ANALYSIS OF MITOCHONDRIAL SIGNALING IN THE REGULATION OF
PROGRAMMED CELL DEATH

by

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A thesis submitted in conformity with the requirements for the degree of
Doctor of Philosophy (Ph.D.).
Graduate Department of Pharmaceutical Sciences, University of Toronto

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ABSTRACT

Analysis of Mitochondrial Signaling in the Regulation of Programmed Cell Death

Doctor of Philosophy, 2011

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The involvement of mitochondrial signaling in mammalian PCD regulation has been examined extensively via biochemical analyses and cellular studies in vitro. However there still exist considerable gaps in our knowledge regarding its contribution in specific tissues and cell types during mammalian development in vivo. In addition, given the numerous pathologic conditions associated with aberrant PCD, modulation of this signaling process represents an attractive target for therapeutic intervention. In this thesis I have therefore examined the regulation of mitochondrion-mediated PCD signaling as it pertains to several forms of developmental and injury-induced cell death.

In the first component of the thesis I have examined the differential sensitivity of Bcl2 on the survival of motor neuron populations from two distinct developmental origins (alpha and gamma motor neurons), demonstrating that gamma motor neurons are preferentially affected in Bcl2 null mice. Thus, Bcl-2 plays a critical in vivo in regulating subtype-specific motor neuron survival during development. In the second study I have demonstrated that a major portion of the neuroprotective effect exerted by the immunophilins cyclosporin A and FK-506 are mediated through calcineurin signaling;
rather than MOMP-mediated events as previously held. Additional findings of this study demonstrated the first neuroprotective effects of the pyrethroid insecticide cypermethrin and calcineurin-mediated control of Bad phosphorylation. Such findings establish a link between calcineurin signaling and mitochondrion-mediated cell survival.

The above studies established critical features of mitochondrion-mediated PCD in regulating survival of several neuronal subpopulations. I therefore followed these studies with an examination of how post-mitochondrial PCD signaling is regulated following MOMP permeabilization. Specifically I examined regulation of the Smac-IAP-caspase axis, investigating how combinatorial deletion of Casp3 and Diablo alter PCD progression in mouse embryonic fibroblasts. Using a series of injury stimuli in the context of biochemical and cellular analyses I have developed a model of how endogenous Smac/DIABLO regulates executioner caspase activity. Collectively these studies elucidate key aspects of mitochondrial signaling during both developmental and injury-induced PCD in vivo.
ACKNOWLEDGEMENTS

My foremost gratitude goes to my thesis advisor Dr. Jeffrey Henderson whom has been much more than a mentor for my doctorate studies, but also a role model and life mentor. Throughout these years I have learned not only lessons of scholarly principles and scientific methods; but also invaluable life lessons as well. Without his ingenious guidance and uncompromising attitude this thesis would not have been possible. I thank him for his patience and confidence as he continued to inspire and motivate me to move forward against what I deemed to be impossible at times. It is indeed an honour to have been trained by him.

I would like to thank my thesis advisory committee members, Drs. James Eubanks, Peter O’Brien and Minna Woo for their insightful comments and constructive criticism throughout the years. As well many thank to Drs. Andrew Elia and Carrie Causing-Henderson for their technical guidance and support throughout my training.

I would also like to thank Canadian Institute of Health Research and Rx&D Health Research Foundation for their scholarship support.

Of course it would have been agonizing to fight this challenging battle without the comradery of my fellow trainees, Drs. Stephanie Ho and Anish Kanungo, and former laboratory technician, Patrick Ng. In addition, I was also fortunate to have interacted with the numerous undergraduate interns whom I have helped train in the laboratory.
Finally, I would like to thank my parents, Patrick and Julianna, my relatives and friends, for their continual support and understanding to help me through this challenging but satisfying journey.
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<tr>
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<tbody>
<tr>
<td>17-AAG</td>
<td>17-(allylamino)-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocase</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease-activating factor 1</td>
</tr>
<tr>
<td>ATG</td>
<td>autophagy-related genes</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting domain domain agonist protein</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BIR</td>
<td>baculovirus inhibitory repeat</td>
</tr>
<tr>
<td>BRUCE</td>
<td>BIR repeat-containing ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CARP</td>
<td>caspase-8 and -10-associated RING protein</td>
</tr>
<tr>
<td>CED</td>
<td>cell death defective</td>
</tr>
<tr>
<td>cFLIP</td>
<td>cellular form of FLICE-inhibitory protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>cIAP</td>
<td>cellular inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CPC</td>
<td>chromosomal passenger complex</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>dARK</td>
<td><em>Drosophila</em> Apaf-1 related killer</td>
</tr>
<tr>
<td>dBorg</td>
<td><em>Drosophila</em> Bcl2 ortholog</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
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<tr>
<td>Debcl</td>
<td>death executioner Bcl-2 homologue</td>
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<td>DED</td>
<td>death effector domain</td>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<td>DIAP</td>
<td><em>Drosophila</em> inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>DIABLO</td>
<td>direct IAP-binding protein with low pI</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signaling complex</td>
</tr>
<tr>
<td>DKO</td>
<td>double knockout</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>drICE</td>
<td><em>Drosophila</em> IL-1β converting enzyme</td>
</tr>
<tr>
<td>Drob</td>
<td><em>Drosophila</em> ortholog of the Bcl-2 family</td>
</tr>
<tr>
<td>Drp1</td>
<td>dynamin-related protein 1</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGL</td>
<td>egg-laying defective</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK-506 binding protein</td>
</tr>
<tr>
<td>FOXO</td>
<td>forkhead box transcription factor O</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence/Förster resonance energy transfer</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HID</td>
<td>head involution defective</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>Hsp90</td>
<td>heat shock protein 90</td>
</tr>
<tr>
<td>IBM</td>
<td>IAP-binding motif</td>
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<tr>
<td>INCENP</td>
<td>inner centromere protein</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of κB (IκB) kinase</td>
</tr>
<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
</tr>
<tr>
<td>IP3R</td>
<td>inositol 1,4,5-triphosphate receptor</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Mfn</td>
<td>mitofusin</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MLS</td>
<td>mitochondrial import sequence</td>
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<tr>
<td>MOMP</td>
<td>mitochondrial outer membrane permeabilization</td>
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<tr>
<td>mPTP</td>
<td>mitochondrial permeability transition pore</td>
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<tr>
<td>Naip</td>
<td>neuronal apoptosis inhibitory protein</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide binding and oligomerization domain</td>
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<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
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<td>PAS</td>
<td>phagophore assembly site</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PCD</td>
<td>programmed cell death</td>
</tr>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>PMSF</td>
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<td>postnatal day</td>
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<td>Puma</td>
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<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>Smac</td>
<td>second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>Timm8</td>
<td>translocase of inner mitochondrial membrane 8</td>
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<td>TNFR</td>
<td>tumour necrosis factor receptor</td>
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<tr>
<td>Tor</td>
<td>target of rapamycin</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
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<td>TRAF</td>
<td>TNFR-associated factor</td>
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<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<tr>
<td>UBA</td>
<td>ubiquitin-associated domain</td>
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<td>UBC</td>
<td>ubiquitin-conjugating domain</td>
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<td>VDAC</td>
<td>voltage-dependent anion channel</td>
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<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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SECTION 1: INTRODUCTION
1.1 RATIONALE AND RESEARCH OBJECTIVES

While our understanding of the molecular details of programmed cell death (PCD) has grown substantially since the first genetic dissection of PCD in *Caenorhabditis elegans* nearly three decades ago, a complete understanding of this regulatory network in mammals has yet to be determined. In many cases, pathologic aspects of PCD have been shown to be intimately connected to a number of human disease states. There is therefore significant interest in understanding and modifying intrinsic responses to PCD.

At the centre of the PCD regulatory network is the power plant of the cell, the mitochondrion. This cellular organelle was linked to PCD regulation for the first time twenty years ago when it was shown to be a principal site of Bcl-2 localization in the cell. Interestingly, involvement of the mitochondrion appears to be a unique property of PCD in vertbrates, as *C. elegans* and *Drosophila Melanogaster* do not require mitochondrial-derived signals. Given its central role in regulating PCD, tremendous research effort has been invested in understanding the involvement of Bcl-2 family proteins at the mitochondria and functional consequences in controlling downstream signaling. As a result a long list of Bcl-2 family proteins have been identified and we now possess a basic molecular understanding of how the interactions are translated into cellular actions. However, there are still a number of regulatory interactions involving these proteins which have yet to be fully deciphered. For example, though it is now recognized that mitochondrial outer membrane permeabilization is critical to many forms of PCD, we have yet to attain a complete understanding of how the release of the various apoptogenic
proteins elicits downstream PCD signaling to ultimately trigger the demise of affected cells.

In this thesis, I have examined those factors which modulate PCD, with a particular focus on the mitochondrial and caspase regulatory checkpoints. Specifically I have examined the supportive role of Bcl-2 in maintaining the survival of a subpopulation of motor neurons during the period of postnatal development. I have delineated mechanisms which promote motor neuron survival following injury, and in doing so elucidated the means by which immunosuppressive agents actually promote motor neuron survival and identified new survival-promoting pharmaceutics in the process. I have pursued mechanisms of post-mitochondrial PCD signaling addressing longstanding issues with respect to the physiologic role of Smac/DIABLO in regulating executioner caspase activity. From these collective studies, I have determined the most significant chokepoints of PCD regulation and designed a real-time high-throughput system to identify optimal small molecule therapeutics for modifying PCD signaling. Taken together, the thesis examines several critical interactions in both developmental and injury-induced PCD signaling. The aim of these studies is to clarify the regulatory influences controlling each of these PCD checkpoints in addition to gaining a better understanding of mammalian PCD.
1.2 REVIEW OF THE LITERATURE

1.2.1 PROGRAMMED CELL DEATH AS A PHYSIOLOGIC PROCESS

Programmed cell death (PCD) was originally recognized by Carl Vogt in 1842 as a natural process which occurs during the physiological development of toad embryos (Clarke and Clarke, 1996). Later examination of insect metamorphosis by (Lockshin and Williams) in 1965 introduced the concept that this process of conversion was “programmed”. In 1972 the term “apoptosis”, used by the Ancient Greeks, was reintroduced in a biomedical context by Kerr and colleagues (1972) to describe programmed cell death as a basic biological process controlling tissue turnover and focal cell deletions during embryonic development. In this seminal review, Kerr and colleagues not only proposed programmed cell death as the principal mode by which cells can control their own demise, but provided a detailed ultrastructural description of this process. Examination of electron micrographs from Kerr and others in embryos as well as healthy and cancerous adult tissues suggested that apoptosis is a common process responsible for a variety of morphological changes in tissues. In particular these investigations observed the formation of condensed cytoplasmic fragments containing remnants of pyknotic nuclei which came to be known as apoptotic bodies. These observations unified the findings in a number of fields, and marked the beginning of an era in PCD research.
1.2.1.1 FUNCTIONS OF PROGRAMMED CELL DEATH

Programmed cell death is a genetically regulated system of cellular suicide which has been conserved throughout metazoan evolution for at least 600 million years (Ayala and Rzhetsky, 1998). Through this process, multi-cellular organisms can eliminate either aberrant or supernumery cells in a manner which largely circumvents inflammatory responses (Carson and Ribeiro, 1993). PCD has been shown to be critical both for the proper development of multi-cellular organisms, and for tissue homeostasis. Because of its importance, PCD is under stringent control and dysregulation of this process can result in developmental abnormalities or pathology in a variety of organ systems.

