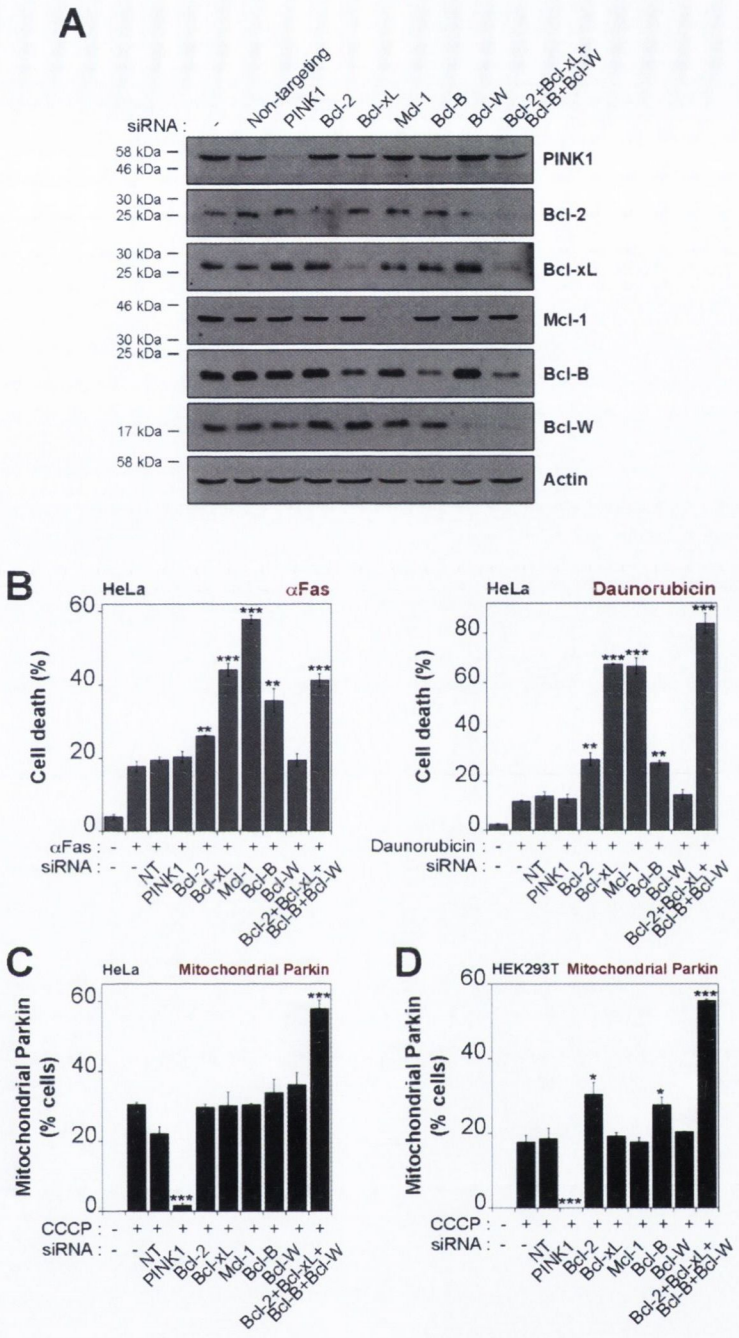

Figure 4.9 Ablation of Bcl-2 family proteins enhances Parkin translocation

(A) HeLa cells were transfected with the indicated siRNAs (200 nM), incubated for 48 hr and cell lysates were analyzed for specific knockdown. (B) HeLa cells transfected with the indicated siRNA (200 nM), as in (A), were treated with 200 ng/ml of anti-Fas for 10 hr or 1 μM of Daunorubicin for 12 hr. Apoptosis was assessed based on morphology. (C) HeLa cells were transfected with the indicated siRNA (200 nM), along with 100 ng of cDNA encoding for Parkin. After 48 hr, cells were then treated with CCCP (4 μM) for 1 hr. Cells were immunostained for Parkin and mitochondria (TOM20 – green) and Parkin translocation to mitochondria was quantified. (D) HEK293T cells were transfected as in (C), followed by treatment with CCCP (10 μM) for 45 min. Cells were immunostained for Parkin and mitochondria (TOM20 – green) and Parkin translocation was quantified. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

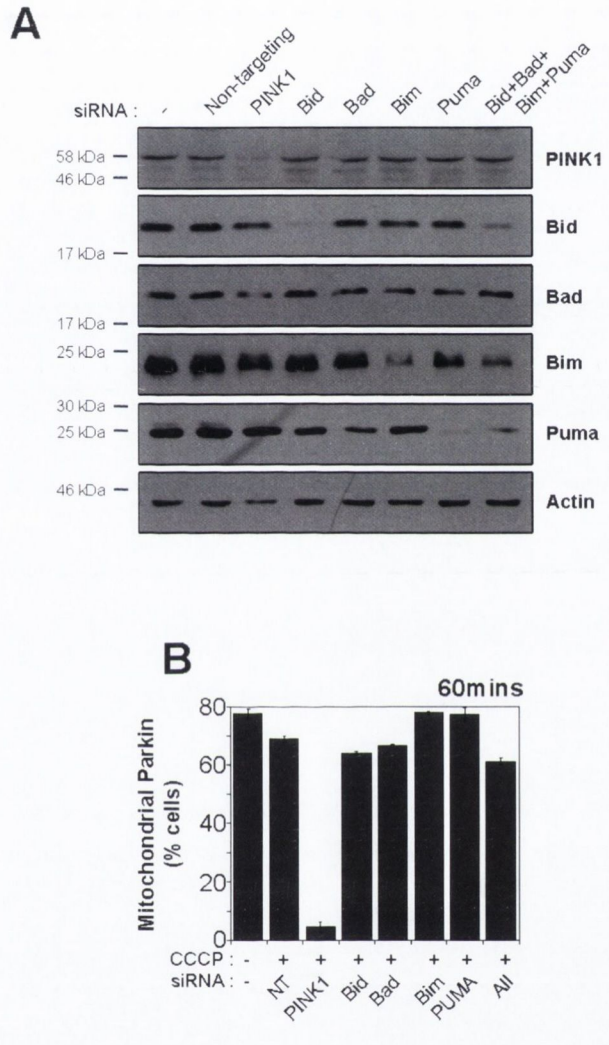

Figure 4.10 Ablation BH3-only protein ablation fails to modulate Parkin translocation.

(A) HeLa cells were transfected with indicated siRNA (200 nM) and incubated for 48 hr. Knockdown efficiency was analyzed by western blotting. (B) HeLa cells were transfected with indicated siRNA (200 nM) along with plasmid encoding for Parkin (100 ng) for 48 hr. Cells were treated with CCCP (5 μM) for 1 hr before being immunostained for Parkin and mitochondria (TOM20 – green). Colocalization between Parkin and mitochondria was then scored. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

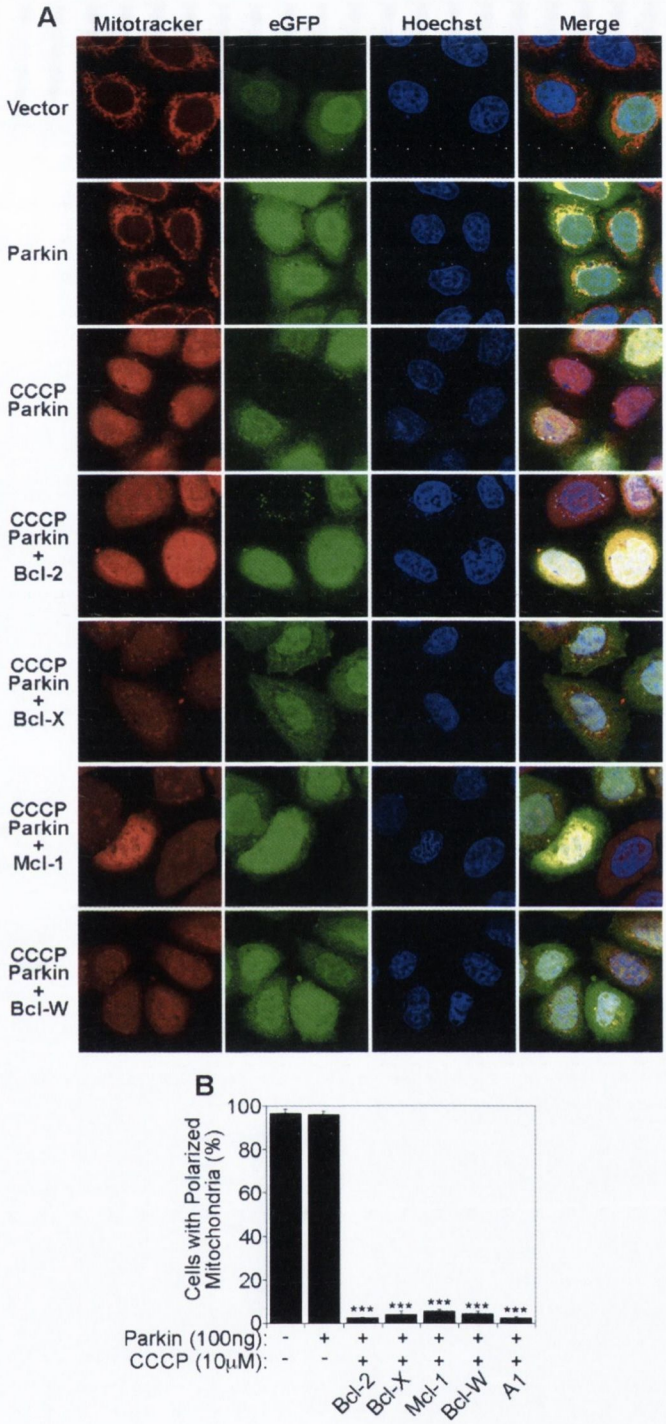

Figure 4.11 Bcl-2 family proteins do not prevent mitochondrial depolarization

(A and B) HeLa-YFP-Parkin cells were transfected with cDNA encoding for indicated Bcl-2 family member (500 ng) along with plasmid encoding for eGFP (50 ng) and incubated for 24 hr. Cells were then treated with CCCP (10 μM) for 3 hr. Cells were stained for polarized mitochondria (Mitotracker – Red) and nuclei were stained with Hoechst (Blue). Cells were analyzed by confocal microscopy and (A) images were acquired or (B) polarized mitochondria were scored in GFP-positive cells. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) 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, *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$.

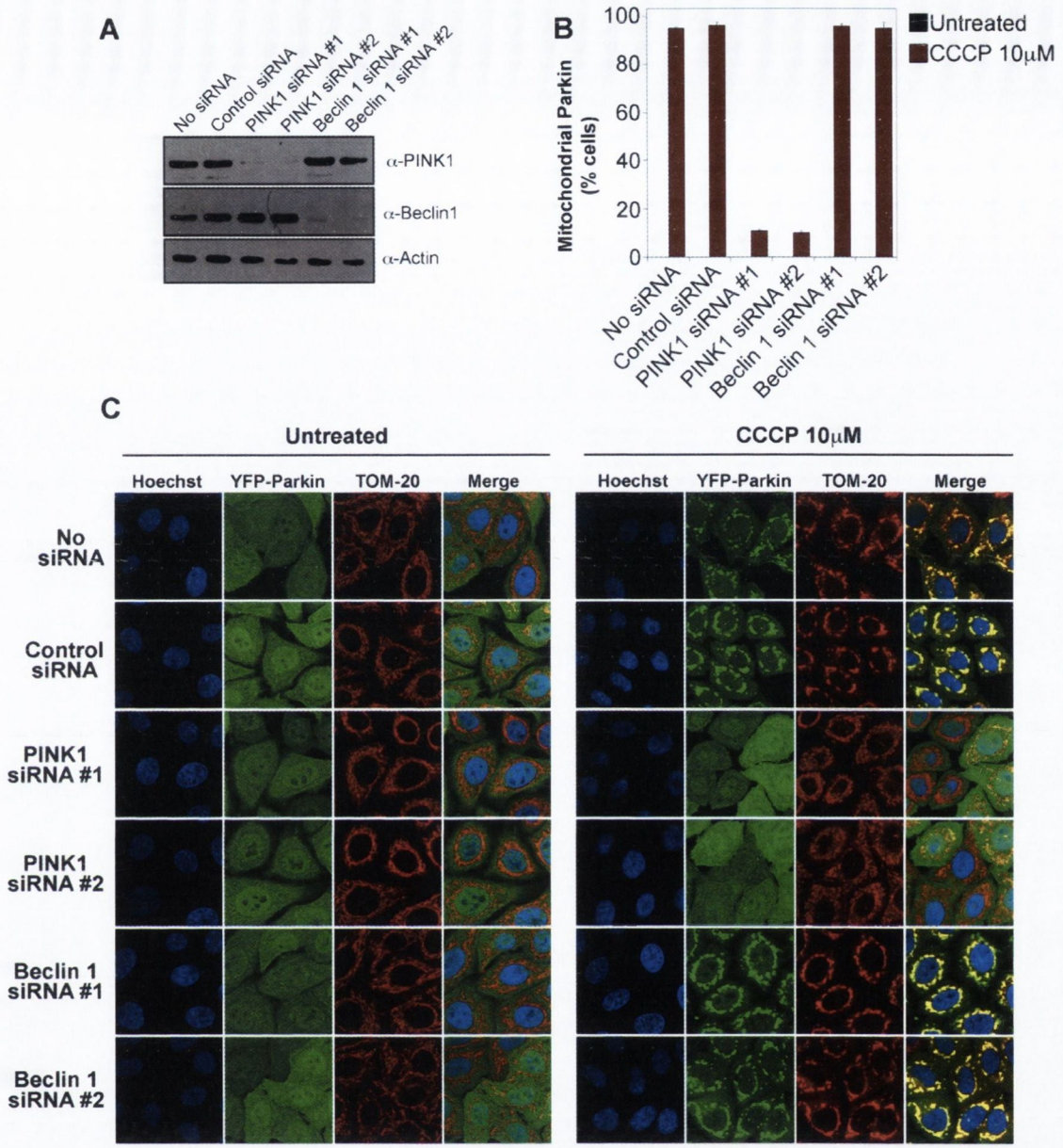

Figure 4.12 PINK1 is required for Parkin translocation to the mitochondria

(A) HeLa-YFP-Parkin cells were nucleofected with indicated siRNA (200 nM) and incubated for 48 h. Cells were lysed and protein knockdown was analyzed by western blot. (B) HeLa-YFP-Parkin cells were nucleofected as in (A) and treated with CCCP (10μM) for 2 hr and YFP-Parkin translocation to the mitochondria was quantified or (C) images acquired. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

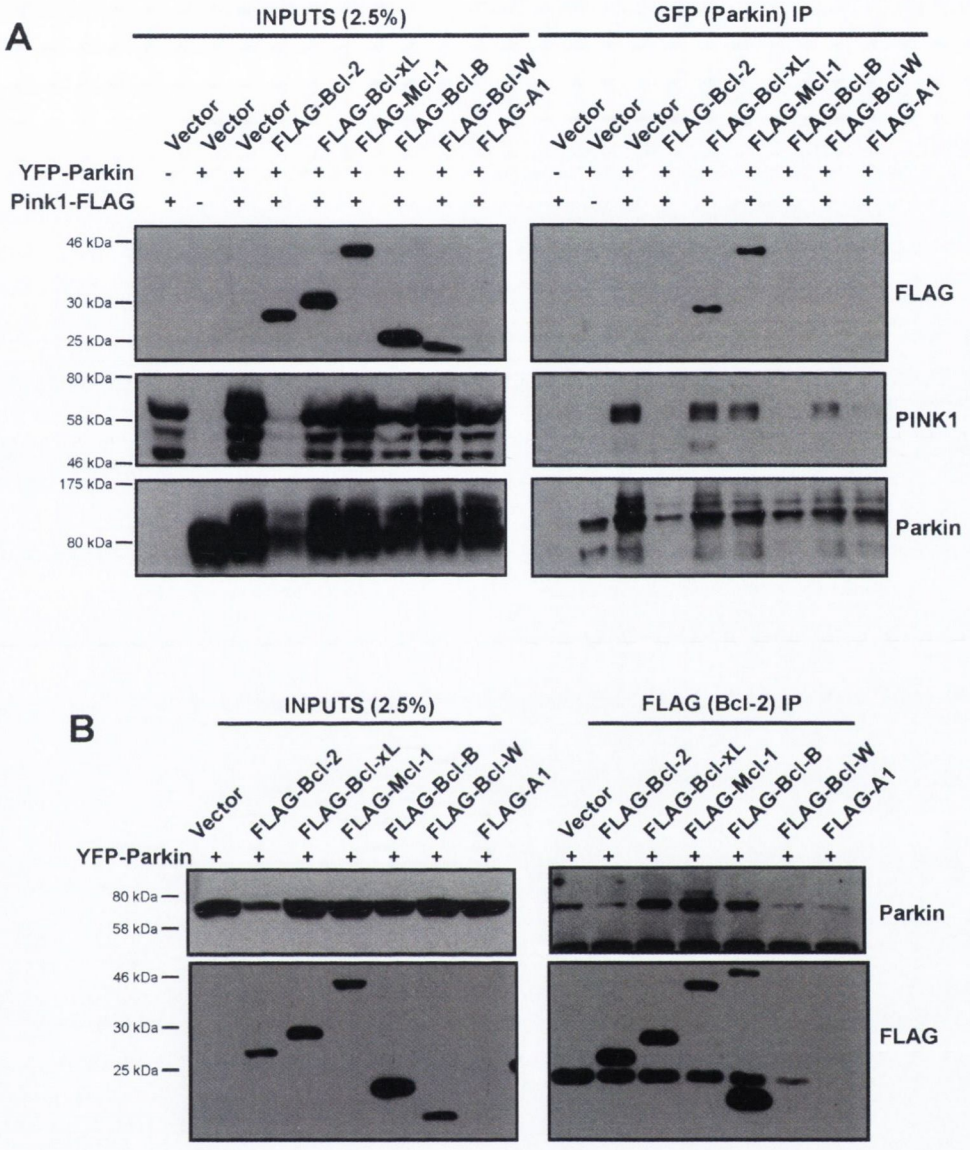

Figure 4.13 Bcl-2 family proteins directly interact with Parkin

(A) HEK293T were transfected with plasmid encoding for YFP-Parkin (3 μg), PINK1 (3 μg) and indicated Bcl-2 family member (3 μg) for 24 hr. Parkin was then immunoprecipitated with anti-GFP antibody (1 μg), followed by western blotting for PINK1 and Bcl-2 family members (FLAG) to investigate specific interaction. (B) HEK293T were transfected with plasmid encoding for YFP-Parkin (3 μg) and indicated Bcl-2 family member (3 μg) for 24 hr. Bcl-2 family proteins were then immunoprecipitated with anti-FLAG antibody (1 μg), followed by western blotting for Parkin to investigate specific interaction.

As PINK1 is the kinase responsible for the activation of Parkin and its subsequent translocation to mitochondria, this places the inhibitory effects of the Bcl-2 family at the PINK1/Parkin interaction. One possibility is that Bcl-2 family proteins antagonize these events through direct interaction. In order to test this, co-immunoprecipitation experiments were performed in 293T overexpressing PINK1, Parkin and the Bcl-2 family proteins.

Immunoprecipitation of Parkin showed a specific interaction between Parkin, PINK1 and Bcl-X_L or Mcl-1 (**Figure 4.13 A**). The reciprocal immunoprecipitations showed a specific interaction between Bcl-X_L, Mcl-1 and Bcl-B and Parkin (**Figure 4.13 B**).

These data suggest that Bcl-2 family proteins antagonize the recruitment of Parkin to damaged mitochondria through binding and preventing PINK1 and Parkin interactions. We suggest that the abundance of Bcl-2 family proteins and expression of specific family members would therefore set a threshold for recruitment to depolarized mitochondria which must be exceeded for mitophagy to occur in different cell types. This would provide a mechanism for preventing deregulated and unnecessary mitophagy of healthy mitochondria within a cell.

4.3 Discussion

Here, we have shown that members of the Bcl-2 family can regulate mitophagy through influencing the translocation of Parkin to depolarized mitochondria. Bcl-xL, Mcl-1 and other members of the pro-survival subset of the Bcl-2 family were found to directly interact with the Parkin/PINK1 complex and to impede stable recruitment of Parkin 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 impact on 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 also regulate the clearance of impaired mitochondria via mitophagy.