1.2.1.2 ROLE OF PROGRAMMED CELL DEATH IN DEVELOPMENT

During embryonic development, tissues are formed via the selective proliferation and deletion of cell populations. Programmed cell death has been demonstrated to be the principal mechanism by which cellular deletion occurs during development. PCD is particularly important in neural tissues which undergo extensive growth prior to connectivity refinements through the removal of suboptimal targets and target-locus matching. It has been estimated that for most regions approximately twice the number of neurons are generated than ultimately incorporated into the network architecture (Lance-Jones, 1982; Oppenheim et al., 1986). Survival appears to be restricted largely to those neurons which manage to form the most optimal neuroanatomical connections. Optimization of the number of neural connections in many CNS regions appears to involve the regulated expression of neurotrophic factors to maintain cell survival. Consistent with this, exogenous overexpression of neurotrophic factors during
development has been shown to be capable of rescuing neurons which would otherwise be fated to die (Hassankhani et al., 1995; Nguyen et al., 1998; Oppenheim et al., 1995; Yip et al., 1984). The absence of such signals is one means by which PCD is initiated (Kuan et al., 2000).

In a similar manner, B and T lymphocytes undergo PCD-mediated positive and negative selection during development of the immune system (MacDonald, 1989; Melchers et al., 1995). To develop adaptive immunity, immunoglobulin and T cell receptor (TCR) repertoires are produced through rearrangement and diversification of their respective VDJ gene segments (Willerford et al., 1996). Negative selection ensures that there is minimal recognition of self-antigens by either immunoglobulins or T cell receptors, while positive selection is utilized primarily by T lymphocytes to ensure that they express functional TCRs capable of binding to major histocompatibility complexes (MHC) for future foreign antigen presentation. Thus PCD operates to support immune surveillance by eliminating non-functional and self-recognizing lymphocytes through the process of programmed cell death (Linette and Korsmeyer, 1994; Strasser and Bouillet, 2003). Failure to do so can result in pathologies such as autoimmunity and cancer (Kuhtreiber et al., 2003).

Programmed cell death is also involved in a number of other processes which include tissue restructuring (such as the removal of interdigital webbing) and metamorphosis (Daish et al., 2004; Mills et al., 2006; Wei et al., 2001). Consistent with these developmental effects, mutations in a number of PCD signaling proteins have been shown to result in embryonic lethality or failure of specific organ systems (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Kuida et al., 1996; Varfolomeev et al.,
1998; Woo et al., 1998; Yoshida et al., 1998). Through such analyses, we have learned a great deal regarding the nature of PCD signaling pathways.

1.2.1.3 ROLE OF PROGRAMMED CELL DEATH IN NEURAL INJURY

As indicated above, dysregulation of PCD has been implicated in various pathologic conditions ranging from neurodegeneration to cancer (Hanahan and Weinberg, 2000; Yuan and Yankner, 2000). In addition, the role which PCD plays in regulating the response to tissue injury, particularly neural injury, has garnered tremendous interest in recent years as modification of this pathway has been shown to significantly enhance neural repair.

To further our understanding of the contribution of the various components of PCD signaling, genetic manipulation of PCD proteins have been performed in a number of cases to examine their contribution in developmental and neural injury-induced PCD (de Bilbao and Dubois-Dauphin, 1996; Deckwerth et al., 1996; Dubois-Dauphin et al., 1994; Kanungo et al., 2008; Michaelidis et al., 1996; Parsadanian et al., 1998; Sun and Oppenheim, 2003). The contribution of these factors to a variety of experimentally-induced forms of neural injury including stroke (middle cerebral artery occlusion – MCAO; excitotoxicity – kainic acid injection), traumatic CNS injuries and neurodegenerative diseases (Parkinson’s disease – 6-hydroxydopamine injection). For example, it has been demonstrated in numerous studies that anti-apoptotic protein Bcl-2 can dramatically alter survival response in various neural injury models ranging from neonatal facial axotomy to adult middle cerebral artery occlusion (de Bilbao and Dubois-Dauphin, 1996; Dubois-Dauphin et al., 1994; Zhang et al., 2006). While studies such as
these clearly demonstrated that modulation of PCD pathways could alter the response to neural injuries, these findings are of limited therapeutic potential given the difficulty of human gene modification. As a result, pharmacologic modulators of programmed cell death have become of intense interest in biomedical research. Though a number of low molecular weight compounds have been examined for their abilities to rescue injured neurons (Casanovas et al., 1996; Iwasaki et al., 1996; Outeiro et al., 2007; Simonin et al., 2006; Zhu et al., 2002), few have demonstrated significant and reproducible success. Fewer still exhibit satisfactory safety profiles for use in humans. None have thus far been translated for clinical use.

Interestingly, a class of immunosuppressants (acting as immunophilin ligands) have been shown to possess significant neuroprotective properties against a variety of neural insults (Dawson et al., 1993; Springer et al., 2000; Steiner et al., 1997a; Steiner et al., 1997b). The two prototypical immunophilin ligands, cyclosporin A and FK-506, are currently in wide use clinically to alleviate host-versus-graft rejection following organ transplants. Their pharmacokinetic properties and clinical safety profiles of these drugs have been extensively studied. Despite this, the molecular mechanism underlying their neuroprotective properties remains elusive. While the molecular mechanisms underlying the immunosuppressive properties of these drugs [calcineurin-mediated dephosphorylation of nuclear factor of activating T cells (NFAT)] are well understood, I will argue that the longstanding model explaining their neuroprotective effects [blockade of mitochondrial permeability transition pore (mPTP)] is incorrect.

As described below, these findings have significant impact toward the development of small molecule neuroprotective agents.
1.2.2 CHARACTERISTICS OF PROGRAMMED CELL DEATH

Prior to the discovery of programmed cell death it was believed that the principal process regulating tissue homeostasis was the rate of cellular proliferation, and that cell death was only a passive process which occurred following irreversible cell damage such as membrane disruption, mitochondrial dysfunction, accumulated damage to DNA, proteins and organelles (i.e., cell death is irreversible). However it was later recognized that an actively regulated mode of cell death existed which occurred in response to specific physiologic stimuli. Morphological analysis of cells undergoing this “programmed” cell death distinguished it from necrosis and in the past two decades tremendous effort has been focussed on dissecting the molecular mechanisms regulating this signal transduction process.

1.2.2.1 MORPHOLOGICAL AND MOLECULAR CHARACTERISTICS

In contrast to necrosis which usually manifests an early loss of plasma membrane integrity, PCD involves progressive sequestration of cellular organelles forming packets of cytoplasmic fragments called apoptotic bodies (Kerr et al., 1972). These apoptotic bodies are taken up and degraded by surrounding cells through phagocytosis (Figure 1), thus allowing cell death to proceed largely in the absence of an inflammatory response. Thus, PCD is believed to represent a developmental alternative to necrosis in which specific critical cell populations can eliminate supernumery cells in a manner which avoids the secondary consequences of inflammation (Carson and Ribeiro, 1993).
Figure 1. Morphological Distinctions Between Apoptosis and Necrosis

Several features distinguish cells morphologically between PCD and necrosis. During PCD, cells destined to die first undergo cellular shrinkage and chromosomal condensation in a process known to be caspase-3-dependent (Woo et al., 1998). Subsequently blebbing of the plasma membrane ultimately results in the formation of apoptotic bodies. These cell fragments are eventually removed through the process of phagocytosis. Due to the maintenance of membrane integrity throughout this process, an inflammatory response is not provoked. In contrast cells undergoing necrosis swell as ionic and osmotic gradients begin to collapse. Eventually the plasma membrane is breached and becomes leaky, leading to disruption of cellular organelles. In the final stages, cellular and nuclear lyses occur releasing cellular constituents into the surrounding environment triggering inflammation.
Necrosis

- cell swelling
- leaky membrane, blebbing
- cellular and nuclear lyses, inflammation

PCD

- cell shrinkage, chromosome condensation
- apoptotic bodies formation
- phagocytosis, inflammatory response averted

Adapted from
Van Cruchten and Van Den Broeck, *Anat Histol Embryol*, 2002
During PCD, activated caspases cleave a large number of cellular substrates (Luthi and Martin, 2007). Among these are key cytoskeletal and regulatory components of the cell [e.g., vimentin, glial fibrillary acidic protein (GFAP), poly (ADP-ribose) polymerase (PARP), inhibitor of caspase-activated DNase (ICAD)] resulting in the observed cellular compaction (Brancolini et al., 1997; Byun et al., 2001; Caulin et al., 1997; Chen et al., 2003a; Kothakota et al., 1997; Mashima et al., 1999; Schmeiser and Grand, 1999). The organelle compaction which occurs as a result of caspase activity makes nucleoplasm of apoptotic cells appear dense when viewed by histologic stains. The DNA and chromosomal fragmentation which occurs during this process is not random, but rather occurs in a regulated manner as can be seen through electrophoresis as is often referred to as DNA laddering (Enari et al., 1998; Sakahira et al., 1998; Wyllie, 1980). The specific form of DNA cleavage which occurs during PCD can be seen using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Gavrieli et al., 1992). While this form of DNA cleavage is not the only kind to occur during PCD, its unique nature has proven useful in identifying apoptotic cells in situ.

A further molecular feature which occurs during the early phase of PCD is the displacement of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Normally, phosphatidylserine is concentrated along the cytoplasmic surface of the cell. Once redistributed to the extracellular surface it serves as a phagocytic signal to surrounding cells such as macrophages for engulfment (Fadok et al., 1992). In the field of PCD, phosphatidylserine redistribution is frequently examined by using labelled Annexin V, a phospholipid-binding protein with a high affinity for phosphatidylserine (Andree et al., 1990). Like TUNEL, annexin V is often used to identify apoptotic cells in situ.
situ (though typically it is applied to free cells in solution for flow cytometric analysis) (Koopman et al., 1994). The development of such molecular markers has allowed us to develop a detailed picture of the nature and extent of PCD in mammals over the last two decades.