Accumulating evidence suggests that the role of Bcl-2 family proteins in cellular homeostasis is much broader than previously appreciated (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, as well as the pro-survival 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 (Delivani et al., 2006; Martinou and Youle, 2011; 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 (Gomes et al., 2011; Rambold et al., 2011; Twig et al., 2008). 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 (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 (Elgendy et al., 2011; Pattingre et al., 2005).

It is striking that many of the processes that members of the Bcl-2 family have been linked with 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), connectivity (via effects on fission/fusion dynamics), turnover (via mitophagy), as well as ATP synthesis (via effects on the respiratory chain). It is also highly compelling that perturbation of many of these processes individually can impact on the others. For example, inhibition of autophagy is well known to sensitize towards apoptosis, conversely, inhibition of apoptosis increases the likelihood of autophagy (Maiuri et al., 2007b). Similarly, mitochondrial networks fragment during apoptosis and enforced mitochondrial fusion has been reported to delay apoptosis (Autret and Martin, 2009).

It is perhaps highly significant that Bcl-2 family proteins have been implicated as regulators of all of the processes described above, suggesting an overarching role for members of this family as regulators of mitochondrial fitness. Thus, Bcl-2 family proteins may police mitochondrial network integrity, 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, 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.

There is a growing body of evidence which places a much greater importance on the role of the Bcl-2 family in maintenance and monitoring of both mitochondrial and cellular homeostasis (Autret and Martin, 2009). The role of the Bcl-2 proteins in the regulation

and initiation of apoptosis is clearly established, in which their role in regulating the permeability of the outer mitochondrial membrane is of vital importance. In addition to this, the Bcl-2 family have been demonstrated to regulate mitochondrial fission and fusion dynamics which may be important in isolation of damaged mitochondria through increased fission or decreased fusion. The Bcl-2 family also regulates autophagy through their interaction with Beclin-1 and can also regulate PINK1/Parkin-independent forms of mitophagy in erythrocytes.

Here, we demonstrate a novel function of the Bcl-2 in yet another aspect of mitochondrial biology where the Bcl-2 family negatively regulates PINK1 and Parkin-dependent mitophagy. The increasing number of cell and mitochondrial processes which are regulated by the Bcl-2 family suggests that the Bcl-2 family are likely to function as global regulators of mitochondrial network homeostasis rather than just regulators of apoptosis.

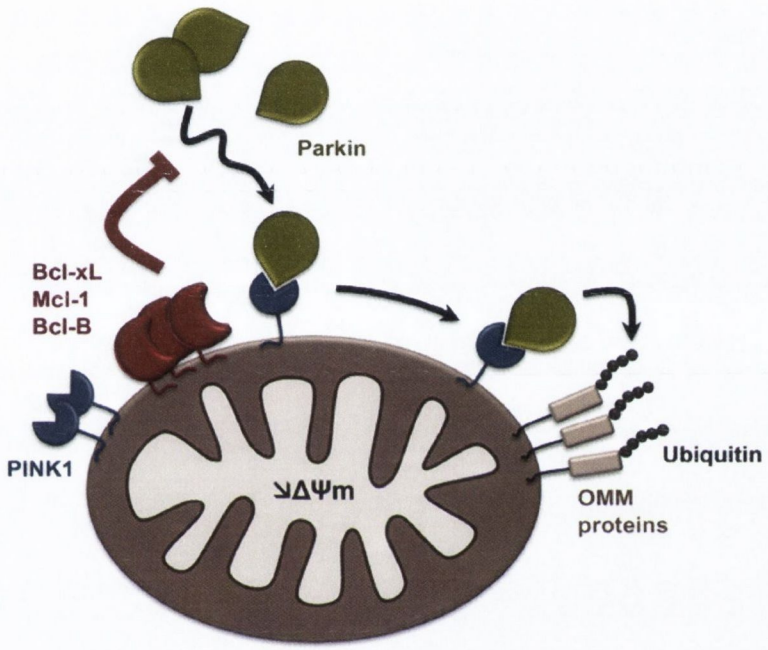

Figure 4.14 Proposed model for Bcl-2 inhibition of Mitophagy

After mitochondrial damage/depolarization, PINK1 accumulates on the outer mitochondrial membrane. PINK1 phosphorylates and activates Parkin in the cytosol inducing its translocation to the damaged mitochondria. The Bcl-2 family can interact with Parkin and prevent Parkin translocation to the mitochondria in a method similar to their prevention of Bax translocation to the mitochondria.

Chapter V

Parkin Sensitizes Towards Apoptosis Induced by Mitochondrial Depolarization Through Promoting Degradation of Mcl-1

5.1 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., 2010a). 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 (Berger et al., 2009; Darios et al., 2003; Ekholm-Reed et al., 2013; MacCormac et al., 2004; Sun et al., 2013; Wang et al., 2013a; Yang et al., 2005). It has been suggested that Parkin blocks cell death in response to a variety of stimuli. .However, many of these studies utilized pro-apoptotic 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 (Muller-Rischart et al., 2013). Furthermore, the latter report also suggested, rather surprisingly, that Parkin inhibits apoptosis in a PINK1-independent manner (Muller-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, which are well established to promote Parkin activation during mitophagy (Narendra et al., 2008; Van Humbeeck et al., 2011; Vives-Bauza et al., 2010). In sharp contrast to the claims that Parkin suppresses apoptosis, here we report that Parkin dramatically sensitized cells towards 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 pro-apoptotic 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 pro-survival 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.

5.2 Results

5.2.1 Parkin fails to suppress apoptosis in response to diverse pro-apoptotic stimuli

HeLa cells are sensitive to numerous pro-apoptotic 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; Sheridan et al., 2008). 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 anti-apoptotic 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 pro-apoptotic stimuli. However, as shown in **Figures 5.1 A and B**, Parkin failed to provide any protection towards apoptosis induced by Fas, TRAIL, cisplatin, etoposide, daunorubicin or actinomycin D. In sharp contrast, Bcl-xL exhibited robust anti-apoptotic effect towards all of the pro-apoptotic stimuli used (Figures 5.1 A and B). Surprisingly however, transient expression of Parkin dramatically sensitized towards apoptosis induced by the mitochondrial depolarizer CCCP (**Figures 5.1 A and B**).

5.2.2 Parkin selectively sensitizes towards mitochondrial depolarization-initiated apoptosis

To ask whether the increased sensitivity towards mitochondrial depolarization-induced apoptosis was reproducible in other cell types, we repeated the preceding experiments in two additional cell lines. As illustrated in **Figures 5.2 A** and **B,** Parkin failed to exhibit any anti-apoptotic activity towards a diverse panel of extrinsic or intrinsic pro-apoptotic stimuli in either MCF-7 or SH-SY5Y cells.

However, once again, there was a clear sensitization towards apoptosis induced by mitochondrial depolarization (CCCP) in both cell lines (**Figures 5.2 A and B**). Of the cells that failed to die after exposure to mitochondrial depolarization agents after 24 h, the majority of these displayed features of mitophagy (**Figure 5.3 A and B**).

Of note, the sensitizing effects of Parkin towards mitochondrial depolarization-induced apoptosis was highly selective and was not observed in response to any of the other pro-apoptotic stimuli used (**Figures 5.1 A, 5.2 A and B**). Furthermore, the pro-apoptotic effects of Parkin were readily observed over a wide range of CCCP concentrations (**Figure 5.4 A**). 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 5.4 B**, Parkin also greatly increased sensitivity towards apoptosis induced by the Streptomyces toxins valinomycin, as well as the combination of Oligomycin and Antimycin A (**Figure 5.4 C**). 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 4.3 F**), as well as Parkin translocation to mitochondria (**Figures 4.3 A-E**). In contrast, treatment with Fas, TRAIL, cisplatin, daunorubicin, etoposide or actinomycin D, failed to activate Parkin or stabilize PINK1 (**Figure 5.5 A and B**). Thus, expression of Parkin sensitized three independent cell lines towards apoptosis induced by mitochondrial depolarization, but not other pro-apoptotic stimuli.

Parkin-dependent cell death in response to mitochondrial depolarization exhibited stereotypical features of apoptosis, such as activation of multiple pro-apoptotic caspases and proteolysis of caspase substrates (**Figure 5.6 A**), as well as DNA fragmentation (**Figure 5.6 B**). Furthermore, Parkin-dependent apoptosis induced by mitochondrial depolarization was readily attenuated with the poly-caspase inhibitor Q-VD (**Figure 5.7 A and B**).

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

5.2.3 Mitochondrial depolarization-induced apoptosis is enhanced by Parkin in a PINK1-dependent manner

As shown in **Figure 5.8 A**, PINK1 is stabilized in response to mitochondrial depolarization (Narendra et al., 2010b; Vives-Bauza et al., 2010). However, PINK1 stabilization on its own, was not sufficient to sensitize towards apoptosis induced by CCCP (**Figures 5.8 A and**

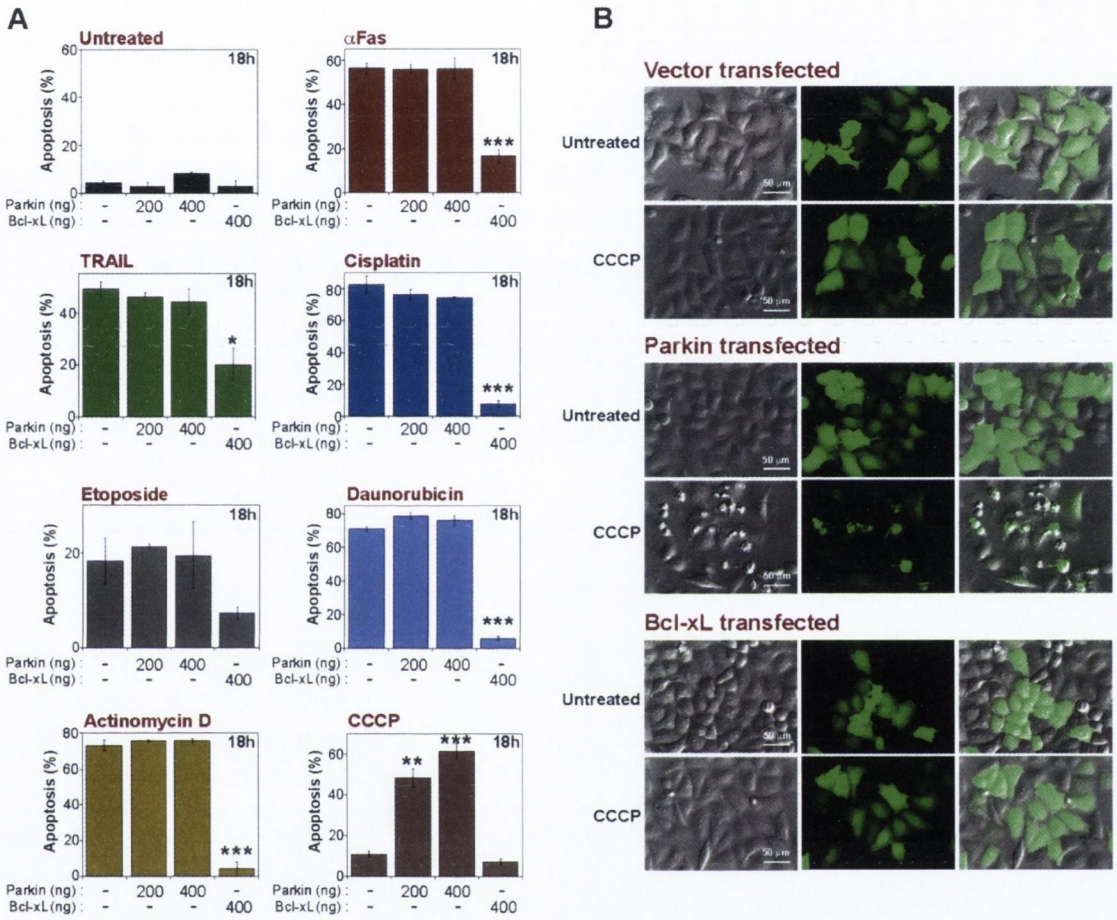

Figure 5.1 Parkin enhances mitochondrial depolarization-initiated cell death.

(A and B) HeLa cells were transfected with a plasmid encoding for eGFP (50 ng) along with the indicated amount of Parkin or Bcl-X_L cDNA for 24 hr. Cells were treated with anti-Fas CH11 (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 then quantified in GFP-positive cells based on cell morphology by phase contrast microscopy (A) and images were acquired (B). Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

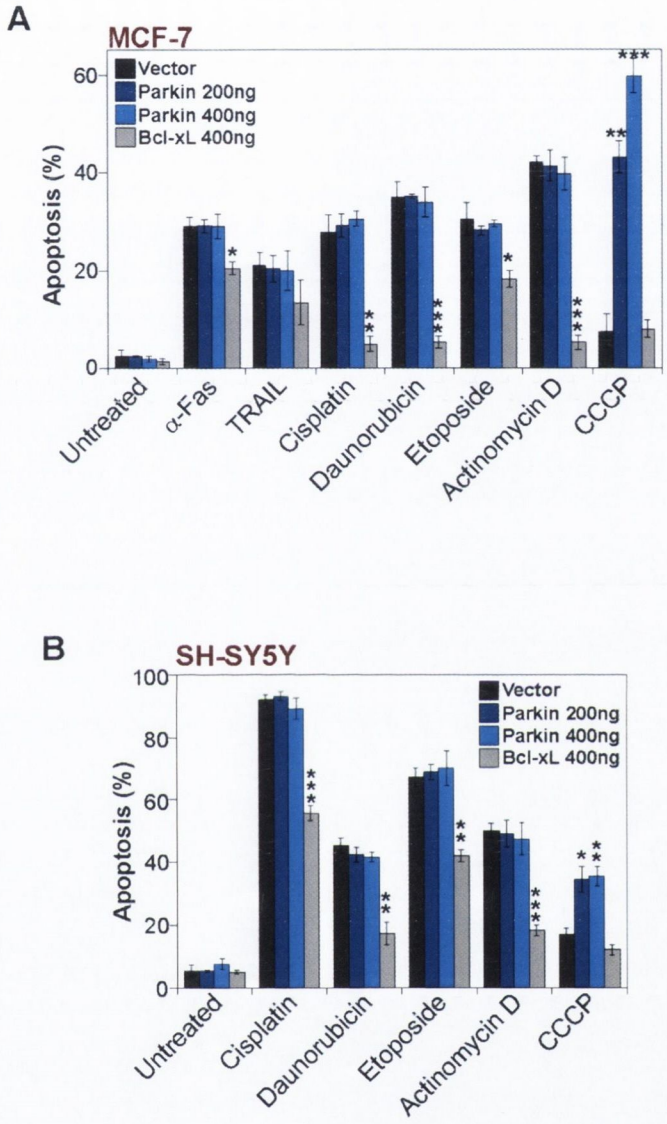

Figure 5.2 Parkin enhances mitochondrial depolarization-initiated cell death in multiple cell types

(A and B) MCF-7 (A) and SH-SY5Y (B) were transfected as in (A) and treated with anti-Fas CH11 (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 scored among GFP-positive cells based on cell morphology. Results from a representative experiment are shown. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

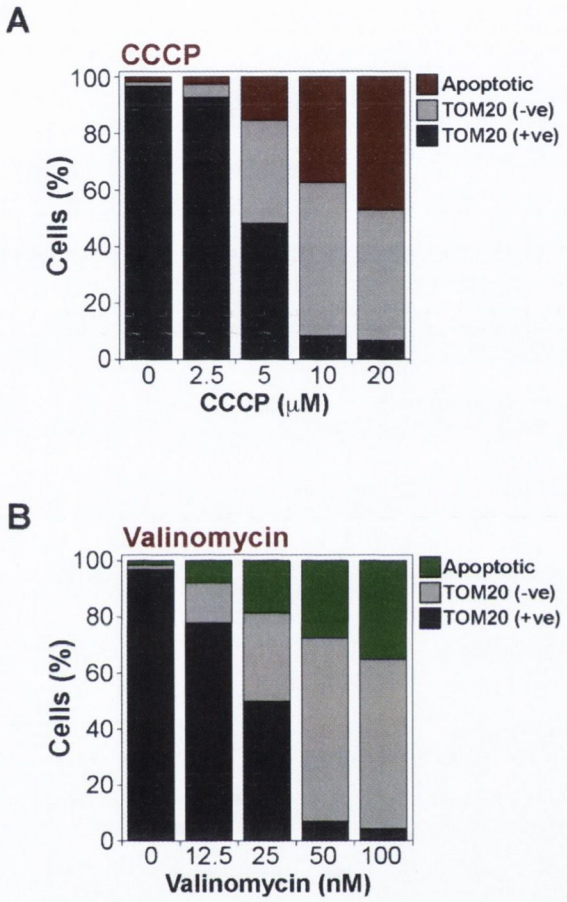

Figure 5.3 Mitophagy versus Cell death

(A and B) HeLa-YFP-Parkin cells were treated with indicated doses of either (A) CCCP or (B) Valinomycin. Cytospins of cells were prepared after 24 hours of treatment and cells were stained with TOM20 to identify mitochondria. Cells were then scored for either apoptotic based on morphology or mitophagic (TOM20-negative). (Figure kindly provided by Dr. Emilie Hollville).

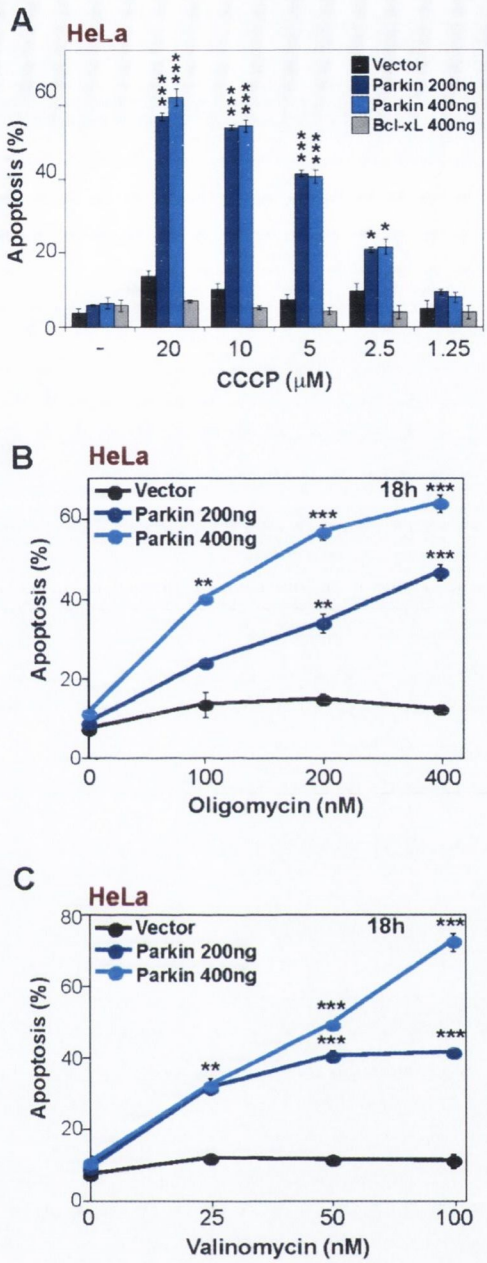

Figure 5.4 Parkin enhances mitochondrial depolarization-initiated cell death by multiple depolarizors

(A) 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 in the presence or absence of CCCP dose curve for 18 hr and cell death was counted among GFP-positive cells based on cell morphology.(B and C) HeLa cells were transfected with a plasmid encoding for eGFP (50 ng) along with Parkin cDNA for 24 hr. Cells were treated with the indicated dose of Valinomycin (B) or the indicated dose of Oligomycin and Antimycin A (500 nM) (C) for 18 hr. Cell death was estimated among GFP-positive cells based on cell morphology.

Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

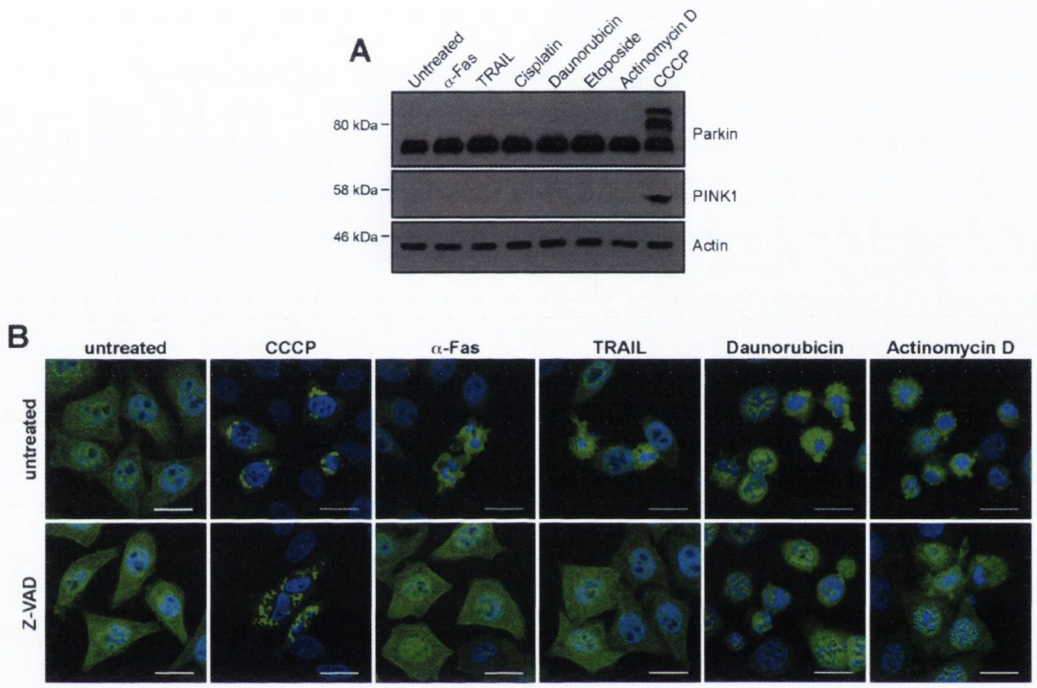

Figure 5.5 Mitochondrial depolarization is required for PINK1 stabilization and Parkin activation

(A) HeLa-YFP-Parkin cells were treated with anti-Fas CH11 (200 ng/ml), TRAIL (50 ng/ml), Cisplatin (50 μM), Etoposide (100 μM), Daunorubicin (5 μM), Actinomycin D (5 μM) or CCCP (10 μM) for 12 hr. Parkin activation and PINK1 stabilization were analyzed by western blotting. (B) HeLa-YFP-Parkin cells were treated with CCCP (10 μM), anti-Fas (200 ng/ml), TRAIL (50 ng/ml), Daunorubicin (5 μM) or Actinomycin D (5 μM) for 12 hr. Cells were then analyzed by confocal microscopy for Parkin translocation to mitochondria and images were acquired. Scale bars represent 20 μM.

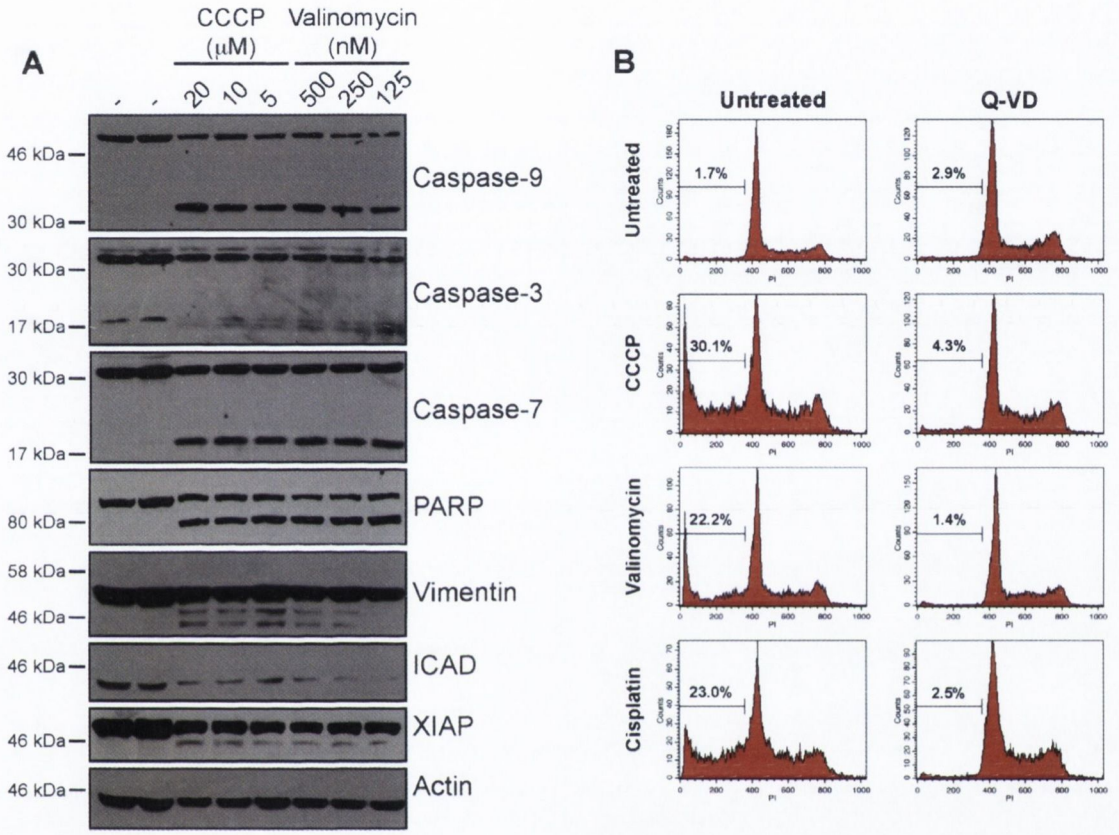

Figure 5.6 Parkin-initiated Cell death has biochemical characteristics of Apoptosis

(A) HeLa-YFP-Parkin cells were either left untreated or treated with indicated doses of CCCP or Valinomycin for 24 hr. Cell lysates were prepared and biochemical markers of apoptosis were analyzed by western blotting. (B) HeLa-YFP-Parkin were treated with CCCP (10 μM), Valinomycin (50 nM) or Cisplatin (50 μM) in the presence or absence of Q-VD-OPh (10 μM) for 20 h. DNA content was analyzed by flow cytometry (I would like to thank Dr. Emilie Hollville for the provision of the data on DNA content analysis).

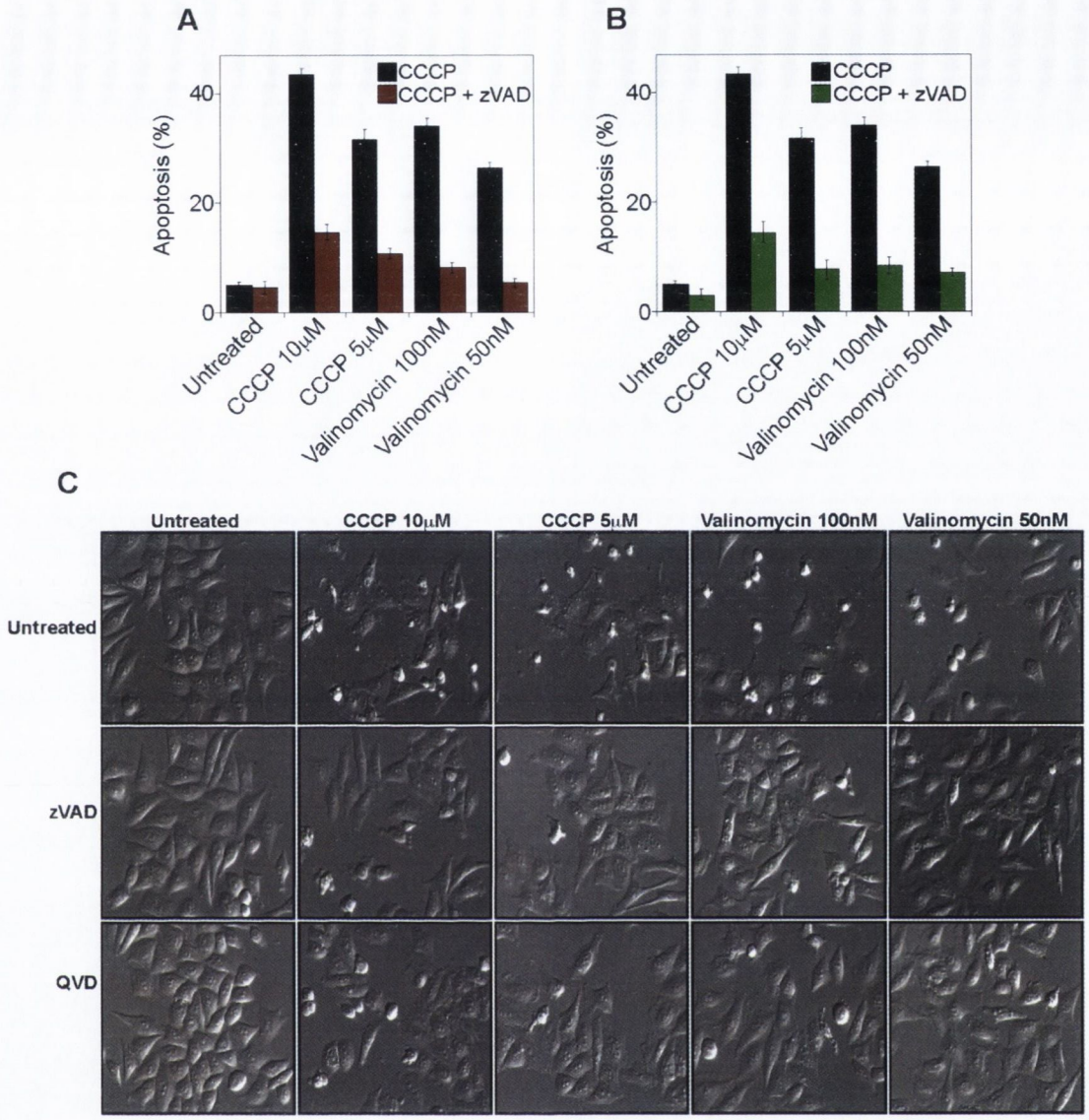

Figure 5.7 Parkin-initiated Cell death is Caspase-dependent

(A and B) HeLa-YFP-Parkin cells were treated with indicated doses of CCCP or Valinomycin in the presence or absence of zVAD-fmk (A) or QVD (B) for 24 hr. Apoptosis was scored based on morphology. (C) HeLa-YFP-Parkin cells were treated as in (A and B) and images were acquired by phase contrast microscopy. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

B). 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 siRNA-mediated knockdown of the latter. As shown in **Figure 5.9 A**, 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 5.9 B - C**, 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. PINK1 silencing can be seen effectively blocking Parkin-induced apoptosis over a broad range of times. The ablation of PINK1 completely reversed all apoptotic morphological changes observed following Parkin expression and mitochondrial depolarization (**figure 5.10 A and B**).

5.2.4 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, we wondered whether Parkin activation in the absence of mitochondrial depolarization was sufficient to promote apoptosis.

Co-expression 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 (**Figure 5.11 A - D**). PINK1/Parkin-dependent cell death was readily inhibited using the poly-caspase inhibitor Q-VD (**Figure 5.12 A)** and exhibited stereotypical features of apoptosis such as caspase activation (**Figure 5.12 B**), caspase substrate proteolysis (**Figure 5.12 B**), and DNA fragmentation (**Figure 5.12 C**).

We also explored whether the Ub ligase activity of Parkin was required for CCCP-induced apoptosis by using two different Parkin mutants, one of which ($Parkin^{C431F}$) failed to translocate to depolarized mitochondria, while the $Parkin^{R275W}$ mutant did so but failed to

promote mitochondrial ubiquitination (**Figures 5.13 A - D**). As illustrated in **Figure 5.14 A and B**, both Parkin mutants were impaired in sensitizing towards CCCP or Valinomycin-induced apoptosis, despite being as efficiently expressed as wild type Parkin (**Figure 5.13 A**).

5.2.5 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 5.15 A**) and are susceptible to apoptosis induced by mitochondrial depolarization (**Figure 5.15 B - D**). 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 5.15 E**).

5.2.6 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 (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 pro-survival Bcl-2 family members, such as Bcl-xL, Mcl-1 and Bcl-2 (Willis et al., 2007; Yang et al., 1995). 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 pro-survival 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 pro-survival Bcl-2 family proteins.

To explore this, we co-expressed 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

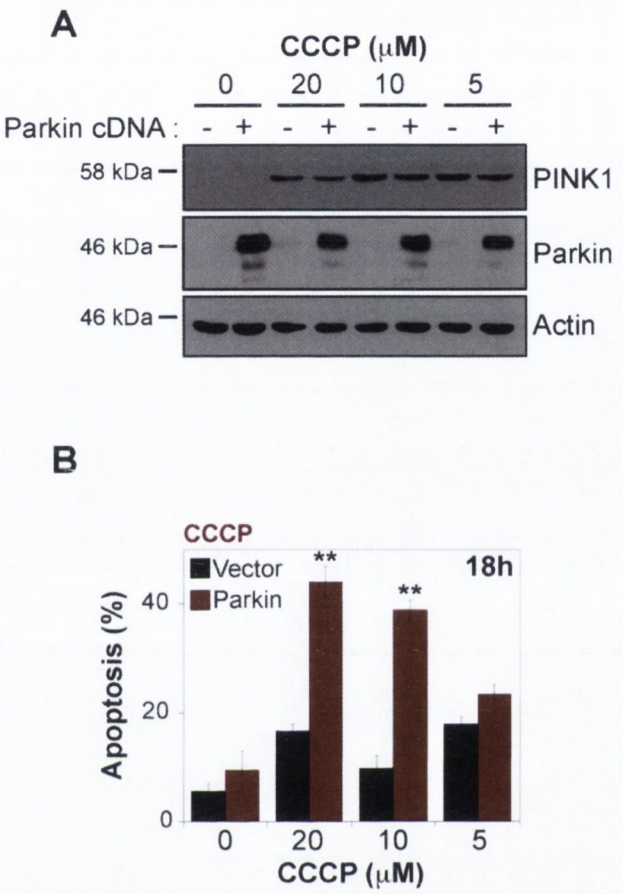

Figure 5.8 PINK1 stabilization alone is insufficient for Parkin-induced apoptosis

(A) HeLa cells were transfected with either empty vector or cDNA encoding for Parkin (100 ng) and incubated for 24 hr. Cells were then treated with indicated dose of CCCP for a further 18 hr. PINK1 stabilization and Parkin expression were analyzed by western blot. (B) HeLa cells were transfected and treated as in (A) and apoptosis was counted based on morphology. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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, *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$.

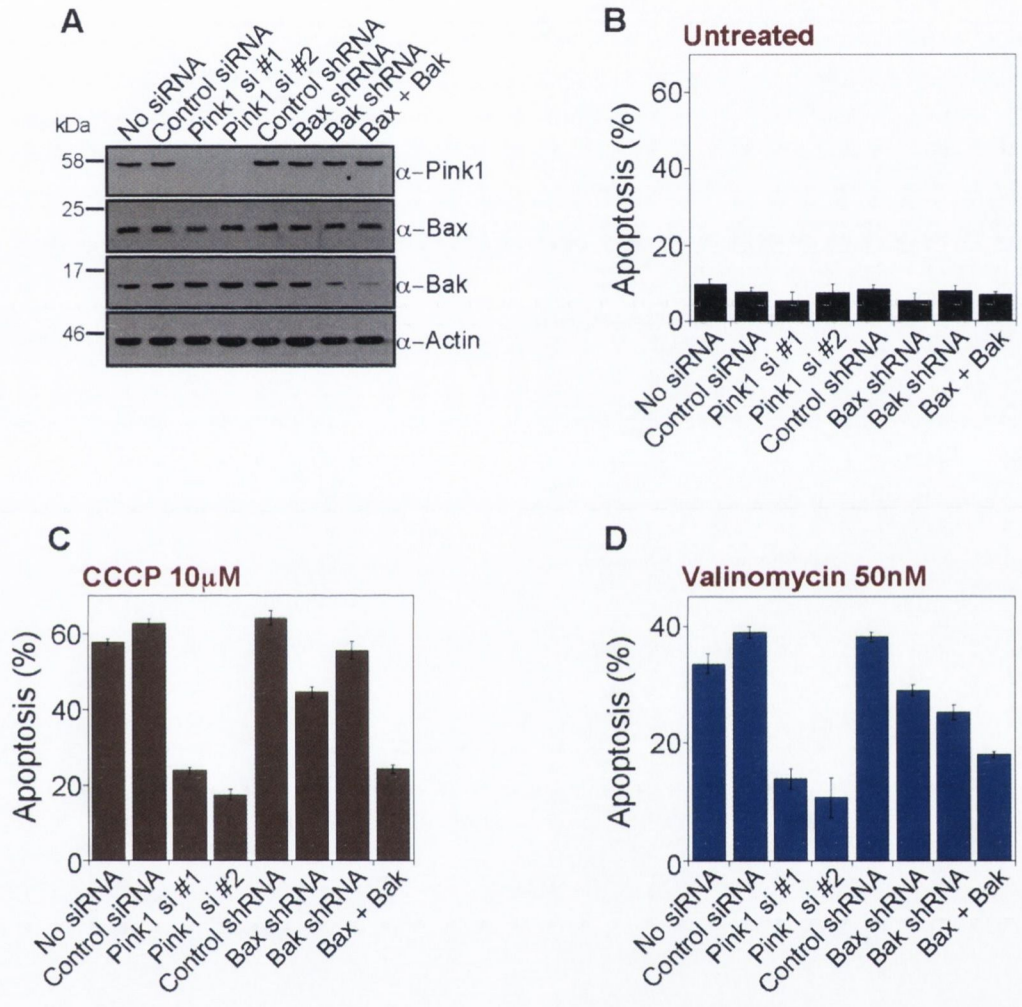

Figure 5.9 PINK1 is required for Parkin-induced apoptosis

(A) HeLa cells were transfected with PINK1 siRNAs (200 nM) or Bax/Bak shRNA (500 ng) and incubated for 24 hr. Cells were then transfected with cDNA encoding for Parkin (100 ng) along with cDNA encoding for eGFP (50 ng) for a further 24 hr. Cells were then treated with CCCP (10 μM) for 16 hr and cell death was counted based on morphology in GFP-positive cells. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

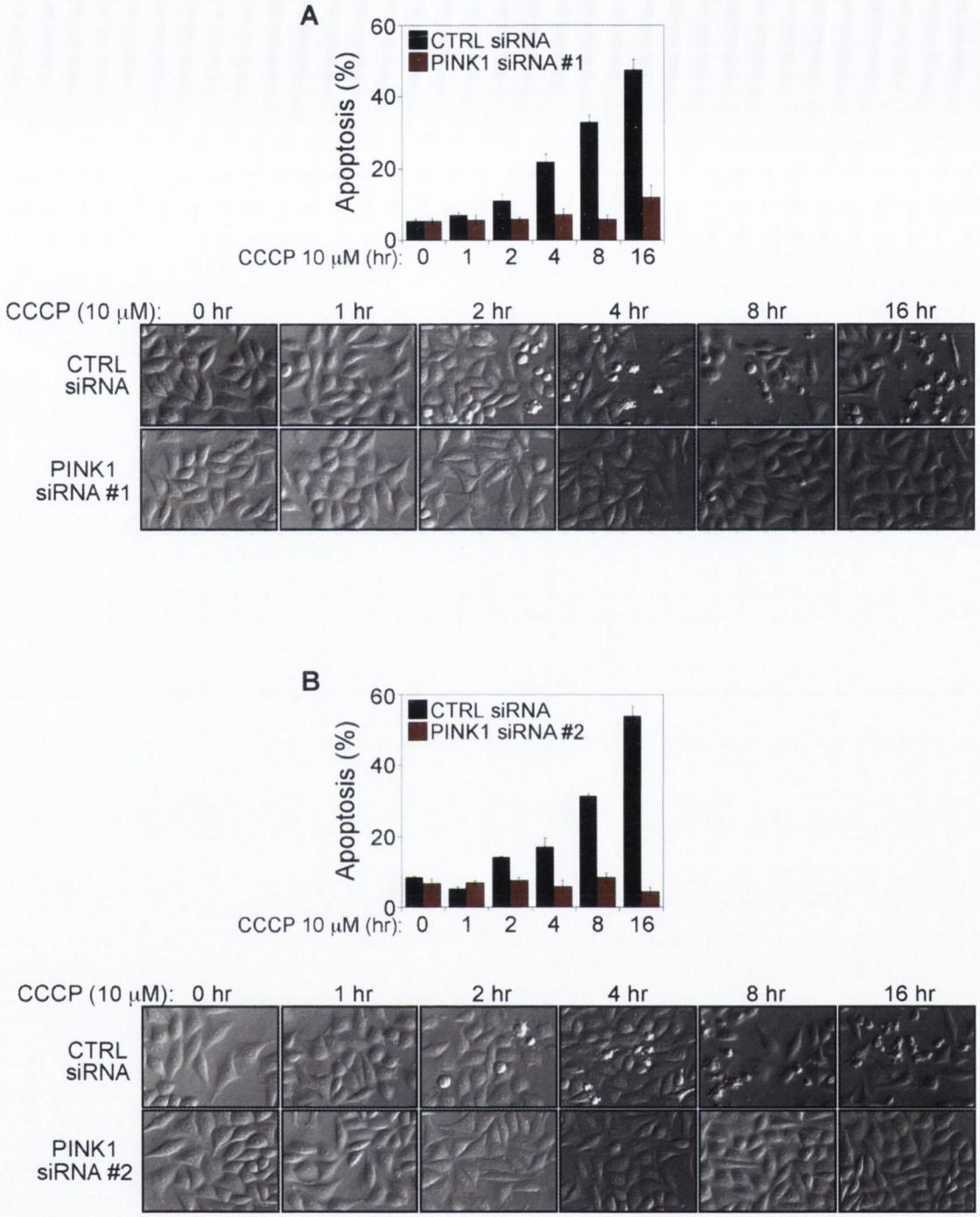

Figure 5.10 PINK1 ablation protects against Parkin-induced apoptosis over a range of times