1.2.2.2 DIFFERENCES WITH OTHER FORMS OF CELL DEATH

Although traditionally viewed as a distinct mode of regulated cell death in multicellular organisms, in recent years several alternative pathways with features overlapping that of PCD have emerged. Principal among these are necroptosis and autophagy. Necroptosis and necrosis are generally believed to involve an oxidative burst with mitochondrial membrane hyperpolarization. Lysosomal and plasma membrane permeabilization are also common elements of these two pathways of cellular destruction (Degterev et al., 2005). There is evidence to suggest that necroptosis is an alternative mechanism by which cells can signal for elimination when primary modes of cell death (such as PCD) are abrogated through pathologic inhibition (e.g., caspases can be inhibited by viral proteins such as p35) (Cho et al., 2009). Mechanistically, necroptosis is typically induced through stimulation of cell surface death receptors such as TNFR1 and Fas. Receptor-interacting serine-threonine kinase 1 (RIPK1/RIP/RIP1), a downstream effector of death receptor signaling has been demonstrated to exert a vital role in both PCD and necroptotic signaling (Degterev et al., 2008; Hitomi et al., 2008; Wang et al., 2008) (Figure 2). Importantly, RIPK1 has been shown to be a target substrate of caspase-8, therefore suggesting that activation of the extrinsic pathway can potentially avert initiation of necroptosis (Lin et al., 1999). Small molecule compounds called necrostatins
Necrosis has traditionally been viewed as a passive and unregulated process which occurs as a result of irreversible cell damage. More recently however, evidence has emerged that necroptosis is a regulated process, exhibiting morphological similarities with necrosis. Necroptosis was first described to be initiated in cells by TNF-α treatment with concurrent blockade of caspases (Degterev et al., 2005; Hitomi et al., 2008). Subsequent work has demonstrated that necroptosis can also be stimulated by signaling from other death receptors such as Fas (Geserick et al., 2009). The initiation of death receptor signaling by the binding of death ligands (e.g., TNF-α) begins with the formation of complex I which includes TRADD, TRAF2, cIAP-1, NEMO (NFκB essential modulator), and CYLD (cylindromatosis). Once formed this complex promotes the formation of other protein complexes (Complex IIa and IIb), initiating distinct signaling pathways, each with different outcomes. As illustrated in the figure, RIP1 is involved in both Complex IIa and IIb, depending on its association with FADD and caspase-8, or RIP3. It remains to be determined what factors regulate which complex is formed under a specific set of cell death condition.
Adapted from Christofferson and Yuan, *Curr Opin Cell Biol*, 2009
have been utilized in recent years to demonstrate the role which necroptosis can play in
the cellular damages elicited during neural injuries (Degterev et al., 2005; Li et al., 2008;
You et al., 2008). More recently RIP3 has been suggested to be involved in both PCD
and NFκB signaling induced by TNF-α, and was shown to be a critical effector of
necroptosis (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). Aside from its
identification as a principal downstream interactor of RIPK1, it has been demonstrated
that RIP3 can orchestrate changes in metabolic enzymes to regulate ROS production
triggered by TNF-α, thus providing further support for a role in mediating necroptosis
(Zhang et al., 2009). As necroptosis has thus far only been demonstrated to be initiated by
death receptor activation, it will be interesting to determine whether necroptosis can be
activated via other modes of stimulation and which cellular signals ultimately discern
between PCD and necroptosis.

In contrast to the whole cell destruction seen in PCD and necroptosis, the process
of autophagy seeks to repair and/or replace damaged organelles and proteins (Klionsky,
2007; Mizushima, 2007) (Figure 3A). Nutrient starvation-induced autophagy has been the
principal form examined to date but other triggers such as hormones, growth factors and
pathogen infection can also induce this pathway. The process of autophagy is regulated
by a family of approximately 30 related genes called autophagy-related (ATG) genes
(Klionsky et al., 2003). Many of these genes have been conserved through evolution (He
and Klionsky, 2009). Protein-protein interactions between ATG proteins typically occur
at phagophore assembly sites (PASs) which serve as the initiation points for phagophore,
an expanding membrane envelope which ultimately engulfs portions of the cytoplasm.
This consequently leads to the formation of a double-membraned vesicle called the
Autophagy is a catabolic process designed to degrade cellular components within lysosomes during periods of nutrient starvation or cellular stress. Variants are known such as macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Some variants have been described in terms of the organelles they target (i.e., mitophagy – mitochondria, pexophagy – peroxisome, reticulophagy – endoplasmic reticulum). (A) Macroautophagy involves the production of a double-membraned vesicle (autophagosome) which engulfs cytoplasmic components destined for degradation by subsequent fusion with lysosomal vesicles. Microautophagy in contrast, involves the targeted degradation of specific cellular components by direct transfer into lysosomes. In CMA, most substrate proteins contain a consensus sequence which facilitates interaction with heat shock protein of 70 kDa (HSP70) and other cochaperone proteins. This protein complex binds to the lysosomal protein LAMP-2A and stimulates the unfolding and translocation of substrate proteins into the lysosome. Degraded materials are subsequently transferred back into the cell cytosol where they can be reused. (B) In macroautophagy, phagophore membrane nucleation and assembly depends upon interactions with specific Atg proteins. Specifically Bcl-2 and Bcl-xL can interact with beclin 1 (Atg 6); which is critical for this process. Expansion of this phagophore membrane consequently result in autophagosome formation. Hence anti-apoptotic Bcl-2 family proteins are capable for regulating (at least partially) the progression of autophagy. Thus the various modes of cell death (PCD, necroptosis and autophagy) are at some level inter-related, though details of the precise mechanisms are as yet not completely understood.
Adapted from Mizushima et al., *Nature*, 2008
autophagosome. Subsequent fusion of lysosomal vesicles with the autophagosome results in the degradation of trapped cellular components (Figure 3B). Microscopic examination of cells undergoing autophagy typically reveals the formation of large vacuole-like autophagosomes.

A key regulatory element in the initiation of autophagy is the target of rapamycin (Tor) in yeast and its mammalian homologue (mTOR). Classified as a phosphatidylinositol kinase homologue, Tor and mTOR phosphorylate specific ATG proteins to inhibit autophagy induction (Blommaart et al., 1995; Noda and Ohsumi, 1998). As is implied by their names, rapamycin was found to suppress the functions of Tor and mTOR, and can thus trigger autophagy (Sabatini et al., 1994).

Mainly considered to be a pathway evolved to promote survival during times of nutrient starvation and degradation of damaged organelles and proteins, dysregulation of this process leading to excess autophagy has recently been implicated in various pathologic conditions (Levine and Kroemer, 2008). In contrast, induction of autophagy via pharmacologic means by rapamycin and other novel autophagy enhancers has been suggested as a potential therapeutic strategy for neurodegenerative conditions associated with protein aggregations and mitochondrial dysfunction (e.g., Huntington’s and Parkinson’s diseases) (Berger et al., 2006; Sarkar et al., 2007a; Sarkar et al., 2007b; Williams et al., 2008). However recent evidence demonstrating an anti-aging function for rapamycin may complicate its suggested role as modulator of neurodegenerative diseases (Harrison et al., 2009; Selman et al., 2009). As such, there is currently controversy regarding the exact functions of autophagy in mammalian systems and its role both under normal physiologic and pathologic conditions.
Although traditionally viewed as the only mode of controlled cell death, PCD is currently joined by necroptosis and autophagy. Still in their respective infancy stages, much remains unknown regarding necroptosis and autophagy. Indeed, their connections with PCD remain ambiguous while many still question their physiologic significance. Furthermore, they appear to share, at least at a minimal level, some common signaling components with PCD; thus suggesting a certain degree of intersection between these processes. Thus continued research is required to elucidate the underlying regulatory mechanisms and their relations to PCD.

1.2.3 PROGRAMMED CELL DEATH PATHWAYS

1.2.3.1 EVOLUTION OF PROGRAMMED CELL DEATH

The PCD pathway was originally identified in *Caenorhabditis elegans* through the identification of mutations which altered neural cell number during development in *C. elegans* (Horvitz et al., 1983). The eventual identification of a linear pathway involving the genes *egl-1*, *ced-9*, *ced-4* and *ced-3* which regulated the process of cell death (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Hengartner et al., 1992) and the subsequent identification of homologues of these genes in other organisms such as fruit fly and mice demonstrated the evolutionary conservation of this pathway (Yan and Shi, 2005). Despite operating under a common structural framework, the details by which these PCD proteins work differ significantly among different organisms (Oberst et al., 2008) (Figure 4).

In *C. elegans* the oligomerization of CED-4 (a homologue of Apaf-1) triggers the activation of CED-3 (a homologue of caspase-3) (Yang et al., 1998), which initiates the
**Figure 4. Evolutionary Conservation of PCD Signaling**

The first molecular dissection of a PCD pathway was performed in the nematode *Caenorhabditis elegans*. As illustrated in the figure this pathway consists primarily of four proteins: EGL-1, CED-9, CED-4, and CED-3. Subsequent research efforts identified homologues of these proteins in organisms such as humans and mice. Interestingly, though CED-9 homologues have been identified in *Drosophila*, a requirement of these proteins within the framework of PCD has yet to be identified. In contrast, a novel PCD regulatory pathway is observed in *Drosophila* involving Reaper, Hid, Grim, and Sickle (commonly referred to as RHG proteins). These proteins ultimately regulate the activity of DRONC (caspase-9 homologue) and Drice (caspase-3 homologue), through their inhibitory interactions with DIAP1/2.

In mammals there is evidence of both systems. Pro- and anti-apoptotic Bcl-2 family protein interactions ultimately regulate the permeability of the mitochondrial outer membrane. During PCD, permeabilization allows proteins such as cytochrome c and Smac/DIABLO to be released into the cell cytosol where they initiate the post-mitochondrial aspects of PCD. Released cytochrome c functions to induce conformational change in Apaf-1, providing an activation platform for caspase-9. This in turn acts to enhance executioner caspase activity (caspase-3/6/7). Smac/DIABLO promotes this process through suppression of inhibitor of apoptosis proteins (IAPs) in a manner similar to RHG proteins. As seen in the figure, though the regulation of PCD is conserved between the organisms shown, regulation of the pathway has grown more complex in mammals.
Adapted from Riedl and Shi, *Nat Rev Mol Cell Biol*, 2004
destruction of cells destined to die (Miura et al., 1993; Yuan et al., 1993). Normally direct interaction between CED-9 (a homologue of Bcl-2) and CED-4 suppresses the apoptotic functions of CED-4 in the absence of apoptotic signals (Chinnaiyan et al., 1997; Spector et al., 1997). Following the initiation of PCD, the inhibitory functions of CED-9 are suppressed by EGL-1, a BH3-only protein which is regulated primarily through transcription (Conradt and Horvitz, 1998; del Peso et al., 1998). In *C. elegans* this pathway was determined to control the fate of at least 131 cells during development (Horvitz et al., 1983; Liu and Hengartner, 1999).

While basic functions are retained, this pathway appears to be regulated in a somewhat different manner in the fruit fly (Figure 4). In *Drosophila* the RHG proteins directly regulate the activity of IAPs by binding to BIR (baculovirus inhibitory repeat) domains. This interaction relieves IAP-mediated suppression of caspases (Hay et al., 1995), allowing PCD to proceed (Chen et al., 1998; Kaiser et al., 1998; Meier et al., 2000; Song et al., 1997). Though two Bcl-2 homologues have been identified in *Drosophila* (*Drosophila Bcl2* ortholog 1 – dBorg-1/death executioner Bcl-2 homologue – Debcl/*Drosophila* ortholog of the Bcl-2 family-1 – Drob-1; dBorg-2/buffy), controversy remains regarding their precise role in *Drosophila* PCD. No BH3-only proteins have been identified to date in *Drosophila*, further highlighting the mechanistic differences in PCD between these two organisms.

### 1.2.3.2 MAMMALIAN PROGRAMMED CELL DEATH PATHWAYS

As one might expect, mammals exhibit greater specialization compared to *C. elegans* and *Drosophila* in their PCD pathway. Interestingly it appears that regulation of
mammalian PCD utilizes aspects of both *C. elegans* and *Drosophila* regulatory systems, relying principally on the balance of protein-protein interactions between Bcl-2 family members to regulate the progression of PCD. These interactions promote or inhibit the release of the PCD signaling proteins cytochrome c and Smac/DIABLO (Du et al., 2000; Liu et al., 1996; Verhagen et al., 2000; Zou et al., 1997). In comparison to simpler organisms cytochrome c is not believed to be involved in the regulation of PCD in *C. elegans* (Shi, 2008), and its role in *Drosophila* remains controversial (Arama et al., 2003; Arama et al., 2006; Dorstyn et al., 2004; Dorstyn et al., 2002; Mendes et al., 2006; Yu et al., 2006).

Several PCD pathways have evolved in mammals to regulate PCD in order to respond to the multitude of survival signals which a cell may receive. Two principal PCD pathways have been identified to date which respond to signals initiated either within the cell or as a result of external stimuli (Adams, 2003) (Figure 5). Both transcriptional and post-translational modifications play important roles in regulating mammalian PCD (Puthalakath and Strasser, 2002).

### 1.2.3.3 THE INTRINSIC PATHWAY

As the name implies this pathway primarily responds to cellular stresses initiated within the cell, such as inadequate trophic support, DNA damage, or excessive calcium influx (Adams, 2003; Puthalakath and Strasser, 2002). These stress signals are transmitted via different pathways to the level of the mitochondria where they influence the balance of Bcl-2 family proteins. In many cases one or more BH3-only proteins are involved in mediating these signals or altering conformation of pro-apoptotic Bax or Bak.
Figure 5. Principal Mammalian PCD Pathways

Mammalian PCD is primarily regulated by two interconnecting pathways. A number of cellular stresses can initiate the intrinsic pathway through the activation of BH3-only proteins. These BH3-only proteins interact with additional Bcl-2 family proteins to (most frequently) promote permeabilization of the mitochondrial outer membrane. Following this process, cytochrome c and several other mitochondrial proteins are released into the cell cytosol where they can elicit their pro-apoptotic functions. Cytochrome c binds to the WD40 domain present on Apaf-1, initiating a conformal change, dramatically enhancing the rate of caspase-9 activation. Caspase-9 can in turn initiate other downstream executioner caspases.

In contrast, the extrinsic pathway is activated through the binding of external death ligands such as TNF-α and Fas ligand to the death receptors TNF receptor (TNFR) and Fas. Ligand binding leads to the formation of the death-inducing signaling complex (DISC), which subsequently activates initiator caspases-8 (caspase-8 and -10 in humans). Activated caspase-8 can cleave the BH3-only protein Bid to truncated Bid (tBid), ultimately promoting executioner caspase activity. Alternatively, caspase-8 can directly activate caspase-3. Activation of these caspases initiates the irreversible phase of PCD in which cellular components are degraded and packaged into apoptotic bodies.
The intrinsic pathway is often referred to as the mitochondrial PCD pathway due to the critical involvement of this cellular organelle.

Perhaps the most important PCD protein released from the mitochondria during cell death is cytochrome c (Kluck et al., 1997; Rosse et al., 1998; Yang et al., 1997). Cytochrome c is a heme-containing protein (holo-cytochrome c; apo-cytochrome c does not contain the heme moiety) which normally participates in mitochondrial electron-transport chain during oxidative metabolism to generate ATP (Li et al., 2000b). Upon its release into the cytosol, holo-cytochrome c interacts with and activates Apaf-1 (Hu et al., 1998; Li et al., 1997; Zou et al., 1997). In its monomeric form Apaf-1 is believed to assume a compact conformation sequestering the nucleotide binding and oligomerization domain (NOD) (Acehan et al., 2002; Riedl et al., 2005; Yu et al., 2005). The interaction of one cytochrome c with the WD40 domain on Apaf-1 facilitates structural release of NOD, promoting a conformation which promotes Apaf-1 oligomerization (Riedl et al., 2005; Yu et al., 2005) (Figure 6A). While it has been demonstrated that apoptosome assembly requires the presence of dATP/ATP, hydrolysis of the high-energy phosphodiester backbone is not required for oligomerization, as a nonhydrolyzable ATP analog (ADP-CP) also promotes apoptosome formation (Jiang and Wang, 2000; Reubold et al., 2009). Thus, it has been proposed that cytochrome c disrupts the interaction between WD40 and NOD, while the exchange of ADP for ATP or dATP promotes uncoupling of this interaction via the \( \gamma \)-phosphate moiety. By unlocking both of these molecular locks, apoptosome formation can occur independent of the order which the interactions occur (Figure 6B).
Figure 6. Apoptosome Formation and Structure

(A) Apaf-1 contains multiple domains including CARD, nucleotide binding and oligomerization (NBD/NOD), and WD40 domains. Prior to activation Apaf-1 is believed to exist in a monomeric form as a result of autoinhibition between the WD40 and NOD/CARD domains. (B) The current model of apoptosome formation involves two steps regulating autoinhibition of Apaf-1: one between WD40 and NOD, the other between NOD and the ADP. Under physiologic conditions, there is unlikely to be a special requirement for dATP given the equivalence of dATP and ATP in promoting apoptosome formation. (C) Three dimensional electron density map and model obtained by electron cryomicroscopy. The assembled mammalian apoptosome exhibits a seven-fold rotational symmetry. The CARD is located at the rotational centre to recruit caspase-9. (D) Electron photomicrographs of apoptosomes and caspase-9 in solution. Many apoptosomes exist as face-to-face dimmers (arrowheads).
Adapted from
Acehan et al., Mol Cell, 2002
Reubold et al., J Biol Chem, 2009
Yu et al., Structure, 2005
When fully assembled, the apoptosome is a protein complex of approximately 1 MDa, composed of seven Apaf-1 subunits binding cytochrome c and dATP/ATP (Cain et al., 2000) (Figure 6C-D). In the multimeric conformation each Apaf-1 subunit contains an accessible caspase recruitment domain (CARD) located at the N-terminus. This domain engages procaspase-9 to promote its catalytic activity (Srinivasula et al., 1998; Zou et al., 1999). It was originally believed that recruitment of procaspase-9 to the apoptosome served to enhance auto-catalytic activation (Srinivasula et al., 1998). However, more recent studies have demonstrated that caspase-9 activity (i.e., caspase-3 cleavage) is 2000 times greater for the procaspase-9/apoptosome complex than that of cleaved caspase-9 released from the apoptosome (Rodriguez and Lazebnik, 1999; Srinivasula et al., 2001). It has since been shown that proximity-induced dimerization of procaspase-9 is required and sufficient for its activity (Pop et al., 2006).

1.2.3.4 THE EXTRINSIC PATHWAY

The extrinsic pathway mediates a variety of PCD responses resulting from death signals imparted by effector cells (Johnstone et al., 2008; Strasser et al., 2009; Wilson et al., 2009). The binding of tumour necrosis factor alpha (TNF-α), Fas ligand (FasL/Apo-1) and TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L) can each lead to trimerization of their respective receptors [TNF-α receptor, Fas/CD95/Apo-1 and Death Receptor 4/5 (DR4/5)] (Papenfuss et al., 2008) (Figure 7). Homotypic interactions between death domains (DDs) located on the cytoplasmic portion of Fas or DR4/5 and FADD (Fas-associated death domain protein), or between DDs of TNF receptor 1 (TNFR1) and TRADD (TNF receptor-associated death domain protein) result in
Figure 7. Death Ligands and Their Receptors

To date six death receptors have been identified, termed respectively as death receptor (DR) 1-6. Physiologic ligands for all death receptors have been identified. Death domains of these receptors promote interaction with similar domains on TRADD or FADD.
Adapted from
Wehrli et al., J Invest Dermatol, 2000
recruitment of these death adaptor proteins (Wilson et al., 2009) (Figure 8). In addition to a DD, FADD also possesses a death effector domain (DED), which promotes homotypic interactions with similar domains on procaspase-8 and -10 (Tibbetts et al., 2003). Several structural studies have demonstrated the critical role of FADD oligomerization in the formation of active signaling complexes (Hughes et al., 2009; Scott et al., 2009) (Figure 8). These signaling complexes, termed the death-inducing signaling complex (DISC), function in a similar manner to the apoptosome to recruit procaspases and promote proximity-induced dimerization (Boatright et al., 2003; Donepudi et al., 2003; Muzio et al., 1998). However unlike caspase-9, activated caspase-8/10 released from the DISC retains optimal levels of catalytic activity and can process procaspase-3/6/7 into their activated forms (Yin et al., 2006). The potential for active caspase-8 outside the DISC may be the reason for the existence of additional negative regulators of caspase-8 activation such as FLICE-inhibitory protein (FLIP) (Scaffidi et al., 1999; Srinivasula et al., 1997).

In contrast to FasL and TRAIL, TNF-α signaling differs in that two distinct signaling complexes are involved: one which functions to activate the NF-κB pathway and another which activates procaspase-8 through FADD (Harper et al., 2003; Micheau and Tschopp, 2003). NF-κB signaling is initiated by membrane-bound complex I consisting of TNFR1, TRADD, RIPK1 and TNF receptor-associated factor 2 (TRAF2) (Micheau and Tschopp, 2003). The formation of complex I results in lysine-63 (K63) polyubiquitination of RIPK1 (Bertrand et al., 2008; Ea et al., 2006; Li et al., 2006; Mahoney et al., 2008; O'Donnell et al., 2007). The newly added polyubiquitin chain enables interactions with proteins containing polyubquitin chain recognition domains
Figure 8. FADD Structure and Oligomerization

Activation of caspase-8 occurs through association with an activating platform, generally termed the death-inducing signaling complex (DISC) via homotypic death domain (DD) interactions and death effector domains (DED). (A) Crystal structure of the Fas/FADD death domain interface provides a molecular insight into DISC formation and clustering which is necessary for caspase-8 activation. (B) Comparison of Fas between its unbound and FADD DD-bound form demonstrated the opening of Fas to reveals two new interaction interfaces, one for binding with FADD and the other for binding with another molecule of Fas. (C) Overlay of unbound FADD versus Fas-bound FADD highlighting conformation changes which promote DED interaction with caspase-8. (D) Suggested model of Fas/FADD bridges allowing for the assembly of higher-order clusters to promote DISC formation.
Adapted from Scott et al., Nature, 2009
such as TGF-β-activated kinase 1 (TAK1) and TAK1 binding proteins 2/3 (TAB2/3) complex and the inhibitor of κB (IκB) kinase (IKK) α/β/γ complex (Blonska et al., 2005; Kanayama et al., 2004; Sakurai et al., 1999; Wang et al., 2001). These interactions ultimately results in phosphorylation of IκBα, promoting its ubiquitin-mediated proteasomal degradation, thus freeing NF-κB to regulate target genes (Verma et al., 1995). The K63-ubiquitination of RIPK1 was initially postulated to be mediated by TRAF2 (Lee et al., 2004a), but has been more recently shown to be mediated by cIAP-1/2 (Vince et al., 2009). In addition to RIPK1, NF-κB inducing kinase (NIK), a critical regulator of the non-canonical NF-κB pathway, was also recently demonstrated to be polyubiquitinated for proteasomal degradation by cIAP-1/2 (Grech et al., 2004; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). As will be discussed in further detail below these observations implicate cIAP-1/2 in the regulation of TNF-α-mediated NF-κB activation.

TNF-α signaling also triggers FADD-dependent pro-caspase-8 activation in a FADD-dependent manner (Micheau and Tschopp, 2003). Upon stimulation, complex I proteins dissociate from TNFR1 to promote formation of complex II, which contains RIPK1 and TRADD (Micheau and Tschopp, 2003; Wang et al., 2008) (Figure 2). TRADD subsequently recruits FADD, which in turn engages pro-caspase-8/10 for their activation in a manner similar to that seen for FADD-DISC described above. It was revealed recently that cIAP-1/2 maintains RIPK1 in complex I through polyubiquitination, thus cIAP-1/2 is also involved in regulating formation of complex II and its subsequent activation of pro-caspase-8/10 (Bertrand et al., 2008; Wang et al., 2008).
An additional level of procaspase-8/10 regulation relates to the competitive binding of FLIP to FADD via its DED. FLIP binding reduces the amount of procaspase-8/10 which can be recruited to FADD-DISC, thus diminishing the levels of activation (Irmler et al., 1997; Scaffidi et al., 1999; Thome et al., 1997).