(A and B) HeLa-YFP-Parkin cells were transfected with two independent siRNAs (200 nM) and incubated for 48 hr. Cells were then treated with CCCP (10 μM) and cell death counts and images were acquired at indicated times. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

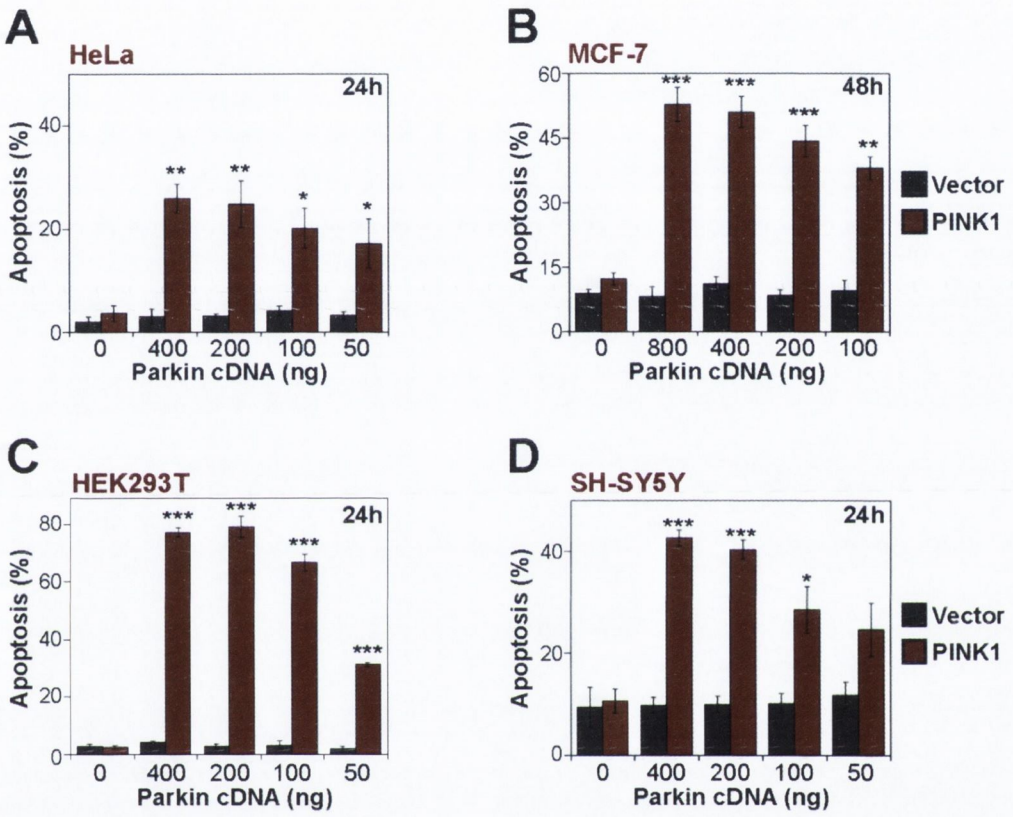

Figure 5.11 PINK1–mediated Parkin activation is sufficient to promote cell death in the absence of mitochondrial depolarization

(A) HeLa, (B) MCF-7, (C) HEK293T and (D) SH-SY5Y cells were transfected with plasmids encoding for eGFP (50 ng) and Parkin (200 ng) along with the indicated amount of PINK1 cDNA for 24 hr or 48 hr. Cell death was counted among GFP-positive cells based on cell morphology. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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, *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$.

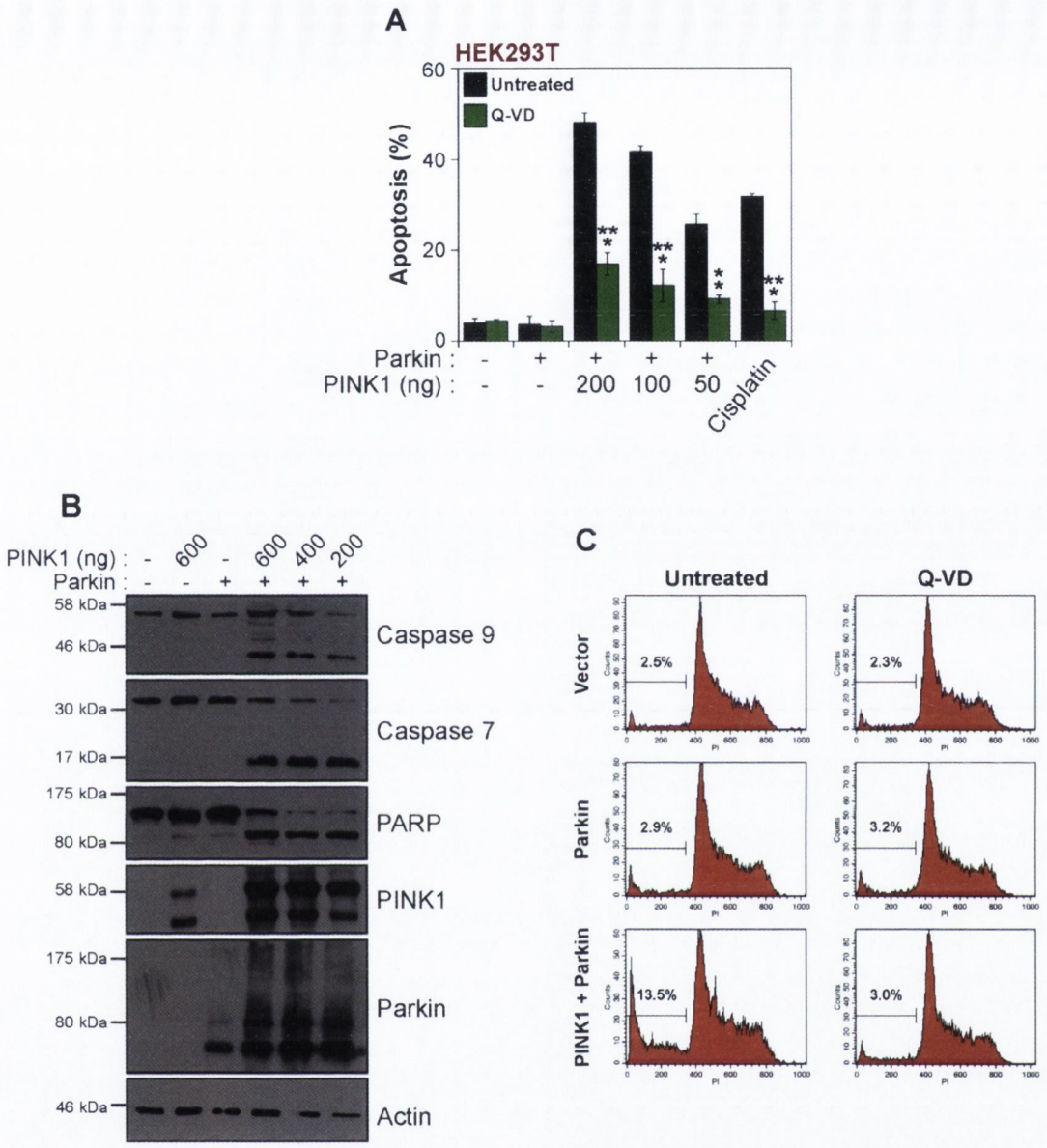

Figure 5.12 PINK1/Parkin-mediated cell death has biochemical characteristics of apoptosis

(A) HEK293T cells were transfected with plasmid encoding for Parkin (200 ng) and indicated amount of PINK1 along with cDNA encoding for eGFP (50 ng) in the presence or absence of Q-VD (10 μM) for 48 hr. HEK293T treated with Cisplatin (50 μM) for 24 hr was used as a positive control. (B) HEK293T cells were transfected with plasmids encoding for YFP-Parkin (300 ng) along with indicated amounts of PINK1 for 48 hr. Lysates were prepared and biochemical markers of apoptosis were analyzed by western blot. (C) HEK293T cells were transfected with plasmids encoding for Parkin (200 ng) and PINK1 (200 ng) in the presence or absence of Q-VD (10 μM). Cells were stained with Propidium Iodide and cell cycle analysis was performed. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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, *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$.

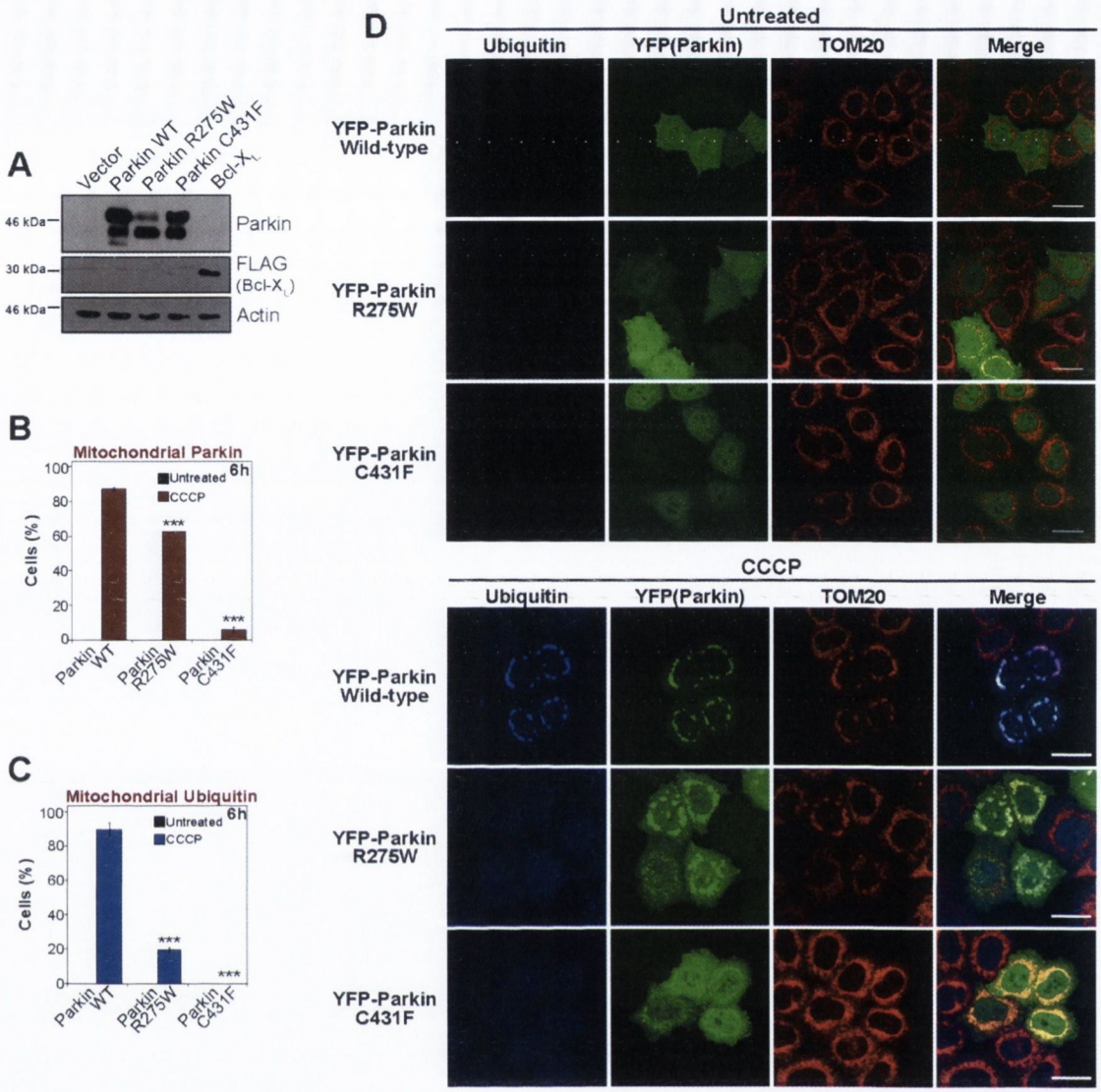

Figure 5.13 Mutations associated with Parkinson's disease lack functions of wild-type Parkin

(A) HeLa cells were transfected with cDNA encoding for Parkin wild-type (500 ng), Parkin R275W (500 ng) or Parkin C431F (500ng) and were incubated for 24 hr. Protein expression of each construct was analyzed by western blotting. (B-D) HeLa cells were transfected as in (A) along with cDNA encoding for eGFP (50 ng) and incubated for 24 hr. Cells were then either left untreated or treated with CCCP (10 μM) for 6 hr. Cells were immunostained for mitochondria (TOM20 – Red) and Ubiquitin (Blue). Cells were analyzed by confocal microscopy for Parkin translocation to mitochondria (B) or Ubiquitin translocation to mitochondria (C) and images were acquired (D). Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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, *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$.

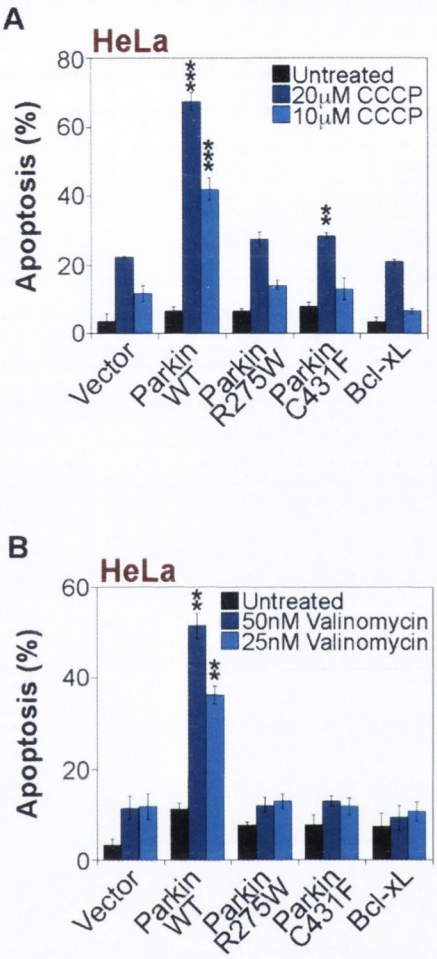

Figure 5.14 Parkin's E3 ligase activity is required for induction of apoptosis

(A and B) HeLa cells were transfected with cDNA encoding for Parkin wild-type (500 ng), Parkin R275W (500 ng) or Parkin C431F (500 ng) along with cDNA encoding for eGFP (50 ng) and incubated for 24 hr. Cells were then treated with indicated dose of either CCCP (A) or Valinomycin (B). Apoptosis was scored based on morphology. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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, *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$.

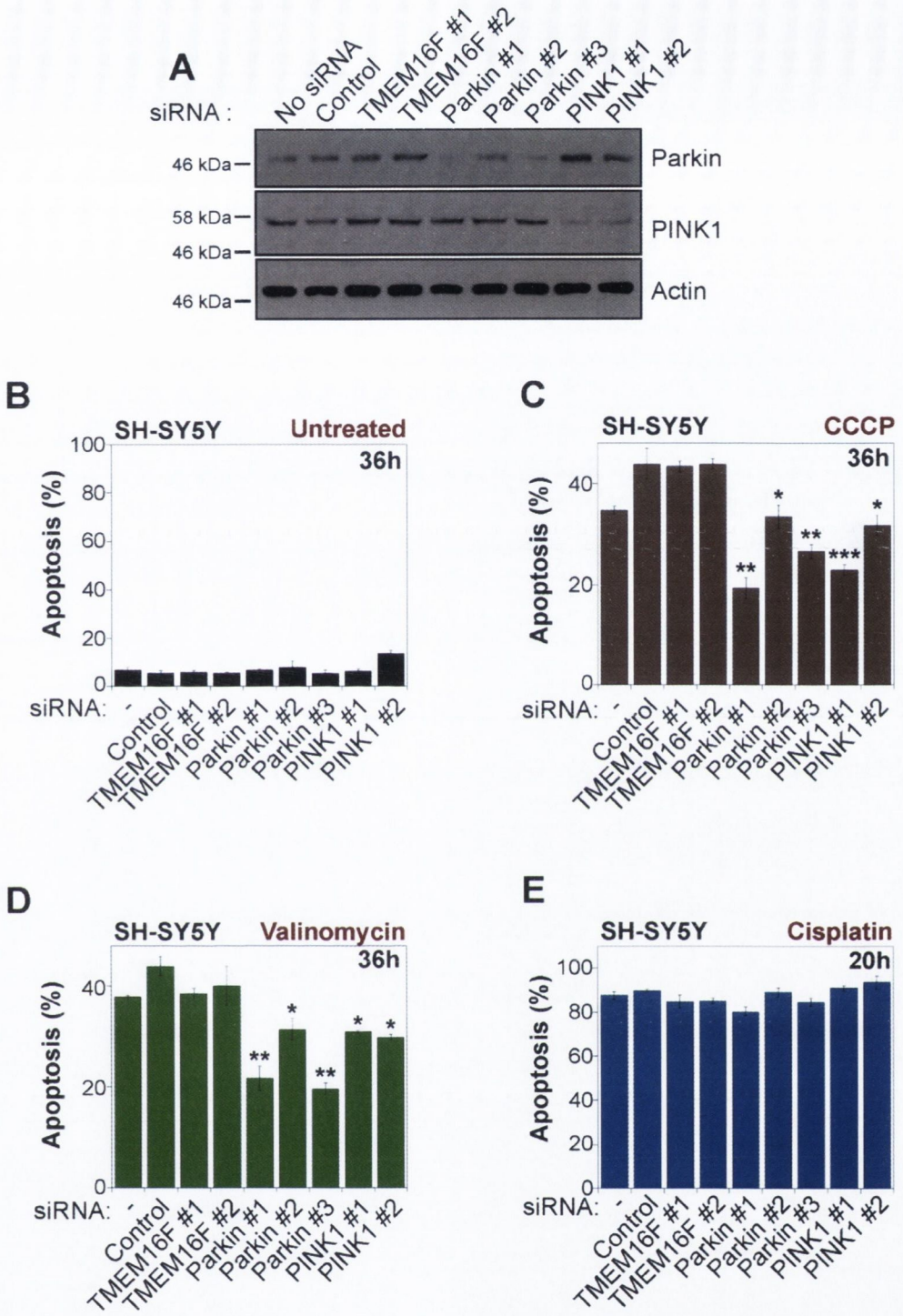

Figure 5.15 Endogenous Parkin and PINK1 can induce apoptosis

(A) SH-SY5Y cells were transfected with siRNA targeted against TMEM16F (200 nM), Parkin (200 nM) or PINK1 (200 nM) and incubated for 48 hr. Cell lysates were prepared and specific knockdown was verified by western blotting. (B-E) SH-SY5Y cells were transfected as in (A) and either left untreated (B) or treated with (C) CCCP (10 μM) for 36 hr, (D) Valinomycin (100 nM) for 36 hr or (E) Cisplatin (50 μM) for 20 hr. Apoptosis was quantified based on morphology. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

Figures 5.16 A - C, 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 5.17 A - C** illustrate, Parkin greatly sensitized towards 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 5.18 A - F** shows, knockdown of Bax or Bak, or both, robustly protected from Parkin/PINK1-dependent apoptosis in response to mitochondrial depolarization.