1.2.3.5 CONVERGENT CHECKPOINTS: THE MITOCHONDRIA

Evidence suggests that the intrinsic and extrinsic pathways evolved independently, and in mammals these systems are capable of independently transmitting PCD signals to bring about cell death. However in mammals it can be seen that those systems subsequently evolved unique cross-regulatory mechanisms to better coordinate their activities. The principal interaction site for these systems is the mitochondria and involves protein-protein interactions between Bcl-2 family proteins which regulate the permeability of the mitochondrial outer membrane. This permeability controls the release of apoptogenic second messengers such as cytochrome c and Smac/DIABLO. The BH3-only protein Bid (BH3 interacting domain death agonist) is one such factor which integrate extrinsic and intrinsic pathway functions. Bid was originally identified as a factor responsible for mediating the release of cytochrome c following caspase-8 activation (Li et al., 1998; Luo et al., 1998). Full-length Bid protein is a 26 kDa cytoplasmic protein which is cleaved to 11 kDa and 15 kDa fragments by caspase-8. Caspase-10 is known to perform a similar function, although Bid possesses an additional caspase-10 cleavage site resulting in the 15 kDa fragment being further reduced to a 13 kDa fragment (Fischer et al., 2006). These carboxyl-terminal 15 kDa and 13 kDa fragments termed tBid (truncated Bid) subsequently translocates to the mitochondria,
where it interacts with Bcl-2 family proteins to facilitate cytochrome c release. Thus caspase-8-dependent cleavage of Bid can trigger MOMP, apoptosome formation, caspase-9 activation and caspase-3/6/7 activity.

The cleavage of Bid has been shown to be critical for specific forms of PCD in vivo such as Fas-induced hepatocellular PCD (Yin et al., 1999). While wild-type mice die within hours following injection of an antibody directed against Fas (Jo2) due to acute liver failure, Bid-deficient mice survive such treatment, demonstrating a form of caspase-8-dependent PCD which is dependent upon MOMP. Subsequent studies into the role of Bid demonstrate that in many forms of PCD, loss of mitochondrial signaling does not eliminate PCD but merely delays it (Bai et al., 2005; Plesnila et al., 2001; Wei et al., 2006; Yin et al., 2002; Zinkel et al., 2003). Interestingly a recent in vivo study involving the IAP family member Birc4 (the gene encoding XIAP) demonstrated that Bid/Birc4 double null animals were “rescued” with respect to Fas-induced hepatotoxicity; in that mice doubly deficient of Bid and XIAP were equally susceptible to hepatocyte cell death triggered by Jo2 compared to wild-type controls (Jost et al., 2009). It was reasoned that in the presence of XIAP, caspase-3 activity is significantly inhibited. However when XIAP inhibition is removed, sufficient caspase-3 activity is available to mediate cellular destruction following Fas stimulation (Jost et al., 2009). Thus in addition to responding to intracellular stresses, the intrinsic pathway also functions to enhance responsiveness of extrinsic pathway signaling through tBid.

In addition to Bid several other BH3-only proteins have been shown to act in PCD signaling through either changes in transcription or via post-translational modifications (Puthalakath and Strasser, 2002). Ultimately these actions link the mitochondria as a
communication hub between the intrinsic and extrinsic pathways to generate the necessary PCD responses.

1.2.3.6 CONVERGENT CHECKPOINTS: EXECUTIONER CASPASES

As mentioned previously, both caspase-9 and caspase-8/10 are capable of cleaving and activating executioner caspases such as caspase-3 and caspase-7 (Stennicke et al., 1998; Yin et al., 2006). Thus despite different activation stimuli, these initiator caspase ultimately trigger a similar set of proteolytic effectors to carry out cell destruction. As such, these executioner caspases provide a critical point of convergence between the intrinsic and extrinsic pathways. Since activation of executioner caspases is generally considered the point of no return with respect to PCD signalling, controlling of this checkpoint is critical for cell survival. Due to the critical nature of this process, a complex network of protein-protein interactions regulate levels of PCD activation to ensure proper regulation at this primary checkpoint. Similar to caspase regulation in Drosophila, executioner caspase activity in mammals is regulated by multiple IAP family members (XIAP, cIAP-1, cIAP-2 and BRUCE/Apollon) which in turn can be inhibited by homologues of RHG proteins such as Smac/DIABLO (Orme and Meier, 2009; Shi, 2004; Verhagen and Vaux, 2002).

1.2.4 BCL2 FAMILY PROTEINS AND MITOCHONDRIA

1.2.4.1 BCL2 FAMILY PROTEINS AND THEIR FUNCTIONS

The prototypical member of the Bcl-2 protein family, B-cell lymphoma 2 (Bcl-2), was originally identified as a protein whose coding sequence is translocated during one of
the most frequent translocations in follicular lymphomas, a recombination between chromosomes 14 and 18 (Tsujimoto et al., 1984). This translocation places BCL2 under control of the immunoglobulin heavy chain locus, resulting in constitutive expression of Bcl-2 in affected lymphocytes (Tsujimoto et al., 1985a; Tsujimoto et al., 1984; Tsujimoto et al., 1985b). The elevated expression of Bcl-2 results in enhanced PCD resistance, ultimately favouring transformation of these cells (McDonnell et al., 1989; Tsujimoto, 1989; Vaux et al., 1988).

As a group Bcl-2 family proteins are characterized by a Bcl-2 homology (BH) motif, a conserved stretch of amino acids consisting of about 9-16 residues (Yin et al., 1994) (Figure 12A). Four distinct BH motifs have been identified, referred to as BH1-4. These BH motifs have been shown to regulate specific protein-protein interactions which occur within Bcl-2 family members, and thus are critical for normal function (Schinzel et al., 2004).

The Bcl-2 protein family can generally be subdivided into three major subgroups based upon functionality and their structural properties (anti-apoptotic, pro-apoptotic, BH3-only) (Figure 9). The anti-apoptotic subgroup generally contains four BH motifs (BH motifs 1-4), with the exception of Mcl-1 which lacks the BH4 motif (Schinzel et al., 2004). This subgroup also includes Bcl-2, Bcl-xL and Bcl-w. The remaining two subgroups are pro-apoptotic in nature, and function to promote the release of cytochrome c and other apoptogenic proteins from the mitochondria. The pro-apoptotic subgroup contains BH motifs 1-3 and is represented by members such as Bax, Bak and Bok (Figure 9). The remaining subgroup of Bcl-2 family proteins contains only a single BH motif (BH3), and is thus referred to as “BH3-only” proteins. Proportionally there are a
**Figure 9. Bcl-2 Family Proteins**

B cell lymphoma 2 (Bcl-2) family proteins are characterized by short sequences of highly conserved residues termed Bcl-2 homology (BH) domains. These proteins can be differentiated into distinct subgroups based on their functional and structural properties. The anti-apoptotic subgroup functions to inhibit PCD and all members with the exception of Mcl-1 generally contain four BH domains. In contrast, pro-apoptotic Bcl-2 family proteins can be divided into two separate groups, the first generally consists of proteins containing containing three BH domains and the second consist of proteins with only a BH3 domain. As noted in the figure, many Bcl-2 family proteins also contain a transmembrane (TM) domain which allows them to associate with the various membranes present in the cell (i.e., mitochondria and endoplasmic reticulum).
Adapted from Youle and Strasser, Nat Rev Mol Cell Biol, 2008
greater number of BH3-only proteins encoded within the mammalian genomes compared
to the subgroups. To date, at least ten BH3-only proteins have been identified in
mammals (Bid, Bad, Bim, Bmf, Hrk, Bik/Blk, Puma, Noxa, Bnip-3 and Mule) (Schinzel
et al., 2004).

Current models describing the function of Bcl-2 family proteins place the BH3-
only proteins as cellular sensors of various PCD signals; in that upon sensing such
signals, they are activated and subsequently interact with other Bcl-2 family proteins to
promote mitochondrial membrane permeability (Puthalakath and Strasser, 2002). It has
been demonstrated that these proteins are regulated in response to specific PCD signals
via both transcriptional control and post-translational mechanisms (Figure 10).
Specifically, the activity of Bcl-2 interacting mediator of cell death (Bim) and Bcl-2
associated agonist of cell death (Bad) are primarily modulated by their phosphorylation
status (Putcha et al., 2003; Seward et al., 2003; Yan et al., 2003; Zha et al., 1996). Under
normal conditions Bim and Bad are phosphorylated at specific serine residues which
impair them with affinity for dynein light chain-1/2 and protein 14-3-3, respectively
(Puthalakath et al., 1999; Zha et al., 1996). These interactions act to sequester these
proteins in the cytosol, away from the mitochondria and other organelles where they can
exert pro-apoptotic functions. Upon PCD signaling these serine residues are
dephosphorylated, lowering the affinity between Bim and Bad with their corresponding
interaction partners. This consequently allows them to translocate to the mitochondria
where they can interact with Bcl-2 family proteins. Other BH3-only proteins such as
Puma (p53-upregulated modulator of apoptosis) and Noxa are regulated principally
through transcriptional control mediated by p53 and FOXO3a (forkhead box transcription
Figure 10. BH3-only Proteins as Sensors of Cellular Stress and Damage

BH3-only proteins are regulated via a variety of mechanisms ranging from phosphorylation to proteolytic cleavage. (A) Bid serve as a convergence point between intrinsic and extrinsic pathways. Following removal of H1 and H2 helices by activated caspase-8/10 or granzyme B, the hydrophobic core (consisted of H5 and H6 helices) becomes more accessible, facilitating for mitochondrial localization. (B) Phosphorylation can also modify function of BH3-only proteins. For example, the phosphorylation-dependent sequestration of Bad and Bim to scaffolding protein 14-3-3 and dynein light chain (DLC), respectively, maintains them from the mitochondria. Following PCD stimulation pro-apoptotic kinases and/or phosphatases such as JNK and calcineurin become activated. These enzymes proceed to modify the phosphorylation status of Bad and Bim. Dephosphorylation of S112 and S136 on Bad reduces its affinity for 14-3-3, promoting translocation to the mitochondria. Conversely phosphorylation of S128 by Cdc20 enhances apoptosis by reducing the interaction with Akt which diminishes phosphorylation at serine-136. Phosphorylation of S155 within the BH3 motif of Bad disrupts interaction with anti-apoptotic Bcl-2 family members. Bim posses intrinsic affinity for DLC. Phosphorylation at threonine-55 disrupts the interface allowing translocation of Bim to the mitochondria where it interacts with other Bcl-2 family proteins. (C) Most BH3-only proteins are upregulated during PCD but the two most clearly associated with transcriptional regulation are Puma and Noxa. Both of these BH3-only proteins were originally identified as p53 target proteins and are upregulated when p53 is activated by DNA damage and other cellular stresses.
Adapted from DeFranco et al., *Immunity: The Immune Response in Infectious and Inflammatory Disease*, 2007
factor O3a) (Nakano and Vousden, 2001; Obexer et al., 2007; Oda et al., 2000; You et al., 2006; Yu et al., 2001a). This regulation allows BH3-only proteins to be activated in a timely manner in response to PCD signaling.

Once activated, BH3-only proteins are available to interact with Bcl-2 family proteins to initiate permeabilization of the mitochondrial outer membrane, facilitating the release of cytochrome c and other apoptogenic factors. There is currently controversy regarding the mechanism by which BH3-only proteins function to accomplish this task (Antignani and Youle, 2006) (Figure 11). In one model (Direct activation), BH3-only proteins are thought to be functionally separable into two subclasses. In this model, tBid and Bim are believed to possess the ability to induce conformational changes in Bax and possibly Bak. Through these interactions they are capable of activating them, enhancing their pro-apoptotic functions (Gavathiotis et al., 2008; Kim et al., 2006; Kim et al., 2009; Kuwana et al., 2005; Lovell et al., 2008). In contrast BH3-only proteins such as Bad and Noxa function primarily to displace the inhibitory interaction of anti-apoptotic Bcl-2 family proteins with Bid and Bim, thus liberating them to interact and activate Bax and Bak (Cheng et al., 2001; Letai et al., 2002). An alternative model (Indirect activation) proposes that all BH3-only proteins act to displace Bax and Bak from inhibitory interactions with anti-apoptotic Bcl-2 proteins, thus allow them to facilitate mitochondrial protein release (Chen et al., 2005; Willis et al., 2007).

Within the Bcl-2 protein family, multiple members of each functional subgroup appear to exist. Therefore the possibility of functional redundancies between Bcl-2 family members of each subclasses must be anticipated. In order to determine the contribution of individual Bcl-2 family members, gene targeting approaches have been employed.
Figure 11. Proposed Models of Bcl-2 Family Protein Interactions

Two different models have been proposed to explain how BH3-only proteins influence MOMP. (A) The Direct Activation model proposes that BH3-only proteins can be subdivided into two functional categories: sensitizers and activators. Under this model activator BH3-only proteins (i.e., tBid and Bim) act to elicit the conformational changes in Bax and Bak required for pro-apoptotic function. Following binding to BH3-only proteins, Bax and Bak can oligomerize to form pores to facilitate MOMP. In this model, the primary function of the anti-apoptotic Bcl-2 family proteins (Bcl-2 and Bcl-xL, etc.) is to prevent the interactions between activator BH3-only proteins and Bax/Bak. Such anti-apoptotic interactions can in turn be suppressed by so-called “sensitizer” BH3-only proteins (shown by Bad and Noxa) thus freeing “activator” BH3-only proteins to activate Bax/Bak. (B) In contrast the Indirect Activation (or Displacement) model proposes that Bax and Bak do not require prior activation for action but are instead maintained in an inactive state by anti-apoptotic Bcl-2 family members. Importantly this model describes only a single group of BH3-only proteins without further functional distinction. However within this model different BH3-only proteins are either selective or promiscuous with respect to binding to anti-apoptotic Bcl-2 family proteins (e.g., Noxa has specificity for Mcl-1 but demonstrates low affinity for Bcl-2, Bcl-xL, and Bcl-w while Bim possesses high affinity for all four proteins). Thus due to the varying degrees by which they can inhibit anti-apoptotic Bcl-2 family members, Bax and Bak may appear functionally dissimilar.
Adapted from Willis et al., Science, 2007
through either overexpression or genetic ablation. Using these approaches, the physiologic function of a number of Bcl-2 family proteins have been determined. At present all major anti-apoptotic Bcl-2 family proteins have been examined via gene knockout approaches (Kamada et al., 1995; Motoyama et al., 1995; Nakayama et al., 1994; Print et al., 1998; Rinkenberger et al., 2000; Veis et al., 1993). While both Bcl2 and Bcl2l2 (Bcl-w) develop to birth (Kamada et al., 1995; Nakayama et al., 1994; Print et al., 1998; Veis et al., 1993), a number of cell populations are affected. In Bcl2 null mice, pleiotropic abnormalities including small size, increased postnatal mortality, polycystic kidneys, apoptotic involution of thymus and spleen, graying in the second hair follicle cycle, reduced numbers of motor, sympathetic and sensory neurons have been observed, and begin to die at 2-3 weeks due to polycystic kidneys (Kamada et al., 1995; Michaelidis et al., 1996; Nakayama et al., 1994; Veis et al., 1993). In contrast, male Bcl-w deficient mice exhibit sterility, with a progressive loss of germ cells, Sertoli cells and Leydig cells beginning at puberty (Print et al., 1998). Conversely Bcl2l1 (Bcl-xL) and Mcl1 null mice die during embryonic development due to massive apoptosis primarily in the central nervous system by E13.5 and during the implantation stage (between E4.0 and E7.5), respectively (Motoyama et al., 1995; Rinkenberger et al., 2000). Since Bcl2l1 and Mcl1 null animals die during development, their functions in various tissues and cell types have been examined via conditional gene targeting approaches (lineage-specific Cre recombinase-mediated ablation). Through these studies Bcl-xL was found to play a critical role in the proper development of substantial nigra and mammary epithelium while Mcl-1 was found to be required for normal lymphocyte and CNS development (Arbour et al., 2008; Opferman et al., 2003; Savitt et al., 2005; Walton et al., 2001).
The pro-apoptotic Bcl-2 family members Bax and Bak are believed to be co-expressed in most cell types, though exceptions are known to exist (Buss et al., 2006; Knudson et al., 1995; Ren et al., 2009). Their key role in regulating PCD is demonstrated by the complete loss of PCD mediated by staurosporine, etoposide, UVC radiation and ER stress inducers (thapsigargin, tunicamycin and brefeldin A) in mouse embryonic fibroblasts and thymocytes derived from Bax/Bak1 double null mice (Scorrano et al., 2003; Wei et al., 2001). However, it was clearly demonstrated previously that their combined functions are absolutely required for PCD signaling, as various primary cells (mouse embryonic fibroblasts, thymocytes, etc.) derived from Bax, Bak1 double knockout animals are incapable of undergoing PCD (Scorrano et al., 2003; Wei et al., 2001).

Due to the frequent co-expression of one or more BH3-only proteins, the majority of animals singly deficient of these proteins undergo normal development and shown nominal phenotypes. (Coultas et al., 2004; Imaizumi et al., 2004; Jeffers et al., 2003; Labi et al., 2008; Ranger et al., 2003; Shibue et al., 2003; Villunger et al., 2003; Yin et al., 1999). Similarly with respect to nerve axotomy and hypoxia-ischemia, most of these animals exhibit only partial protection, suggesting that the involvement of multiple BH3-only proteins in response to a particular injury (Imaizumi et al., 2004; Kiryu-Seo et al., 2005; Ness et al., 2006; Plesnila et al., 2001; Yin et al., 2002). An exception appears to be Bim-deficient (Bcl2l11) animals which accumulate lymphoid and myeloid cells due to an inhibition of apoptosis, succumbing to an autoimmune kidney disease (Bouillet et al., 1999). Interestingly Bim deficiency reverses many of the phenotypes seen in Bcl2 null mice (i.e., graying during the second hair follicle cycle, compromised lymphoid system,
polycystic kidneys), suggesting that those two proteins oppose each other’s function (Bouillet et al., 2001).

1.2.4.2 STRUCTURAL PROPERTIES OF BCL2 FAMILY PROTEINS

Both anti- (i.e., Bcl2 and Bcl-xL) and pro- (i.e., Bax and Bak) apoptotic Bcl-2 family proteins have tertiary structures homologous to bacterial toxins such as colicin A (channel domain) and diphtheria toxin (T domain) (Aritomi et al., 1997; Chou et al., 1999; McDonnell et al., 1999; Muchmore et al., 1996; Suzuki et al., 2000). Such structural similarities have provided insights into how Bcl-2 family proteins may control mitochondrial outer membrane permeabilization (Lazebnik, 2001). The primary function of these bacterial toxins is to form pores on the plasma membranes of affected cells, resulting in a dissipation of ion gradients and an influx of water, ultimately resulting in cell rupture (Lazdunski et al., 1988; Zalman and Wisnieski, 1984). From inference to their structural similarities, it has been suggested that Bcl-2 family proteins may form pores of specific molecular geometries on the mitochondrial outer membrane depending upon their class. Specifically it has been shown that three helices of Bax (α5, α6 and α9) insert into the membrane prior to observation of permeabilization (Annis et al., 2005; Cartron et al., 2005; Dlugosz et al., 2006; Schendel et al., 1997; Yethon et al., 2003). It has been suggested that Bcl-2/Bcl-2 and Bcl-2/Bax oligomers form a closed pore, while Bax/Bax oligomers for permeabilized pore structures.

A distinguishing feature between pro- and anti-apoptotic Bcl-2 family members is the presence of a BH4 motif. It has been demonstrated that removal of the BH4 motif either genetically or through caspase-mediated cleavage of Bcl-2 can convert this protein
from an anti-apoptotic to a pro-apoptotic isoform (Cheng, 1997; Grandgirard, 1998). While the tertiary structure of pro- and anti-apoptotic Bcl-2 family proteins may appear to be superficially similar, the conformational energetics of these proteins are likely distinct due to sequence differences. With respect to how the BH4 motif may affect pro-versus anti-apoptotic function, it is interesting to note that the BH4 motifs of both Bcl-2 and Bcl-xL have been shown to influence the calcium channel inositol 1,4,5-triphosphate receptor in regulating calcium influx within the ER (Rong et al., 2009; White et al., 2005).

With respect to multi-motif pro-apoptotic proteins, Bax normally resides in the cytoplasm where it translocates to the mitochondria during PCD signaling. The structure of soluble Bax indicates that its hydrophobic tail is normally sequestered into a pocket on the protein’s surface (Suzuki et al., 2000). Exposure of this tail segment is required for mitochondrial localization of Bax during PCD (Desagher et al., 1999; Goping et al., 1998; Hsu and Youle, 1998). This conformational change also exposes the first alpha helix (α1) located in the amino-terminus and has been widely utilized as a means to detect Bax activation through the use of an epitope-specific antibody (6A7) (Cartron et al., 2004; Gavathiotis et al., 2008; Kim et al., 2009). The 6A7 epitope comprises amino acids 13-19 of Bax which are normally inaccessible in the unactivated (cytoplasmic) state (Hsu and Youle, 1997). Previously it was believed that activator BH3-only proteins (i.e., tBid and Bim) promoted exposure of the tail-anchor sequence, thereby promoting translocation and oligomerization of Bax. However more recent evidence suggests an additional binding site for BH3-only proteins comprised of α1 and α6 may exist on Bax (Cartron et al., 2005; Cartron et al., 2004) (Gavathiotis et al., 2008) (Figure 12C).
**Figure 12. Structural Features of Bcl-2 Family Protein Interactions**

(A) BH3 motifs of Bcl-2 family proteins. Sequence alignment between BH3 motifs of different Bcl-2 family proteins shows conservation of the central motif. In particular, mutation of the conserved leucine to alanine can completely abolish BH3 motif-mediated interactions. (B) A x-ray crystal structure of Bcl-xL with Bak BH3 peptide. A hydrophobic groove is created by the BH1, BH2, and BH3 motifs (yellow, red, and green, respectively) within Bcl-xL, allowing the Bak BH3 peptide to bind. The general motif is common to an array of Bcl-2 family members (structures of Bcl-xL with Bim BH3 peptide has also been solved). The hydrophobic groove is traditionally viewed as the principle interaction site between Bcl-2 family proteins. (C) Recent studies described an additional interaction site between BH3-only proteins and Bax. The traditional hydrophobic groove within the Bax molecule is shown on the left, while the novel interaction interface is located on the opposite side. The right panel depicts conformational changes induced by the binding of Bim SAHB (stabilized-helices of BCL-2 domain) to Bax. The amino-terminal chain and the loop between helices α1 and α2 prior (green) and following (blue) the binding are shown. The red region in these panels denotes the 6A7 epitope which was traditionally viewed as a marker for Bax activation.
Adapted from
Sattler *et al.*, *Science*, 1997
Consistent with this a point mutation within this binding site (K21E) results in impaired staurosporine-induced PCD (Gavathiotis et al., 2008). Interactions between helices α1 and α9 appear to help confine helix α9 to the hydrophobic pocket, maintaining Bax as a cytosolic monomer (Kim et al., 2009) (Figure 13). Upon PCD stimulation BH3-only proteins like tBid, Bim and Puma (but not Bad) interact with helix α1 relieving its hold on helix α9, which triggers a conformational change to promote translocation to the mitochondria. Bax translocation and homo-oligomerization appear to occur as distinct steps, each of which requires BH3-only proteins acting at two distinct binding sites (Kim et al., 2009). Consistent with this mutations have been made in which Bax is capable of inserting into the mitochondrial membrane but unable to oligomerize (Kim et al., 2009). Hence it has now been demonstrated that exposure of the 6A7 epitope is an indication of mitochondrial translocation only and is not indicative of oligomerization. Furthermore, Bak has been observed to undergo analogous conformational changes during its activation (Kim et al., 2009).