5.2.7 Parkin/PINK1 promote selective degradation of Mcl-1 in response to mitochondrial depolarization

Because pro-survival 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 pro-survival Bcl-2 family proteins expressed in HeLa cells (Mcl-1, Bcl-xL, Bcl-2 and Bcl-W) in response to mitochondrial depolarization. As **Figure 5.19 A and B** shows, Mcl-1 was selectively and rapidly degraded upon CCCP treatment, in tandem with Parkin activation and PINK stabilization, whereas the stability of other pro-survival Bcl-2 family members was largely unaffected under the same conditions. Moreover, knockdown of PINK1, using two independent siRNAs, completely reversed CCCP-induced degradation of Mcl-1 and blocked the activation of Parkin (**Figure 5.19 A and B**). We also wondered if depolarization of mitochondria in a cell line expressing endogenous levels of PINK1 and Parkin also promoted the Mcl-1 degradation. As can be seen in F**igure 5.20 A**, Mcl-1 is degraded following mitochondrial depolarization induced by CCCP in SHSY-5Y. Furthermore, addition of the proteasome inhibitor MG132 blocked the degradation of Mcl-1 seen upon treatment with CCCP (**Figure 5.21 A**). We also explored the fate of Bax and Bak in response to mitochondrial depolarization. As **Figure 5.21 B** 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, as this effect was reversed with the poly-caspase inhibitor zVAD-fmk, whereas Mcl-1 degradation was only minimally affected under the same conditions (**Figure 5.21 B**).

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

5.2.8 Parkin/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 ubiquitination upon CCCP treatment. As **Figure 5.22 A** demonstrates, 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 5.23 A and B**), 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 5.24 A** 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 5.24 B**).

We also asked whether transient expression of Parkin could promote Mcl-1 degradation. 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 5.25 A**) and pulldown 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 5.25 B**).

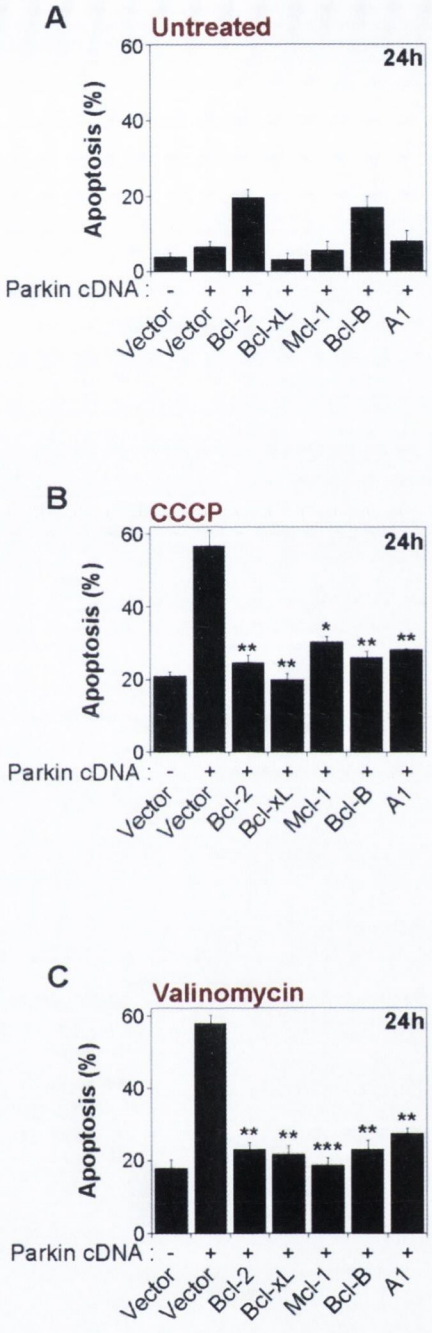

Figure 5.16 Pro-survival Bcl-2 family proteins protect against Parkin-induced death

(A - C) HeLa cells were transfected with cDNA encoding for Parkin (150 ng) and cDNA encoding for indicated Bcl-2 family member (450 ng) along with plasmid encoding for eGFP (50 ng) and were incubated for 24 hr. Cells were then either (A) left untreated, (B) treated with CCCP (10 μM) or (C) treated with Valinomycin for 24 hr. Cell death was scored based on morphology. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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, *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$.

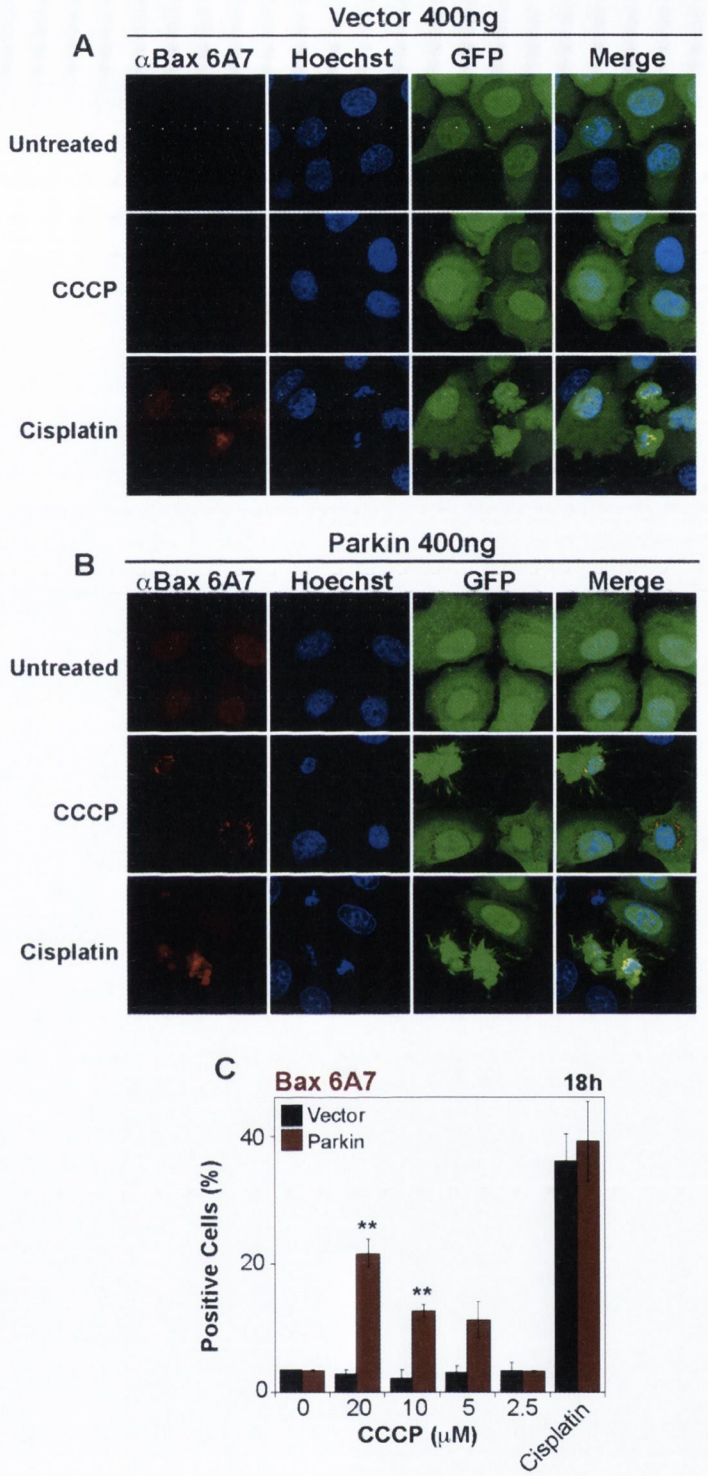

Figure 5.17 PINK1/Parkin-induced cell death promotes Bax activation

(A and B) HeLa cells were transfected with either (A) empty vector or (B) cDNA encoding for Parkin (400 ng) along with plasmid encoding for eGFP (50 ng) and were incubated for 24 hr. Cells were then treated with CCCP (10 μM) for 24 hr. Cells were immunostained with anti-active Bax (Red) and images were acquired by Confocal microscopy. (C) HeLa cells were transfected, treated and immunostained as in (A and B) and cells positive for active Bax were counted in GFP-positive cells. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

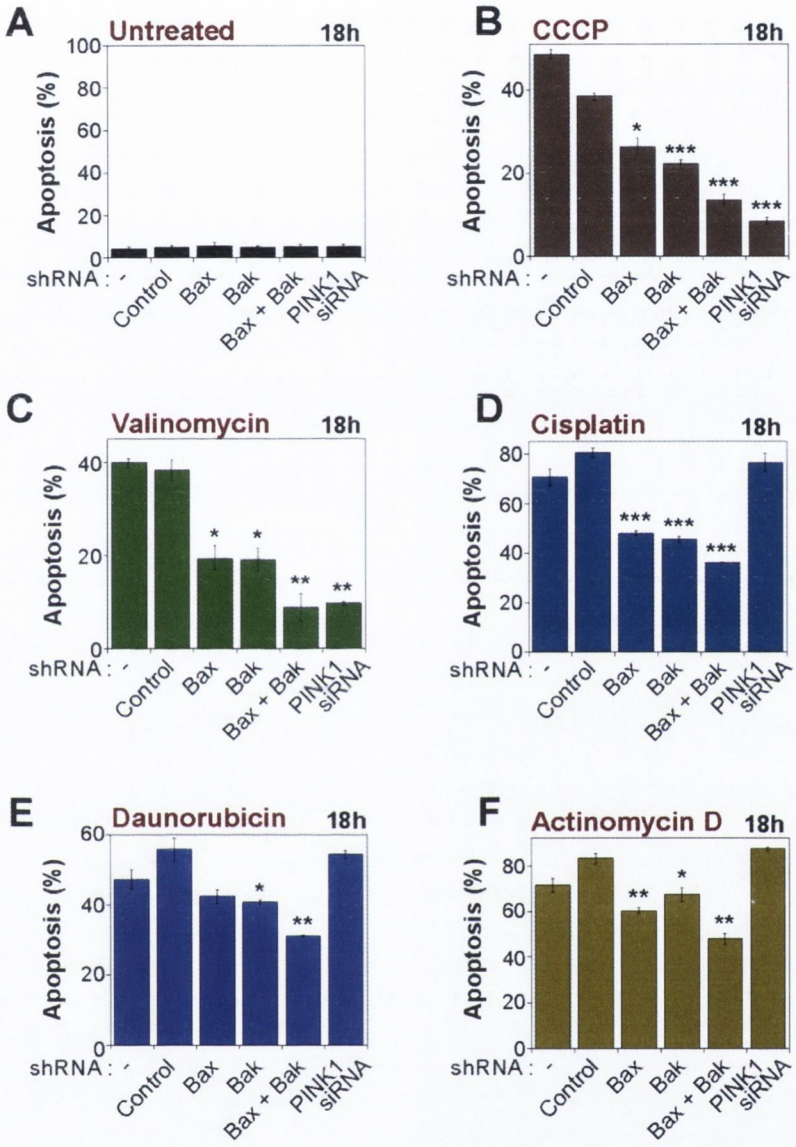

Figure 5.18 Parkin-induced apoptosis requires Bax and Bak activation

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 h. Cells were then transfected with Parkin cDNA (200 ng) and incubated for a further 24 h before either being (A) left untreated, (B) treatment with CCCP (10 μM), (C) Valinomycin (50 nM), (D) Cisplatin (50 μM), (E) Daunorubicin (5 μM) or (F) Actinomycin D (5 μM) for 18 h. Cell death was scored among GFP-positive cells based on cell morphology.

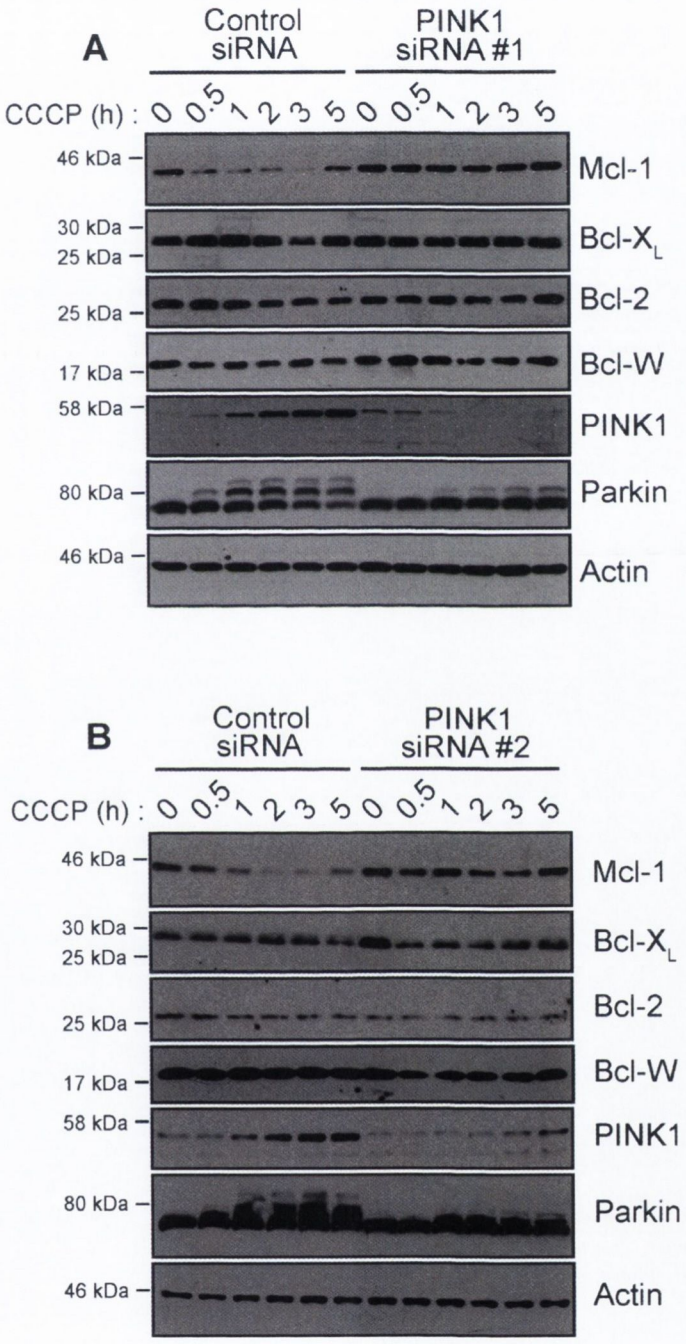

Figure 5.19 Parkin activation leads to the selective and rapid degradation of Mcl-1

(A and B) HeLa-YFP-Parkin cells were nucleofected with two independent siRNAs (200 nM) targeted against PINK1 and were incubated for 48 hr. Cells were treated with CCCP (10 μM) for indicated time. Cell lysates were prepared and Bcl-2 family protein expression was analyzed by western blotting.

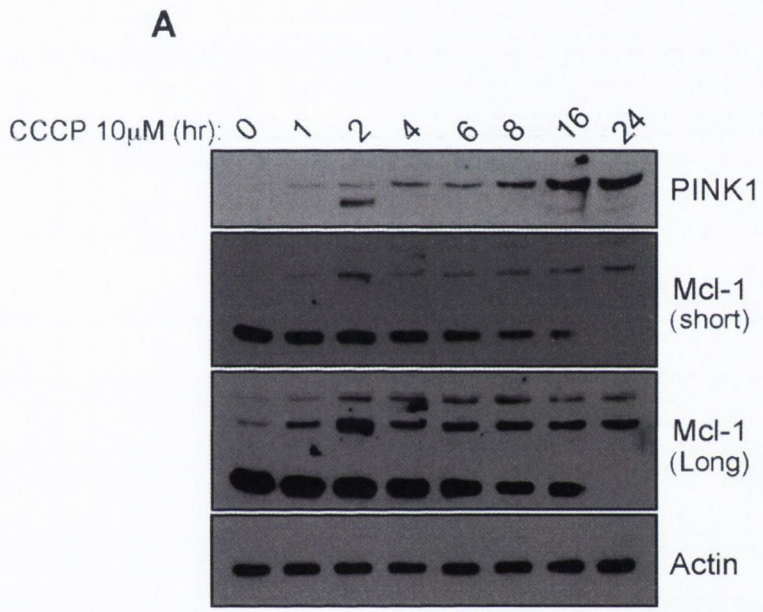

Figure 5.20 Mcl-1 degradation occurs in cells expressing endogenous Parkin

SH-SY5Y cells were treated with CCCP (10 μM) for indicated timepoints. Cell lysates were prepared and Mcl-1 degradation was analyzed by western blotting.

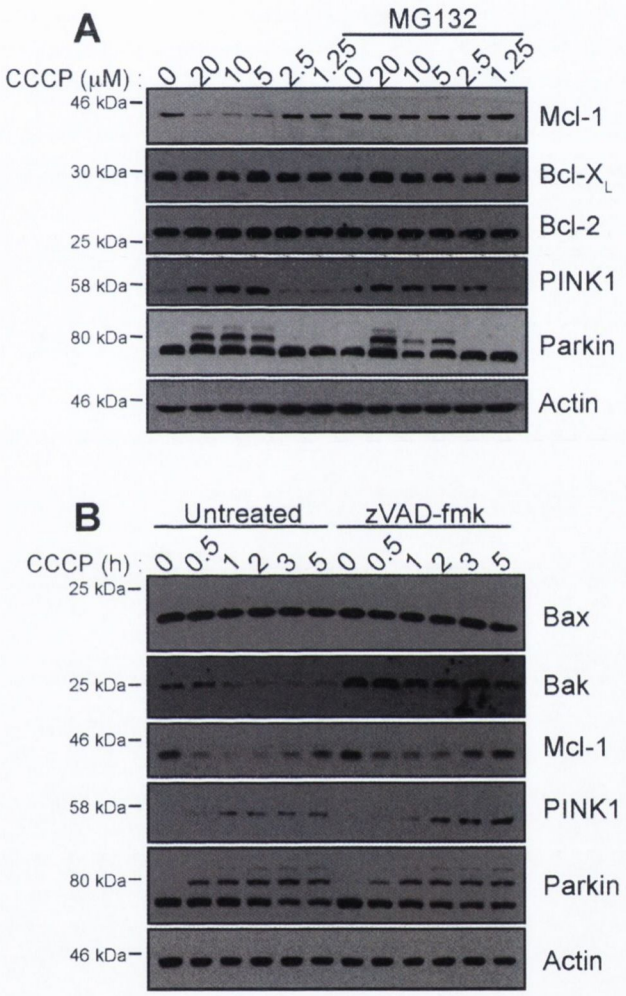

Figure 5.21 Parkin-induced Mcl-1 degradation is proteosome-dependent and caspase-independent

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

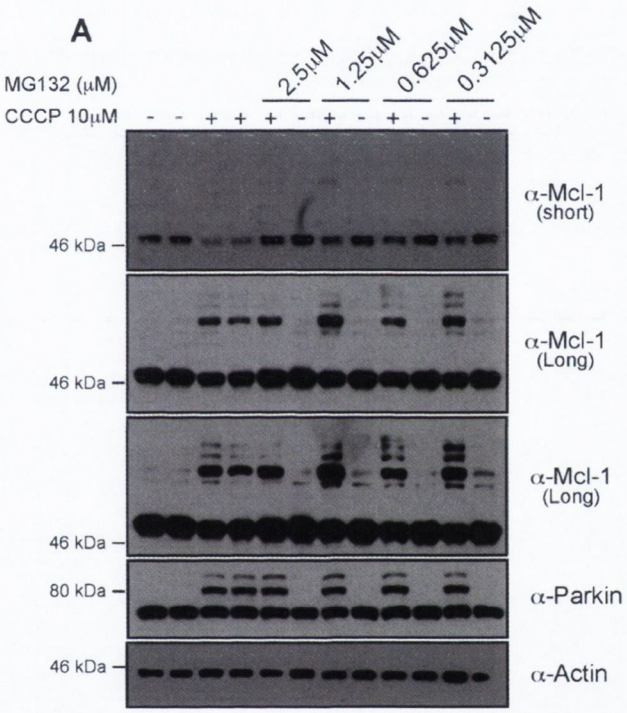

Figure 5.22 Parkin activation leads to appearance of higher molecular weight forms of Mcl-1

(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 h. Mcl-1 expression was analyzed by western blotting.

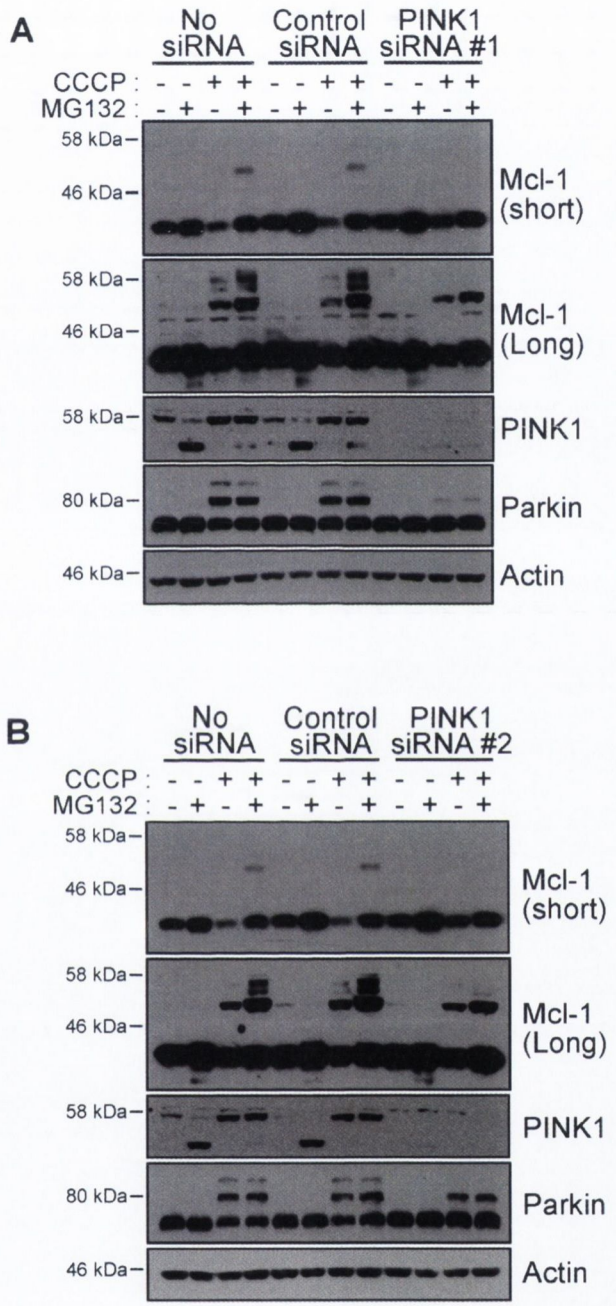

Figure 5.23 Mcl-1 modifications are dependent on a functional PINK1/Parkin pathway

(A and B) Hela-YFP-Parkin were nucleofected with two independent siRNAs (200 nM) targeted against PINK1 and incubated for 48 hr. Cells were then treated with CCCP (10 μM) for 1 hr in the presence or absence of MG132 (10 μM). Cell lysates were prepared and Mcl-1 modifications were analyzed by western blotting.

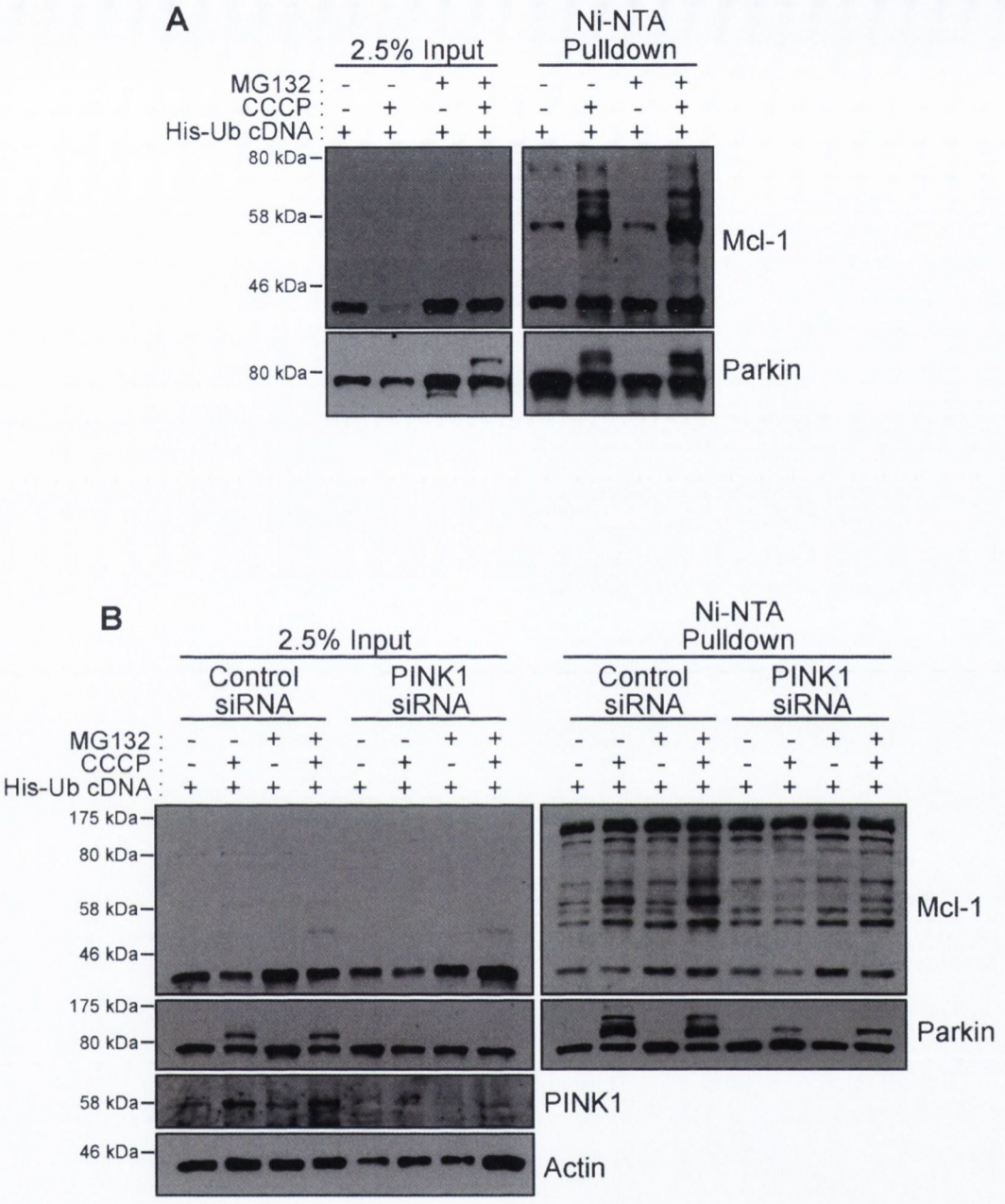

Figure 5.24 Mcl-1 is ubiquitinated in response to Parkin activation

(A) HeLa-YFP-Parkin cells were transfected with a plasmid encoding for 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 using Ni-NTA beads and Mcl-1 ubiquitination was analyzed by western-blotting. (B) HeLa-YFP-Parkin cells were transfected with either non-targeting control siRNA or siRNA targeted against PINK1 (PINK1 siRNA #1) for 24 hr. Cells were then transfected with a plasmid encoding for 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 using Ni-NTA beads and Mcl-1 ubiquitination was analyzed by western-blotting.

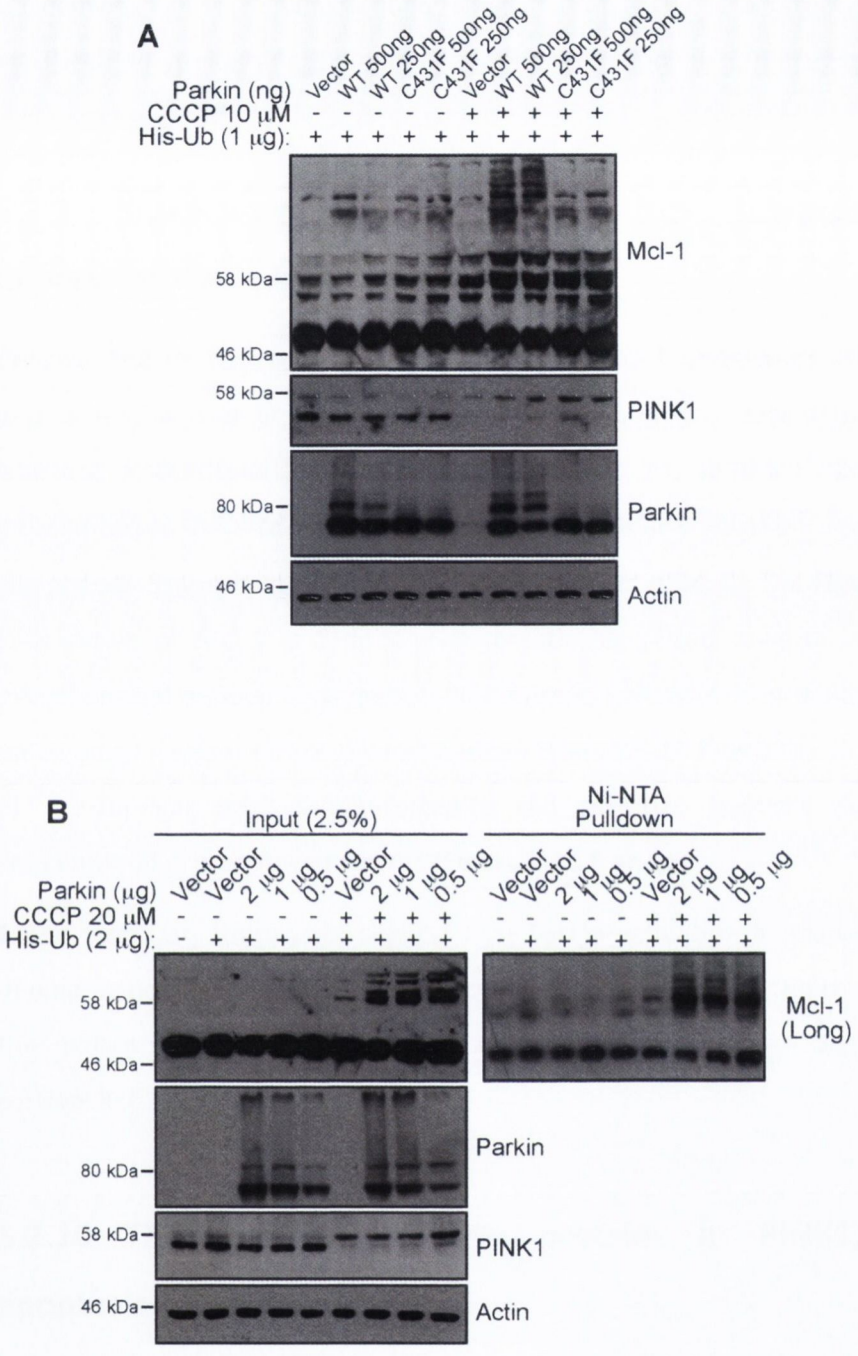

Figure 5.25 Mcl-1 Ubiquitination can be enhanced by Parkin overexpression

(A) HeLa-YFP-Parkin cells were transfected with a plasmid encoding for 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 using Ni-NTA beads and Mcl-1 ubiquitination was analyzed by western-blotting. (B) HeLa-YFP-Parkin cells were transfected with either non-targeting control siRNA or siRNA targeted against PINK1 (PINK1 siRNA #1) for 24 hr. Cells were then transfected with a plasmid encoding for 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 using Ni-NTA beads and Mcl-1 ubiquitination was analyzed by western-blotting.

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

5.2.9 Knockdown of Mcl-1 sensitizes towards 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 towards mitochondrial depolarization-induced apoptosis. As controls, we also knocked down other members of the pro-survival subset of the Bcl-2 family (Bcl-2 and Bcl-X). As **Figure 5.26 A - D** shows, knockdown of Mcl-1 in SHSY5Y cells greatly sensitized towards 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 5.26 C and D**). In contrast, knockdown of pro-survival Bcl-2 family proteins did sensitize towards apoptosis induced by conventional pro-apoptotic agents (**Figure 5.26 E and F**)

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

5.2.10 The role of BH3-only proteins in PINK1/Parkin-mediated apoptosis

As shown above, we have demonstrated a key role for Mcl-1 in the regulation of PINK1/Parkin-mediated apoptosis; however, knockdown of Mcl-1 alone in the absence of mitochondrial depolarization was insufficient for initiation of apoptosis suggesting that activated Parkin also modulates other processes involved in the intrinsic pathway of apoptosis.

A balance between anti-apoptotic Bcl-2 family members and pro-apoptotic BH3-only family members is vital for maintaining the viability of the cell. We have demonstrated that Parkin has a clear role in specifically degrading Mcl-1 during in response to

mitochondrial depolarization but it may also regulate members of the pro-apoptotic BH3-only family members. To explore this, we ablated expression of the BH3-only family members individually using siRNA-mediated knockdown in HeLa-YFP-Parkin. We then induced mitochondrial depolarization using either CCCP or Valinomycin to ask whether removal of any of the BH3-only family would provide protection against PINK1/Parkin-mediated apoptosis. As can be seen in **Figure 5.27 A – C**, knockdown of BAD provided a modest protection against PINK1/Parkin-mediated apoptosis, however, this was only seen with one of the two siRNAs tested. Following this result, knockdown of individual BH3-only proteins were analyzed by western blotting **(Figure 5.27 D)** and neither siRNA targeted against the BAD protein appeared to fully knockdown. As the pro-apoptotic function of BAD has been clearly characterized and there was a functional inhibition of apoptosis following BAD knockdown (**figure 5.27 B**), it is possible that the antibody used is not recognizing BAD, however, further work will be required to elucidate the involvement of the BH3-only proteins in PINK1/Parkin-mediated apoptosis.

Interestingly, Parkin has been shown to interact with the 14-3-3 proteins and the latter proteins are known to inhibit the pro-apoptotic function of BAD suggesting that BAD, may play some role in PINK1/Parkin-mediated apoptosis.

5.2.13 The role of reactive oxygen species in PINK1/Parkin-mediated apoptosis

Mitochondrial depolarization is known to release of large amounts of reactive oxygen species into the cytosol. Generation of reactive oxygen species by mitochondria can surpass the cytosolic antioxidant capacity resulting in oxidation of proteins and possible alteration of their function.

This is important in relation to Parkin function as E3 ligase function is regulated by deubiquitinases and deubiquitinases are known to be inhibited by high levels of cytosolic ROS (Lee et al., 2013). It is therefore possible that ROS released from mitochondria following mitochondrial depolarization could act to inhibit deubiquitinases involved in negatively regulating Parkin function. Thus, we also sought to investigate a role for reactive oxygen species in PINK1/Parkin-mediated apoptosis. To this end, HeLa-YFP-Parkin cells were incubated with the reactive oxygen scavenger N-acetyl-cysteine (NAC) before

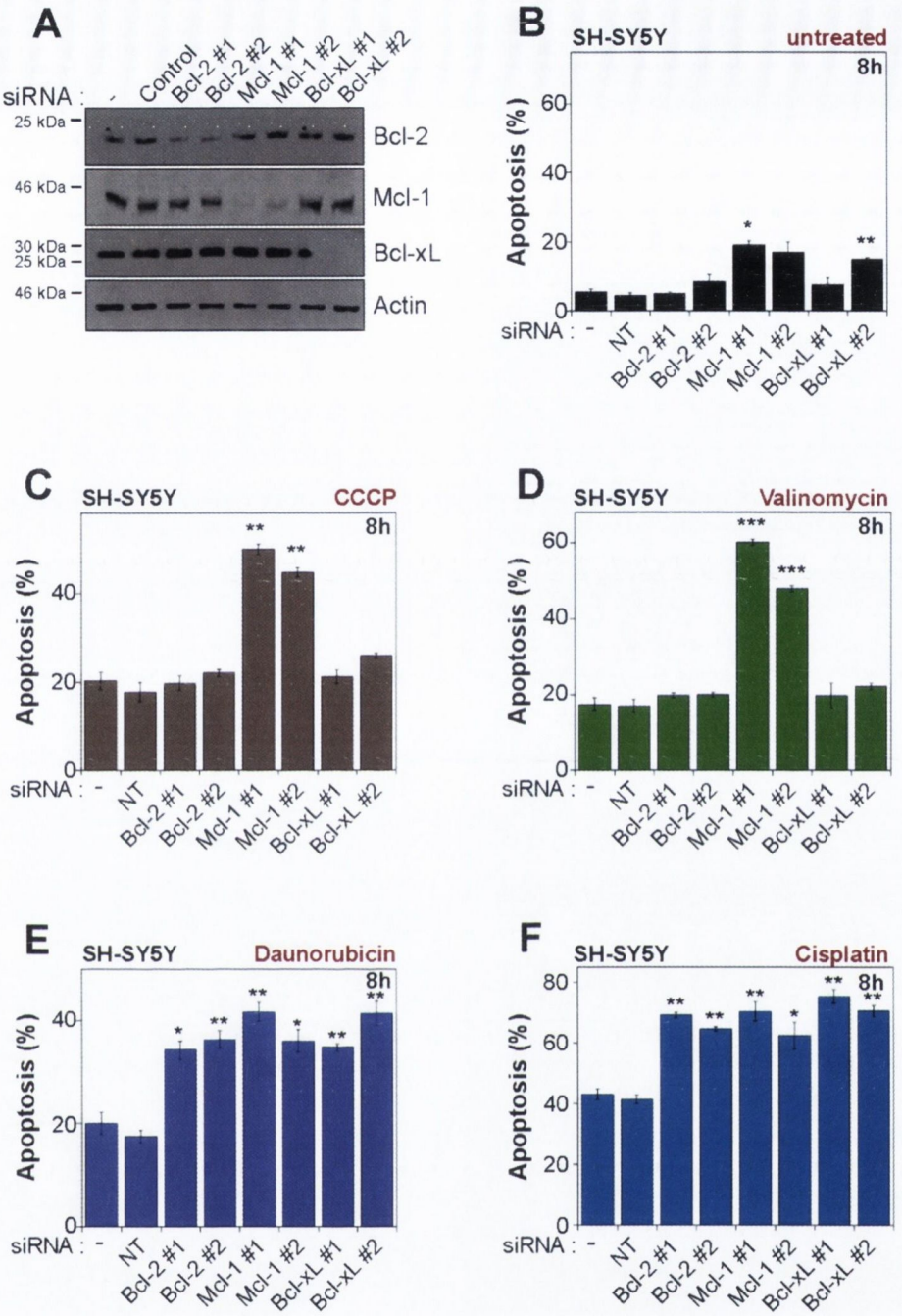

Figure 5.26 Mcl-1 ablation specifically enhances PINK1/Parkin-mediated apoptosis

SH-SY5Y cells were transfected with siRNA (200 nM) targeted against indicated Bcl-2 family member and incubated for 48 hr. (A) Lysates were prepared and specific knockdown was analyzed by western blotting. Cells were either (B) left untreated or treated with (C) CCCP (10 μM), (D) Valinomycin (100 nM), (E) Daunorubicin (5 μM) or (F) Cisplatin (50 μM) for 8 hr. Apoptosis was scored based on morphology. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) 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 ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001.