The interaction between BH3 motifs among Bcl-2 family members is central to the regulation of PCD, as a point mutation within this motif (e.g., Bax L63) can abolish apoptotic function (Luo et al., 1998; Wang et al., 1998; Zha et al., 1997) (Figure 12B). The importance of this leucine within the BH3 motif has been demonstrated for virtually all Bcl-2 family proteins. Even relatively conservative substitutions at this site (leucine to alanine) can completely abolish function (Zha et al., 1997). Proximal to this residue, a number of others have been shown to be conserved to a lesser extent and to regulate interaction specificity. Consistent with this, different BH3-only proteins bind to anti-apoptotic Bcl-2 family members with varying affinities (Certo et al., 2006; Chen et al.,
Figure 13. Proposed Steps in the Activation and Oligomerization of Bax and Bak

(A) Activator BH3-only proteins such as tBid, Bim, and Puma bind to the amino-terminus of Bax inducing conformational changes exposing the amino-terminal helix α1 and carboxyl-terminal helix α9. (B) Disruption of these interactions exposes the hydrophobic transmembrane promoting translocation to the mitochondria. (C) Following translocation, homoligomerization occurs as a distinct step involving activator BH3-only proteins. Furthermore, a second interaction between activator BH3-only proteins at the Bax BH1 motif. (D) Similar interactions have been demonstrated for Bak activation and homooligomerization. (E) It was previously shown that multi-motif Bcl-2 family proteins can bind to the mitochondrial outer membrane via a hydrophobic transmembrane domain residing in helix α9. (F) This is followed by subsequent insertion of helices α5 and α6 into the membrane which are critical for pore formation. (G) Illustrated in the figure are two different models for Bax homooligomerization. In the first (2 to 4), Bax monomers associate with the mitochondrial outer membrane and oligomerize prior to the membrane insertion of helices α5 and α6. In the second model (2’ to 4’), Bax monomers insert helices α5 and α6 together with helix α9 prior to oligomerization. A recent study of insertion kinetics support a model in which membrane insertion is the rate-limiting step in the process (2-4) and occurs prior to homooligomerization.
Adapted from
Annis et al., EMBO J, 2005
Kim et al., Mol Cell, 2009
Kim et al., Mol Cell, 2004
For example Bad exhibits high affinities for Bcl-xL and Bcl-2 but negligible binding to Mcl-1 (Certo et al., 2006; Chen et al., 2005). Conversely Noxa has a high affinity for Mcl-1, but not for Bcl-2 or Bcl-xL (Chen et al., 2005; Kim et al., 2006; Willis et al., 2005). In contrast to Bad and Noxa, Bim binds all anti-apoptotic Bcl-2 proteins with high affinity (low nanomolar dissociation constants). BH3 motif swap experiments support the contention that the amino acid sequence within the central core of the BH3 motif is key to defining the interaction avidities (Chen et al., 2005).

Additionally serine residue S155 within the Bad BH3 motif can be regulated by phosphorylation (Datta et al., 2000; Tan et al., 2000). When phosphorylated, the charge and steric hinderance significantly reduce its affinity for Bcl-xL and other proteins (Datta et al., 2000; Tan et al., 2000).

While most studies have focussed upon Bax as the principal mediator of MOMP, numerous experiments have demonstrated the functional equivalence of Bak (Wei et al., 2001) (see section 1.2.4.1). Unlike Bax, Bak is constitutively associated with the mitochondrial outer membrane (Wei et al., 2000). Two recent structural studies of Bak by Dewson and colleagues (2009; 2008) demonstrated that similar to Bax, two molecular interfaces are involved in its activation and homo-oligomerization (Figure 14). In the proposed model, Bak in the unactivated state exist in a conformation which positions its BH3 motif towards the hydrophobic core. However upon activation a conformational change occurs which exposes this BH3 motif, allowing it to undergo heterodimerization with anti-apoptotic Bcl-2 family proteins (e.g., Bcl-xL and Mcl-1), or to form symmetric homodimers (Dewson et al., 2008) (Figure 14A). An independent dimerization interface formed between helix \( \alpha 6 \) means that Bak can form higher order homo-oligomers.
Figure 14. Bak Conformational Changes and Mechanism of Activation

Unlike Bax, Bak is associated with the mitochondrial prior to PCD activation. (A) Prior to activation, Bak sequesters its BH3 motif similarly to Bax. Following interaction with BH3-only proteins it undergoes a conformational change exposing its BH3 motif. This BH3 motif either interacts with anti-apoptotic Bcl-2 family members such as Bcl-xL and Mcl-1 (BH3 capture), or promotes homodimerization (reciprocal binding). Thus depending on its interaction partners, pro-survival heterodimers or apoptotic homodimers may be formed. (B) Due to the presence of a secondary dimer interface, high-order oligomers of Bak may be formed via interaction with helix α6. As such, both the BH3:hydrophobic groove and α6:α6 interactions are required for Bak homooligomerization and pore formation.
Adapted from Dewson et al., Mol Cell, 2008
Dewson et al., Mol Cell, 2009
(Dewson et al., 2009) (Figure 14B). It is presently unclear whether hetero-oligomerization can occur between activated Bax/Bak monomers \textit{in vivo}.

Interestingly the BH3 motif of multi-motif Bcl-2 family members appear to have a defined tertiary structure, while the BH3 motif of BH3-only proteins appears intrinsically disordered. An exception to this is the BH3-only protein Bid which has a tertiary structure quite similar to that of multi-motif Bcl-2 family proteins (Chou et al., 1999; McDonnell et al., 1999). In contrast, other BH3-only proteins appear to be intrinsically unstructured (Hinds et al., 2007). It has therefore been postulated that (A) Bid had originated as a Bax-like protein but has since evolved into a BH3-only protein, having lost sequence conservation in the BH1 and BH2 motifs, (B) other BH3-only proteins subsequently evolved from Bid with a loss of stabilization of their tertiary structure in order to promote particular energetic parameters of the system but adopt localized structural changes in the BH3 motif upon binding to other Bcl-2 family proteins.

1.2.4.3 RELEASE OF PROTEINS FROM MITOCHONDRIA

A recent study attempted to probe the sequence of molecular events beginning from the interaction between Bax and BH3-only proteins to the release of fluorophores from liposomes (Lovell et al., 2008). It was observed in this study that (1) the interaction between Bax and tBid required the presence of membrane (Gonzalvez et al., 2005; Lucken-Ardjomande et al., 2008), (2) Bax membrane insertion is the rate-limiting step leading up to membrane permeabilization, (3) membrane permeabilization occurs rapidly following oligomerization of Bax, (4) Bcl-xL prevents membrane permeabilization by
competing with Bax for recruitment to the membrane by tBid (Billen et al., 2008) and (5) BH3-only proteins like Bad can bind to Bcl-xL releasing tBid, allowing it to recruit Bax for membrane insertion and oligomerization.

It has been proposed that release of mitochondrial proteins could occur via the mitochondrial permeability transition pore (mPTP) (Kroemer et al., 2007), minimally comprised of Bax, VDAC, ANT and cyclophilin D (Figure 15). Early genetic and biochemical evidence suggested a requirement for ANT and VDAC in the pro-apoptotic functions of Bax (Marzo et al., 1998; Shimizu et al., 1999). However more recent studies have drawn these findings into question (Baines et al., 2005; Baines et al., 2007; Kokoszka et al., 2004; Nakagawa et al., 2005). In addition, VDAC2 appears to inhibit Bak in vivo as shown by a recent study of Vdac2/Bak1 DKO animals (Ren et al., 2009). Cells lacking VDAC2 exhibits greater Bak oligomerization and higher PCD sensitivity (Cheng et al., 2003). Consistent with this, ectopic VDAC2 overexpression inhibited Bak activity and cell death. While deletion of Vdac2 in the thymus accelerated thymocytes death during development and following PCD stimulation. Concurrent deletion of Bak1 led to a reversion of this phenotype, confirming the critical interaction between these proteins in vivo (Ren et al., 2009). In contrast concurrent deletion of Bax (Vdac2/Bax DKO cells) did not rescue the observed Vdac2 deficiencies. Consistent with this, Vdac1/Vdac3 DKO MEFs with concurrent siRNA-mediated knockdown of Vdac2 support an inhibitory role on Bak (Baines et al., 2007).

Both ANT and cyclophilin D have previously been examined via genetic deletion studies in mice. Several forms of PCD have been observed to occur normally in each of these knockouts (Baines et al., 2005; Kokoszka et al., 2004; Nakagawa et al., 2005). Thus
Figure 15. The Mitochondrial Permeability Transition Pore

Suggested structure of the mitochondrial permeability transition pore (MPTP), consisting minimally of VDAC, ANT, and cyclophilin D (CypD). (A) Under physiologic conditions MPTP facilitates the transport of ions and metabolites from the cytoplasm into the mitochondrial matrix. (B) Factors such as increased cytoplasmic calcium levels or reactive oxygen species (ROS) can cause a substantially increase in conductance due to formation of the MPTP, resulting in a massive influx of water into the mitochondrial matrix due to its higher osmolarity, ultimately resulting in mitochondrial swelling and rupture. Though there is strong evidence for interactions between MPTP and Bcl-2 family proteins, the precise role of MPTP in PCD is still unclear. Several commonly used modulators of MPTP are depicted in the diagram: atracyloside (ATR), bongkrekic acid (BKA) and cyclosporin A (CsA).
Adapted from Zhivotovsky et al., *Cell Death Differ*, 2009
these findings suggest that the molecular target of cyclosporin A is not cyclophilin D and mPTP inhibition. As will be discussed in section 3.2, a common signaling mechanism involving calcineurin inhibition is responsible for the neuroprotection mediated by cyclosporin A and FK-506.