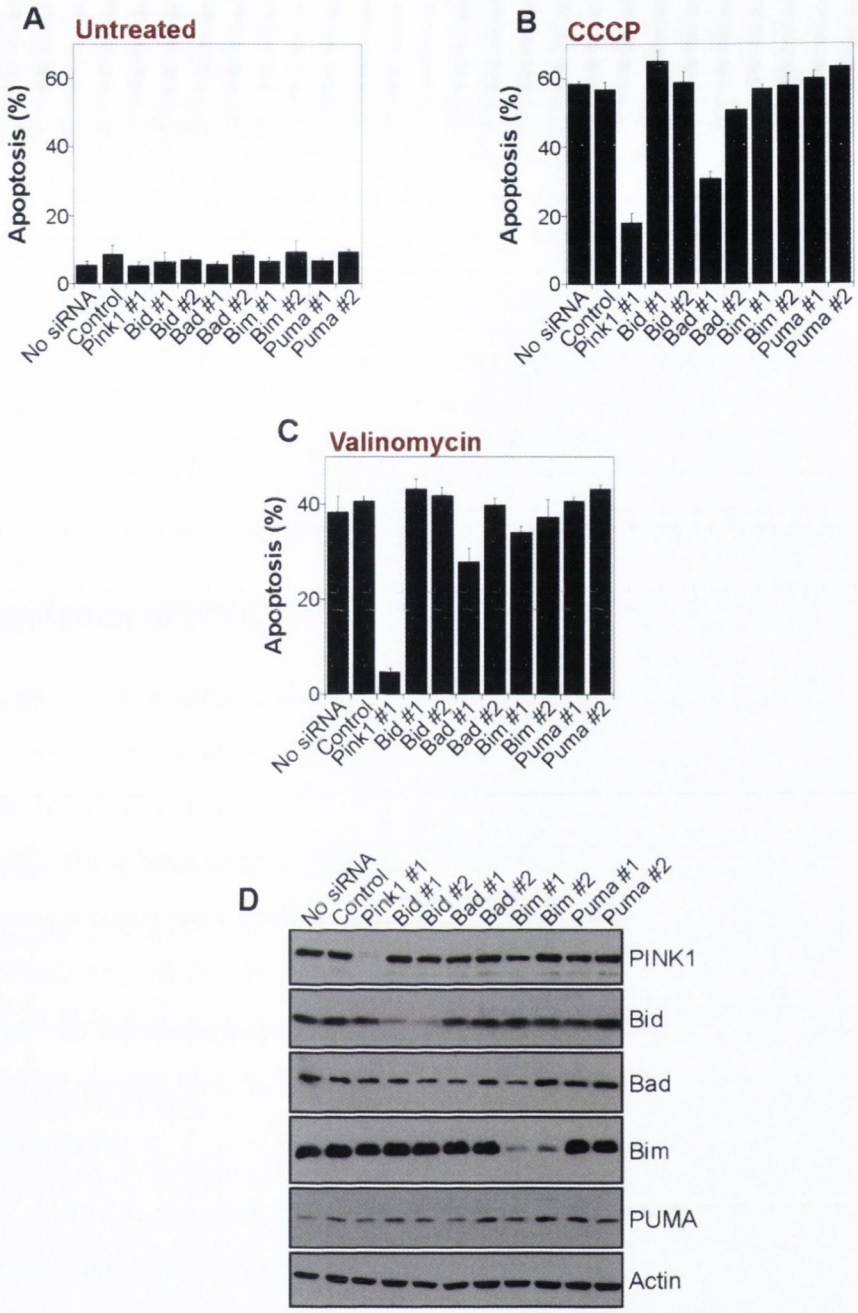

Figure 5.27 Bad is involved in PINK1/Parkin-mediated mitophagy

(A – C) HeLa-YFP-Parkin cells were nucleofected with siRNAs (200 nM) targeted against members of the BH3-only family members and incubated for 48 hr. Cells were then either (A) left untreated, (B) treated with CCCP (10 μM) or (C) Valinomycin (100 nM) for 24 hr. Apoptosis was quantified based on cell morphology. (D) Cell lysates were prepared and specific knockdown was analyzed by western blotting. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

induction of mitochondrial depolarization using CCCP. Following 24 hours of co-incubation, cells treated with NAC were completely protected from PINK1/Parkin-mediated apoptosis (**Figure 5.28 A and C**). Furthermore, removal of reactive oxygen species using NAC only provided protection against PINK1/Parkin-mediated apoptosis and failed to protect against apoptosis induced by chemotherapeutic agents (**Figure 5.28 B**). While this does not directly implicate deubiquitinases in PINK1/Parkin-mediated apoptosis, it does demonstrate that an oxidation event is absolutely required for PINK1/Parkin-mediated apoptosis. Further work is required in this area to elucidate the unique function of ROS in PINK1/Parkin-mediated apoptosis.

5.2.14 Regulation of PINK1/Parkin-mediated apoptosis by IAPs

Previously we have extensively described the molecular mechanisms required for PINK1/Parkin-mediated apoptosis to occur. However, a common feature in biology is bidirectional regulation to protect against either uncontrolled growth or uncontrolled death of cells. There have been a number of screens in the literature in which Parkin has been shown to interact with IAPs or that Parkin function is regulated by IAPs (Hasson et al., 2013; Sarraf et al., 2013). Thus, we wished to explore whether IAPs were also involved in regulating PINK1/Parkin-mediated apoptosis. HeLa-YFP-Parkin cells were pre-treated with the IAP-antagonist, BV6, followed by induction of mitochondrial depolarization using CCCP or Valinomycin.

The addition of BV6 accelerated apoptosis in HeLa-YFP Parkin cells following mitochondrial depolarization, suggesting a role for IAPs as negative-regulators of PINK1/Parkin-mediated apoptosis (**Figure 5.29 A – C**). As negative regulators of PINK1/Parkin-mediated apoptosis, Parkin may regulate the stability or function of the IAPs to promote apoptosis. Parkin and XIAP were found to interact with each by co-immunoprecipitation (data not shown). As Parkin is an E3 ligase, we wondered whether Parkin could modulate the abundances of the IAP proteins during mitochondrial depolarization. To this end, HeLa-YFP-Parkin cells were treated with CCCP over a 5 hour timecourse and abundances of the IAP proteins were analyzed by western blot. While there was no obvious modulation of any of the IAP proteins there was an increase in the activation of NFκB as demonstrated by increased degradation of the NFκB inhibitor, IκB (**Figure 5.30 A**). IAPs are known regulators of NFκB and XIAP has been reported to promote the non-canonical NFκB pathway by enhancing

the interaction of TAK and TAB1 leading to activation of the IKK complex and degradation of IκB (Lu et al., 2007). It is conceivable that during mitochondrial depolarization its activity it increased, either through potential modifications (phosphorylation, ubiquitination or oxidation) or through a reduction in activity of its deubiquitinases. Increased XIAP activity could promote survival of cells with mild mitochondrial depolarization through activation of NFκB through the non-canonical pathway and allow time for the cell to remove depolarized mitochondria through mitophagy resulting in full recovery of the cell. XIAP activity could be vital in the determination of whether a cell will undergo mitophagy and recover or undergo apoptosis following excessive mitochondrial damage through the fine balance of NFκB activation.

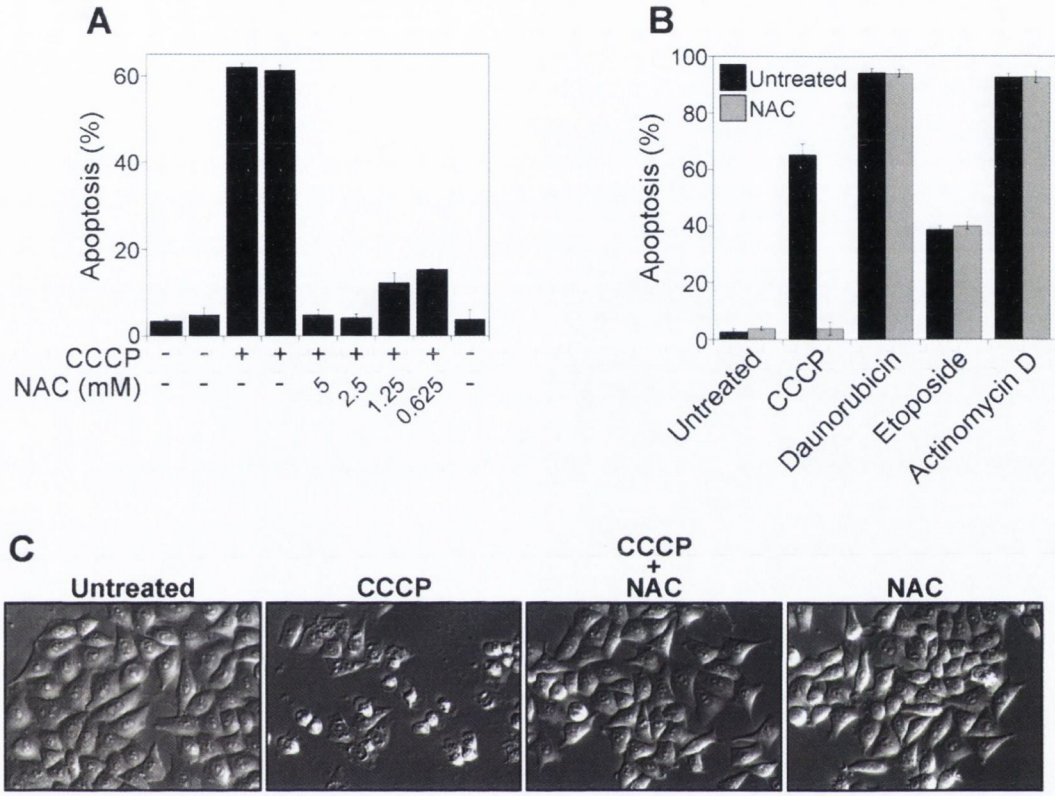

Figure 5.28 PINK1/Parkin-mediated apoptosis is dependent on reactive oxygen species

(A) HeLa-YFP-Parkin cells were either left untreated or treated with CCCP (10 μM) along with the indicated dose of NAC. Cells were incubated for 18 hr and cell death was scored based on morphology. (B) HeLa-YFP-Parkin cells were treated with CCCP (10 μM), Daunorubicin (5 μM), Etoposide (50 μM) or Actinomycin D (5 μM) in the presence or absence of NAC (2.5mM). Cells were incubated for 18 hr and cell death was scored based on morphology and (C) images were acquired by phase microscopy. (D) HeLa-YFP-Parkin cells were either left untreated or treated with CCCP (10 μM) along with the indicated dose of NAC. Cells were incubated for 2 hr and Park

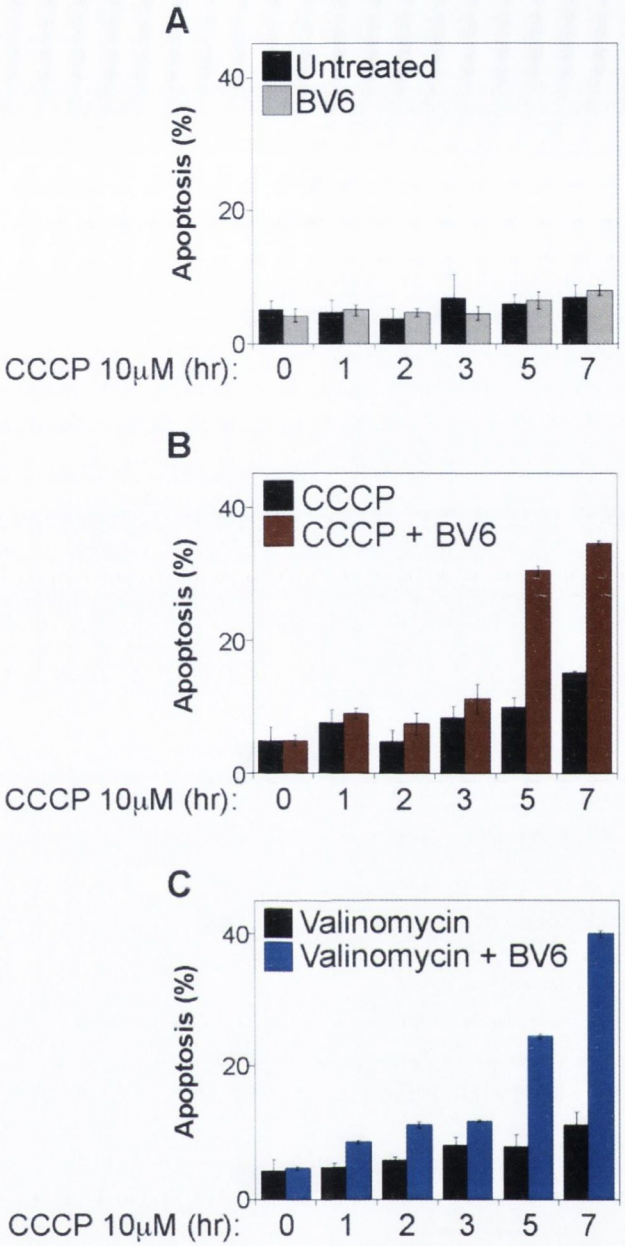

Figure 5.29 IAPs are involved in regulation of PINK1/Parkin-mediated apoptosis

(A - C) HeLa-YFP-Parkin cells were pre-treated with BV6 (2 μM) for 2 hr. Cells were then either (A) left untreated, (B) treated with CCCP (10 μM) or (C) treated with Valinomycin (100 nM) for 16 hr. Cell death was then scored based on cell morphology. Results shown are representative of at least three independent experiments. Error bars indicate standard error of the mean (SEM) of triplicate counts of a minimum of 100 cells.

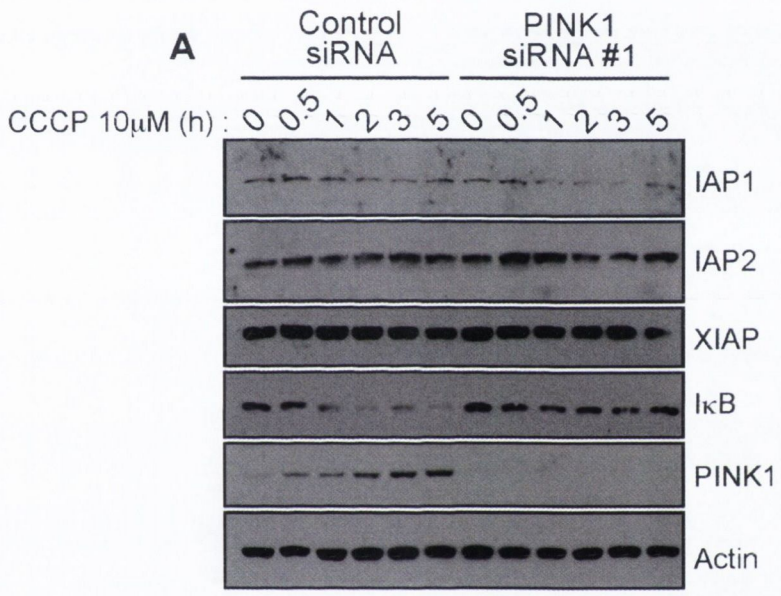

Figure 5.30 PINK1/Parkin-mediated apoptosis does not regulate stability of IAPs but does promote NFκB activation

(A) HeLa-YFP-Parkin cells were nucleofected with siRNA (200 nM) targeted against PINK1 and incubated for 48 hr. Cells were then treated with CCCP (10 μM) for indicated times. Cell lysates were prepared and IAP or IκB stability was analyzed by western blotting.

5.3 Discussion

Here we have shown that, rather surprisingly, Parkin sensitized towards apoptosis induced by mitochondrial depolarization. This effect was highly selective and was not observed in response to a range of additional pro-apoptotic 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 towards 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 pro-survival 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 pro-apoptotic protein that polices mitochondrial integrity and is capable of either initiating repair of mitochondrial networks via mitophagy, or promoting the elimination of critically damaged cells via apoptosis.

While 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 (Berger et al., 2009; Darios et al., 2003; Ekholm-Reed et al., 2013; MacCormac et al., 2004; Sun et al., 2013; Wang et al., 2013a; Yang et al., 2005). 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. Muller-Rischart and colleagues also reported that induction of OPA1 expression by Parkin mediates Parkin anti-apoptotic function in response to staurosporine (Muller-Rischart et al., 2013). 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 pro-apoptotic 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 which 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 pro-apoptotic 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 MEFs, 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 pro-survival 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 pro-survival 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 pro-apoptotic activity (Chen et al., 2013; Purvis et al., 2012). Similarly, Myc activation can lead to cell proliferation or apoptosis depending on the context and Ras activation can promote cell division, cell senescence, or cell death (Elgendy et al., 2011; Evan et al., 1992; Serrano et al., 1997).

Bad is a BH3-only protein which is phosphorylated to increase its interaction with the 14-3-3 proteins and diminish its activity. The 14-3-3 proteins are ubiquitously expressed with their highest expression is in the brain. They have also been shown to interact with wild-type Parkin while failing to interact with Parkin mutations associated with Parkinson's disease (Martin et al., 1994; Sato et al., 2006). However, these studies between Parkin and 14-3-3 proteins pre-date the discovery of PINK1-mediated activation of Parkin. This leaves the actual significance of Parkin and 14-3-3 protein interactions to be determined. It is possible that the 14-3-3 are ubiquitin substrates of Parkin and following interaction with activated Parkin, are ubiquitinated and targeted for proteosomal degradation. Thus, this would a further mechanism whereby Parkin promotes apoptosis. However, further work is required to elucidate the role, if any, that 14-3-3 proteins and Bad play in determining either a mitophagic or autophagic outcome.

Parkin acts as a sensor for mitochondrial damage, however, there is likely many proteins involved in determining the fate of the cell. Here we have demonstrated a role for IAPs as negative regulators of PINK1/Parkin-mediated apoptosis. The IAPs can activate NFκB to promote survival and the balance between pro-survival signals from IAPs along with pro-apoptotic signals from Parkin may determine either cell survival (mitophagy) or cell death (apoptosis). The decision to enter apoptosis is an important one as it is a decision which can only be made once. It is not surprising that there are several pathways involved in making this decision during Parkin activation.

The release of mitochondrial ROS also appears to be essential for this decision to enter apoptosis. The inhibition of ROS does not affect apoptosis in response to cytotoxic agents, however, dramatically protects against PINK1/Parkin-mediated apoptosis. Deubiquitinases are highly sensitive to an increase in ROS and their oxidation inhibits their deubiquitinases activity (Lee et al., 2013). As deubiquitinases are very important in negatively regulating E3 ligases, it is likely that the increase in ROS during mitochondrial damage is required to inhibit deubiquitinases that regulate the degradation of Parkin substrates. This inhibition would enhance the ubiquitination of all Parkin substrates allowing for more robust and sustained signalling during mitochondrial dysfunction.

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.

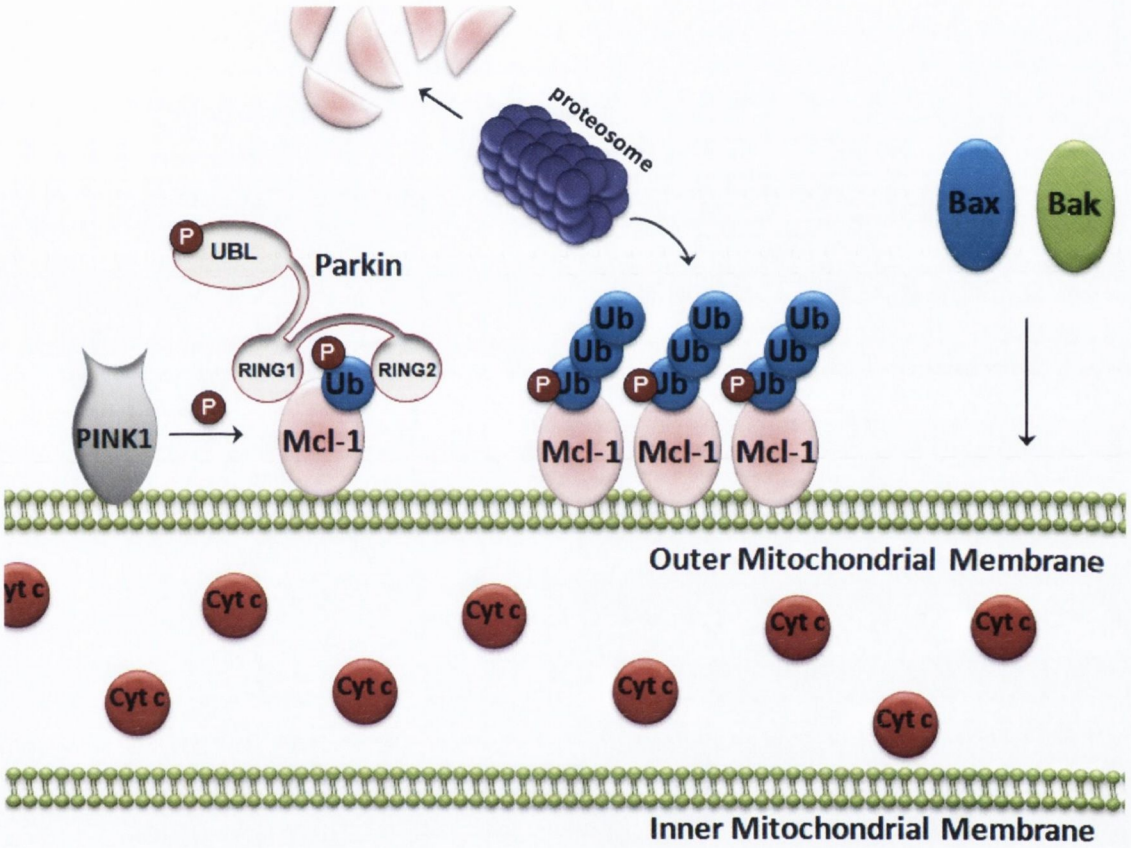

Figure 5.31 Model of Parkin-mediated apoptosis

PINK1 accumulates on the surface of damaged mitochondria where it phosphorylates and activates Parkin. Once activated, Parkin translocates to the mitochondria and ubiquitinates Mcl-1 targeting this pro-survival protein for rapid degradation through the proteosome. This reduces the amount of anti-apoptotic Bcl-2 proteins and reduces their capacity to prevent Bax/Bal oligomerization allowing for apoptosis initiation.

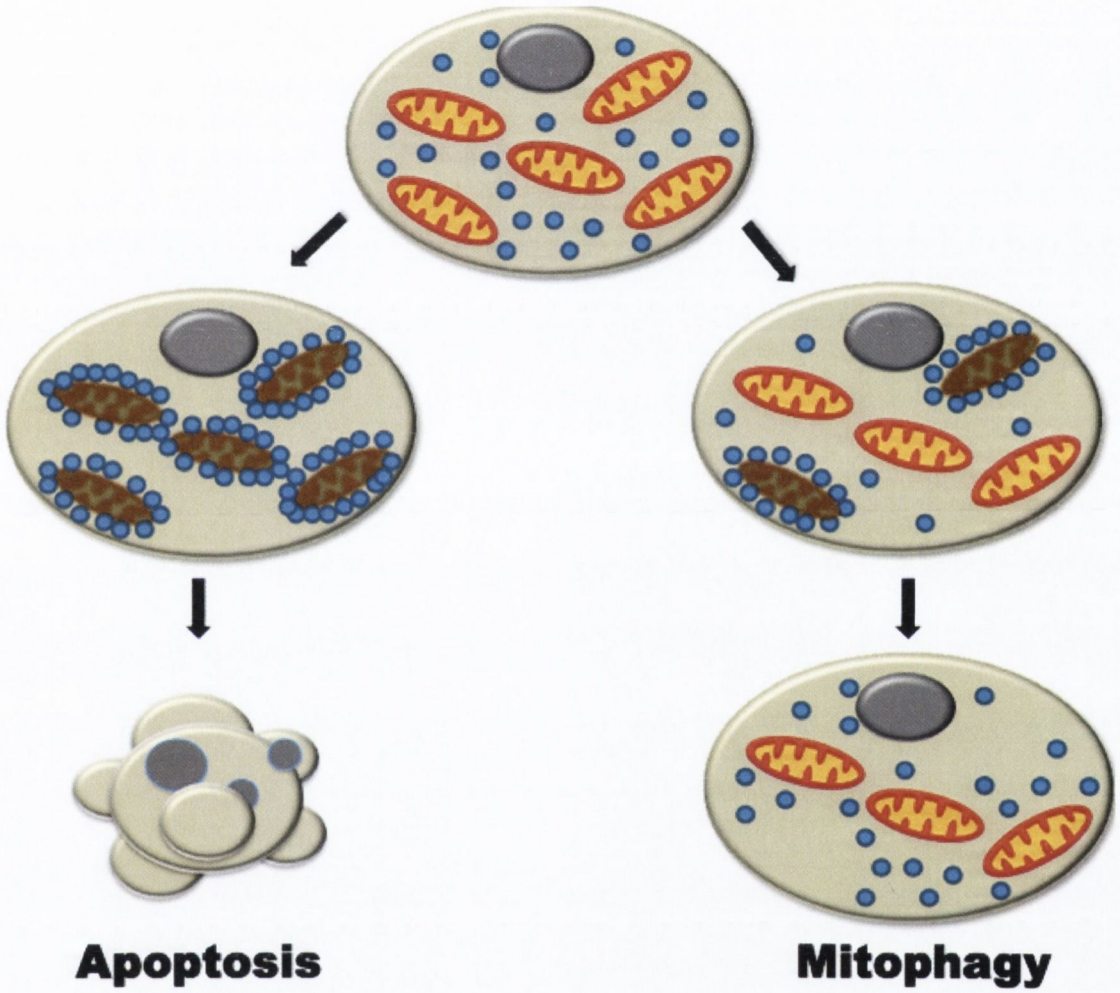

Figure 5.32 Parkin can mediate both cell survival and apoptosis

Cells in which mitochondrial damage occurs can have two outcomes. Cells which experience low mitochondrial damage can have defective mitochondria removed by mitophagy allowing the cell to remain healthy. Cells which experience excessive mitochondria initiate apoptosis to remove the cell.

Chapter V

General Discussion

6.1 Introduction

The purpose of the work described in this thesis was to characterize the role of the Bcl-2 family in maintaining mitochondrial homeostasis through their interactions with the mitophagy related protein, Parkin. Mitophagy is essential for removal of damaged and potentially hazardous mitochondria in order to keep the mitochondrial network healthy. The maintenance of mitochondrial homeostasis requires co-ordination of mitochondrial fission, fusion, motility and turnover (Chen and Chan, 2009; Detmer and Chan, 2007; Knott and Bossy-Wetzel, 2008). Loss of any one of the latter can lead to mitochondrial impairment and has been associated with many human diseases, particularly neurodegenerative diseases. For instance, a mutation in the mitochondrial fusion protein, Mfn2, causes Charcot-Marie-Tooth disease, a neurodegenerative disorder associated with axonal degeneration (Zuchner et al., 2004). Furthermore, mitochondrial network defects are observed in brains of patients with Alzheimer's disease. The cause of neurodegeneration in Alzheimer's remains unknown, however, the amyloid precursor protein that has been implicated in neurotoxicity also shows an enhancement of mitochondrial fission and a breakdown of the mitochondrial network (Cho et al., 2009). Disease-associated mutations in the Huntington's gene is reported to promote mitochondrial dysfunction through diminished function of complex II, an increase in mitochondrial fission and also through defects in mitochondrial calcium buffering potential (Bossy-Wetzel et al., 2008; Wang et al., 2009). Mutations in the mitophagy genes, PINK1 and Parkin, have been found as two of the causative agents in the degeneration of the dopaminergic neurons in Parkinson's disease (Bentivoglio et al., 2001; Kitada et al., 1998). It is clear that maintaining mitochondrial homeostasis is essential for promoting the survival of cells and, in particular, neurons. The Bcl-2 family have already been implicated in regulation of both the fission and fusion dynamics of mitochondria and now we have characterized a novel role for anti-apoptotic Bcl-2 proteins in regulation of mitochondrial turnover through mitophagy. Understanding how the Bcl-2 family influence mitochondrial homeostasis could be vital for the development of treatments for diseases associated with mitochondria dysfunction, such as, neurodegenerative diseases. Furthermore, we also characterized a novel pro-apoptotic function of Parkin which is in contrast to its anti-apoptotic function reported in the literature (Darios et al., 2003; MacCormac et al., 2004; Muller-Rischart et al., 2013; Sun et al., 2013). As Parkin has been

identified as a causative gene of Parkinson's disease, we think that this novel pro-apoptotic function could provide a possible mechanism for neurodegeneration observed in idiopathic Parkinson's disease.

6.2 The Bcl-2 family and regulation of apoptosis

The role of various Bcl-2 family members in the intrinsic pathway to apoptosis has been clearly established with the mitochondria being central to this mode of cell death. The BH3-only proteins act as sensors that detect a variety of stresses in the cell. Following a cellular stress, the BH3-only family can be activated either by transcriptional upregulation or by post-translational modifications such as cleavage or phosphorylation (Luciano et al., 2003; Luo et al., 1998; Villunger et al., 2003; Zha et al., 1996). Once BH3-only protein activity reaches a threshold, they overwhelm anti-apoptotic Bcl-2 proteins through neutralization and promote activation of Bax and Bak. Bax and Bak then oligomerize to form channels and insert into the outer mitochondrial membrane inducing MOMP. The activation of MOMP has two significant consequences, firstly protons leak out through the Bax/Bak channel and the proton gradient (mitochondrial potential) required for production of ATP is lost. As a result, the mitochondria can no longer produce the energy required to fulfil the needs of the cell. Secondly, cytochrome c is released into the cytosol via the Bax/Bak channel were it assembles the apoptosome to initiate apoptosis, however, this second step is dispensable for cell death. For instance, in cells with cytochrome c-dependent capase activation abolished, via inactivation of genes encoding caspase-9 or Apaf-1, MOMP promotes a caspase-independent mode of cell death with morphological features resembling necrosis (Ekert et al., 2004). This indicates that loss of mitochondrial potential is the step that commits a cell to dying with subsequent caspase activation merely acting to reduce the inflammatory potential of the dying cell (Luthi et al., 2009; Martin et al., 2012). The anti-apoptotic Bcl-2 family proteins block apoptosis by maintaining the mitochondria potential through inhibiting Bax/Bak oligomerization and subsequent membrane permeabilization. This function of anti-apoptotic Bcl-2 proteins maintains the integrity of the mitochondria throughout the lifetime of a cell.

6.3 The Bcl-2 family and Mitochondrial morphogenesis

As mentioned previously, cells maintain mitochondrial homeostasis through the coordination of mitochondrial fission, fusion, motility and turnover (Chen and Chan, 2009; Detmer and Chan, 2007; Knott and Bossy-Wetzel, 2008). In healthy cells, mitochondrial predominantly exist as a tubular network and maintain this through a balance of mitochondrial fission and fusion. This dynamic process allows mitochondria to mix their mitochondrial DNA (mtDNA) and other metabolites that is essential for maintaining mitochondrial function. Fragmentation of this network, either through increased fission or decreased fusion, leads to impaired mitochondria and decreased ATP production (Chen and Chan, 2005; Suen et al., 2008). The Bcl-2 family have been implicated in mitochondrial morphogenesis with different family members having opposite effects on the network. During apoptosis, the mitochondrial network becomes fragmented generating numerous small and circular mitochondria (Frank et al., 2001; Karbowski et al., 2002; Sheridan et al., 2008). The fragmentation of mitochondria is caused by Bax activation and is a result of either increased mitochondrial fission or decreased fusion. This fragmentation prevents the exchange of mtDNA or metabolites and leads to impairment of the mitochondria. As Bax promotes mitochondrial dysfunction in both apoptosis and mitochondrial morphogenesis, this suggests that the role of Bax activation is to promote mitochondrial dysfunction in order to favour apoptosis. Concomitantly with this idea, anti-apoptotic Bcl-2 proteins also modulate mitochondrial fission and fusion dynamics but with the opposite effect of Bax. Anti-apoptotic Bcl-2 proteins balance mitochondrial fission and fusion to maintain a tubular mitochondrial network. This allows mixing of mtDNA and metabolites between the network and is essential to maintain the integrity of the mitochondria which ultimately favours cell survival. Bcl-$X^{-/-}$ mice showed increased fragmentation of mitochondrial networks which were rescued by re-introduction of Bcl-X (Berman et al., 2009). Another report demonstrated that cells overexpressing Bcl-2 had mitochondria that were larger and structurally more complex than normal (Kowaltowski et al., 2002). Once again, the anti- and pro-apoptotic Bcl-2 demonstrate opposing effects, however, now it is observed in mitochondrial morphogenesis. Furthermore, anti-apoptotic Bcl-2 proteins again promote survival and the pro-apoptotic BH3-only proteins again promote apoptosis. In both apoptosis and mitochondrial morphogenesis, the anti-apoptotic Bcl-2 proteins promote survival through

maintaining mitochondrial homeostasis while the pro-apoptotic proteins promote apoptosis through decreasing mitochondrial health.

6.4 The Bcl-2 family and Regulation of Mitophagy

The balance between removal of damaged mitochondria and biogenesis of new mitochondria is essential in regulating mitochondrial biomass in order to generate ATP requirements of cells. Again we observed opposing roles for anti- and pro-apoptotic Bcl-2 family members in this process. We demonstrated that the anti-apoptotic Bcl-2 family members can regulate mitophagy through influencing the recruitment of Parkin to mitochondria, thereby setting a threshold for clearance of damaged mitochondria via mitophagy. Clearance of damaged mitochondria in a timely fashion while sparing healthy mitochondria was once again promoting cell survival. In contrast, we found that BH3-only proteins or the BH3-only mimetic, ABT-737, were capable of enhancing Parkin translocation to mitochondria through decreasing this threshold for mitophagy. A lower threshold would lead to excessive mitophagy and decreased cellular health through a reduction in ATP synthesis. The production of ATP through oxidative phosphorylation generates reactive oxygen species that can have damaging effects on the mitochondria, either through oxidation of mitochondrial proteins or by damaging the mitochondrial genome (Ishikawa et al., 2008; Lin and Beal, 2006). Therefore, these potentially damaging mitochondria must be removed before impacting severely on cellular health. Once again, Bcl-2 family members play a role in maintaining the mitochondrial network. The balance between anti-apoptotic Bcl-2 proteins and pro-apoptotic BH3-only proteins is essential to prevent entry into apoptosis, however, during mitophagy this balance sets a threshold allowing specific removal of damaged mitochondria and the maintenance of the mitochondrial health.

While there is redundancy in anti-apoptotic Bcl-2 proteins in inhibiting mitophagy, different family members have varying inhibitory potential. This means that the mitophagy threshold will vary from cell to cell with expression levels of various Bcl-2 family proteins will dictate the threshold in different cell types. Mitochondrial dysfunction is common among many neurodegenerative diseases. Neurons are post-mitotic and must survive for the entire life of an organism. The Bcl-2 expression pattern in neurons may favour neuronal survival over the lifetime, however, at the same time indirectly setting a

high threshold for mitophagy. This could potentially lead to accumulation of mitochondrial network defects over many decades, until the mitochondria can no longer meet the energy requirements of the cell and begin to die. This may explain why mitochondrial dysfunction is so prevalent in neurodegenerative diseases and indicate that correct management of the mitophagy threshold could be a useful tool in the management of neurodegenerative diseases.

6.5 Bcl-2 family proteins and Parkin-induced apoptosis

While the anti-apoptotic Bcl-2 proteins are constantly protecting the mitochondria from MOMP, there are cellular stresses which once sensed by the cell can promote Bax/Bak activation and apoptosis through a reduction in anti-apoptotic Bcl-2 proteins. Interestingly, we have demonstrated that in response to mitochondrial depolarization Parkin can decrease the abundance of the anti-apoptotic Bcl-2 family member, Mcl-1. This decrease reduces the ability of the anti-apoptotic Bcl-2 family as a whole to protect mitochondria from MOMP. Here, in contrast to the cytoprotective role of Parkin we observed during mitophagy, we see that Parkin can in fact promote apoptosis. As demonstrated in cancer, too much anti-apoptotic activity can have detrimental consequences and it appears that Parkin can alter the abundance of Mcl-1 in response to mitochondrial depolarization to induce apoptosis. Bax/Bak activation induces MOMP resulting in mitochondrial depolarization, however, it is the release of cytochrome c which initiates apoptosis. As can be seen in our data, not all mitochondrial depolarization events create channels large enough for release of cytochrome c and subsequent apoptosis. The treatment of HeLa cells with mitochondrial depolarizors does not initiate apoptosis as they lack a functional PINK1/Parkin pathway. As these mitochondria are no longer generating enough ATP, the cells will eventually die through the pro-inflammatory mode of cell death, necrosis. However, in cells with a functional PINK1/Parkin pathway, this depolarization can be sensed by Parkin, which then promotes MOMP allowing for the cell to be removed through the non-inflammatory mode of cell death, apoptosis.

The bidirectional regulation of Parkin and anti-apoptotic Bcl-2 proteins allows the maintenance of mitochondrial integrity while possessing anti-inflammatory properties by promoting apoptosis following mitochondrial depolarization which fails to release cytochrome c.

6.6 PINK1/Parkin rupture of the Mitochondria

There have been other reports which also characterized mitochondrial rupture in response to Parkin activation leading to the death of the cell. Yoshii et al. characterized the mitochondrial morphology during activation of Parkin and found that the mitochondria swell and rupture suggesting that cell death in response to Parkin activation maybe independent of apoptosis (Yoshii et al., 2011). Yoshii et al. draw these conclusions from electron scanning imagery of few mitochondria within a cell and unfortunately do not analyze any molecular markers of apoptosis. Mitochondria swelling and rupture has been observed in cells treated with pro-apoptotic agents such as Etoposide. Etoposide induced apoptosis is Bax/Bak dependent suggesting that mitochondrial rupture and Bax/Bak activation are not mutually exclusive and can occur concurrently (Albihn et al., 2006; Sun et al., 2007).

A recent paper (Liang et al., 2015) also demonstrated the dependency of Bax/Bak in PINK1/Parkin-induced cell death. The study also demonstrated that the deubiquitinases, USP30, antagonizes Parkin ubiquitin ligase function and overexpression can protect cells against PINK1/Parkin-induced cell death. This demonstrates a tight regulatory mechanism to prevent excessive cell death in response to Parkin activation. The group also finds, consistent with our results, that while overexpression of USP30 can protect against PINK1/Parkin-induced cell death, knockdown of Bax and Bak is required to protect against PINK1/Parkin-induced cell death. This futher supports our conclusions that PINK1/Parkin-induced apoptosis is mediated through the activation MOMP and subsequent Caspase activation (Liang et al., 2015).

6.7 Parkin and Parkinson's disease

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic cells within the Substantia Nigra of the mid-brain and approximately 5-10% of cases are inherited while the remaining 90-95% are idiopathic (Dauer and Przedborski, 2003). The discovery of PINK1 and Parkin as two causative genes in inherited Parkinson's disease implicated a failure of mitophagy in the neurodegeneration. To date, the only insight into idiopathic Parkinson's disease is an increase of mitochondrial dysfunction in patients (Lestienne et al., 1991; Lin and Beal, 2006; Schapira et al., 1989). While further

work may implicate mitophagy in the etiology of idiopathic Parkinson's disease, it is possible that the novel pro-apoptotic property of Parkin plays a role in the neurodegeneration. Parkin may act as a sensor for mitochondrial dysfunction, becoming recruited to damaged mitochondria to promote their removal via mitophagy and enhance neuronal survival. Loss of a functional mitophagy pathway in inherited Parkinson's disease would decrease neuronal network health through an accumulation of dysfunctional mitochondria. However, robust or sustained activation of Parkin would promote the pro-apoptotic function of Parkin. The mitochondrial dysfunction observed during idiopathic Parkinson's disease could robustly activate Parkin, thereby promoting its pro-apoptotic activity and promoting neurodegeneration. The specific sensitivity of dopaminergic neurons to defective mitophagy observed in familial Parkinson's disease suggests that these cells require a high turnover of their mitochondria to maintain a functional mitochondrial network in order to provide the ATP requirements of the cell. This high rate of turnover suggests the presence of a lot of damaged mitochondria within dopaminergic neurons. These damaged mitochondria could provide the robust or sustained Parkin activation to induce apoptosis in idiopathic Parkinson's disease. However, further work is required on Parkin-induced apoptosis to elucidate a role, if any, associated with idiopathic Parkinson's disease.

6.8 Bcl-2 Therapeutics for treatment of Parkinson's disease

Through intensive research over the last two decades, the function of the Bcl-2 family members in regulation of apoptosis has been progressively elucidated. More recently, the development of cancer therapies has moved from conventional cytotoxic agents to targeted therapies against cancer promoting proteins. Examples of these targeted therapies are the BH3 mimetics, such as ABT-263, obatoclax and TW-37, which neutralize the anti-apoptotic Bcl-2 proteins (Konopleva et al., 2006; Wilson et al., 2010; Zhai et al., 2006). Further research is required to delineate the role, if any, the Bcl-2 family have in Parkinson's disease. It is possible that alterations in expression levels or post-translational modifications of the Bcl-2 family proteins may alter their balance to maintain mitochondrial homeostasis. The development of targeted therapies against the Bcl-2 family members, in a vein similar to cancer therapies, may allow for the manipulation of

the threshold set by the Bcl-2 family to control of mitochondrial homeostasis and treat neurodegenerative diseases.

The role of Bcl-2 in cancer has been clearly established and chemotherapeutics drugs were developed to overcome the resistance to cell death within cancer. However, neurodegeneration is the excessive/unwanted death of cells and to halt disease progression this deregulated death must be prevented. At present, there are no treatments which can prevent this unwanted death of cells, however, as mitochondrial dysfunction is a common feature to many neurodegenerative diseases, the best strategy may be to maintain mitochondrial homeostasis through manipulation of Bcl-2 family proteins and promote neuronal survival.

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