1.2.4.4 ROLE OF MITOCHONDRIAL PROTEINS IN PROGRAMMED CELL DEATH REGULATION

The mitochondrion is a key player in releasing PCD second messengers from the intermembraneous space. The first such protein to be identified in mammals was cytochrome c, a heme-containing protein loosely associated with the inner mitochondrial membrane which plays an essential role in electron transport and thus ATP generation (Li et al., 2000b). Holo- (heme-containing) cytochrome c was originally designated as Apaf-2 since it was discovered that it could facilitate caspase-3 activation in a dATP-dependent manner (Liu et al., 1996). This mitochondrial protein is now known to function by interacting with Apaf-1 to promote apoptosome formation (Hu et al., 1998; Li et al., 1997; Zou et al., 1999). Though apo-cytochrome c is capable of binding to Apaf-1 it does not promote caspase-9 activation (Martin and Fearnhead, 2002; Martin et al., 2004). Thus holo-cytochrome c provides a specific conformation to promote Apaf-1 oligomerization. Consistent with this a point mutant of cytochrome c which maintain activity with respect to the electron-transport chain but incapable of activating Apaf-1 had been identified (Kluck et al., 2000; Yu et al., 2001b).

A second mitochondria protein designated “second mitochondria-derived activator of caspases” (Smac) and “direct IAP-binding protein with low pI” (DIABLO),
depending upon the research groups which concurrently characterized it, was later
discovered to promote the activation of caspase-3 (Du et al., 2000; Verhagen et al.,
2000). A detailed review of Smac/DIABLO is provided in latter sections (1.2.5.6 and
1.2.5.7).

Other mitochondrial proteins which promote PCD have been described. These
include apoptosis inducing factor (AIF) and endonuclease G (EndoG), both of which
redistribute to the nucleus during PCD (Li et al., 2001; Parrish et al., 2001; Susin et al.,
1999; Susin et al., 1996). AIF is a flavoprotein which plays a role in oxidative
phosphorylation (Pospisilik et al., 2007; Vahsen et al., 2004) and shows homology to
several bacterial ferredoxin or NADH-oxidoreductases (Susin et al., 1999). Upon entry
into the nucleus, AIF is involved in DNA fragmentation and chromosomal condensation
associated with PCD. The precise mechanism by which AIF mediates these nuclear
changes is unclear, although an AIF-DNA interaction is required for apoptotic functions
as AIF mutants which do not bind DNA are defective in mediating PCD (Ye et al., 2002).
It is interesting to note that the *C. elegans* homologue of AIF, WAH1, was previously
shown to interact with and enhance the nuclease activity of CPS-6 (*C. elegans*
homologue of endonuclease G, which is released from the mitochondria during
mammalian PCD) (Wang et al., 2002). Despite this, cooperation between these two
mitochondrial proteins in mammalian PCD has not been observed.
1.2.4.5 MITOCHONDRIAL MEMBRANE POTENTIAL AND PROGRAMMED CELL DEATH

In addition to the release of mitochondrial proteins, pores formed by Bax/Bak or mPTP can also lead to a loss of mitochondrial membrane potential (MMP, ΔΨ) (Kroemer et al., 2007). The MMP is the electrochemical gradient required for oxidative phosphorylation. During electron transport protons are transported across the inner mitochondrial membrane to the intermembraneous space, creating an electrochemical gradient across the inner mitochondrial membrane (lower in pH in the intermembraneous space compared to the matrix). This gradient is utilized for ATP production when protons are allowed to enter back into the matrix through F₁F₀ ATP synthase, converting the potential energy of the proton gradient to ATP (Saraste, 1999). As such when the outer mitochondrial membrane is permeabilized the proton gradient is dissipated, halting ATP production. In addition, the massive influx of anions into the mitochondria results in mitochondrial swelling and eventual rupture of the outer mitochondrial membrane (Kinnally and Antonsson, 2007).

1.2.4.6 EXTRA-MITOCHONDRIAL ROLES OF BCL-2 FAMILY PROTEINS

The mitochondria and endoplasmic reticulum constitute the principal storage sites for intracellular calcium (Petersen et al., 2001) (Figure 16). When mitochondrial function is disrupted following Bax/Bak-mediated MOMP, stored calcium is released into the cytosol, where it triggers a series of catabolic events (Scorrano et al., 2003). Physiologic release of intracellular calcium is taken up by the mitochondria, enhancing oxidative phosphorylation and ATP production (Pinton et al., 2008). However, excessive release of
Figure 16. Calcium Regulation and PCD

Calcium levels are typically tightly regulated within the cell in response to stimuli ranging from neurotransmitter release in neurons to muscle contraction of cardiomyocytes. Calcium also plays a critical role for decisions between life and death. Physiologic levels of intracellular calcium can induce signaling pathways such as NFAT activation for T cell proliferation. Bcl-2 family proteins can regulate calcium currents to prevent calcium overload. In contrast, a variety of cellular stresses can trigger calcium release from the ER into cytosol. Among these, Bik has been shown to translocate to the ER and trigger Bax/Bak-mediated endoplasmic calcium release. This can ultimately result in calcium overload and trigger PCD. Bax and Bak have also been shown to enhance release of endoplasmic calcium stores in that Bax/Bak1 DKO MEFs possess lower resting ER calcium levels than control cells.
Adapted from Rong and Distelhorst, *Annu Rev Physiol*, 2008
intracellular calcium can result in mitochondrial overload, resulting in a failure of the
mitochondria to buffer cytoplasmic calcium, ultimately inducing the activation of
calpains, calcineurin and other calcium-regulated enzymes which can promote PCD
(Newmeyer and Ferguson-Miller, 2003). As illustrated Figure 16, oscillations in
intracellular calcium can be triggered by various signals. These signals induce small
calcium currents between the endoplasmic reticulum and the mitochondria, where it can
increase oxidative phosphorylation and other metabolic processes.

Within the ER, Bcl-2 family proteins such as Bcl-2 and Bcl-xL have been shown
to interact with IP3R through their BH4 motifs to regulate inositol 1,4,5-triphosphate
receptor (IP3R) activity, reducing the normal levels of Ca^{2+} release from ER which can
affect the amount of calcium released during PCD (Hetz and Glimcher, 2008). Consistent
with this, the precise mechanism of these effects remain unclear, however calcium
mobilization during PCD is reduced in Bax/Bak1 DKO MEFs (Scorrano et al., 2003)
(Oakes et al., 2005; Rong et al., 2008; Rong et al., 2009; White et al., 2005). It is believed
that pro-apoptotic Bcl-2 family members such as Bax and Bak actually interrupt the
interaction of Bcl-2 and Bcl-xL with IP3R (Oakes et al., 2005; Scorrano et al., 2003). The
lower level of calcium in the ER result in a reduced amount of calcium released from the
ER to the mitochondria during PCD and therefore a blunted response to PCD is
generated.

In addition to their roles in regulating MOMP, Bcl-2 family proteins also plays a
role in regulating mitochondria dynamics. Though often viewed as static organelles,
mitochondria are extensively interconnected structures which undergo continuous and
dynamic remodelling in the form of mitochondrial fusion/fission (Cerveny et al., 2007).
Homo- and heterotypic interaction of mitofusins such as Mfn1 and Mfn2 (dynamin family GTPases) located on the mitochondrial outer membrane critically regulate the process of fusion (Chen et al., 2003b; Ishihara et al., 2004). In conjunction with Opa1, which regulates mitochondrial tethering and fusion (Cipolat et al., 2004; Song et al., 2009), the dynamin-related protein 1 (Drp1) promotes the process of mitochondrial fission through cooperation with Fis1 (Lee et al., 2004b). The importance of these proteins in mitochondrial dynamics is demonstrated by the lethal phenotypes seen in null mutations of Mfn1, Mfn2, Opa1, or Drp1 in mice (Chen et al., 2003b; Davies et al., 2007; Ishihara et al., 2009), and mutations of these genes in humans have been implicated in neurodegenerative diseases (Alexander et al., 2000; Delettre et al., 2000; Zuchner et al., 2004).

Though evidence supports a connection between Bcl-2 proteins and these regulators of mitochondrial dynamics, the precise functional consequences of such interactions are currently controversial (Autret and Martin, 2009). During PCD, mitochondria undergo extensive fission and it has been suggested that this remodelling may play a significant role in the release of cytochrome c during MOMP (Suen et al., 2008). Consistent with this, a dominant negative mutant of Drp1 (Drp1K38A) which impairs mitochondrial fission was shown to exhibit reduced cytochrome c release and delayed PCD progression (Cassidy-Stone et al., 2008; Estaquier and Arnoult, 2007), consistent with previous studies demonstrating that cristae remodelling within the mitochondria is required for efficient release of cytochrome c as a significant portion of cytochrome c is sequestered within cristae (Figure 17A) (Kim et al., 2004; Scorrano et al., 2002). Paradoxically Bax/Bak1 DKO MEFs, which have been demonstrated to be
Figure 17. Bcl-2 Family Proteins and Mitochondrial Dynamics

Linkage between Bcl-2 family proteins and mitochondrial dynamics. (A) Suggested model showing a substantial portion of cytochrome c stored in mitochondrial cristae, between folds of the mitochondrial inner membrane, increasing the surface area available for oxidative phosphorylation. Opening of cristae during MOMP would thus accelerate cytochrome c release. As demonstrated in the figure, Opa1 oligomers formed with the aid of PARL (presenilin-associated rhomboid-like), regulates cristae openings and restricts cytochrome c exit. It has been shown that Bax/Bak can mediate cristae opening by facilitating the disassembly of Opa1 oligomers. Cristae opening and MOMP have been shown to be independent events though both appear to require Bax/Bak. (B) Bcl-2 family proteins have also been suggested to act as regulators of mitochondrial dynamics. Bax and Bak have been shown to interact with Mfn2 and Drp1, which mediate mitochondrial fusion and fission, respectively. In this model, Bax molecules translocate to the mitochondria and localize to foci on the mitochondrial outer membrane. Drp-1 is believed to function downstream of Bax, perhaps through recruitment to specific foci by Bax, where they mediate membrane pinocytosis facilitating mitochondrial fission.
Adapted from Delivani and Martin, *Cell Death Differ*, 2006
incapable of releasing cytochrome c upon PCD stimulation, were shown to have shorter mitochondria which were reversed when Bax was reintroduced (Cleland et al., 2010; Karbowski et al., 2006), thus implying a role for Bax to induce mitochondrial fusion instead.

Although such data suggest cytochrome c release and mitochondrial dynamics may be distinct processes, they do not preclude that Bcl-2 family proteins may be involved in both processes independently. It has been demonstrated recently that Mfn2 can physically interact with both Bax (Cleland et al., 2010), Bcl-2 and Bcl-xL (Delivani et al., 2006). Moreover, it was observed previously that Bax and Bak are required for Mfn2 to form discrete loci around the mitochondrial outer membrane, and that Bax cooperates with Drp1 and Mfn2 to regulate mitochondrial fission and fusion, respectively (Figure 17B) (Karbowski et al., 2006). A similar role for Bak in mitochondrial dynamics has also been demonstrated (Brooks et al., 2007). Thus Bcl-2 family proteins appear to regulate both processes.

Bcl-2 family proteins are also believed to play a role in the regulation of autophagy (Levine et al., 2008). Though autophagy is principally regulated by ATG proteins, Atg6 (a BH3-only protein also termed beclin 1) has been shown to interact with Bcl-2 and Bcl-xL (Luo and Rubinsztein, 2010; Maiuri et al., 2007b; Oberstein et al., 2007; Pattingre et al., 2005). The interaction between beclin 1 and Bcl-2/Bcl-xL are not pro-apoptotic in nature and occur under physiologic conditions in a nutrient-dependent manner (presence or absence of amino acids in the growth medium) (Ciechomska et al., 2009; Pattingre et al., 2005). Under conditions of sufficient nutritional support, Bcl-2 or Bcl-xL interact with beclin 1 (Figure 18), sequestering it from other Atg proteins in order
Figure 18. Beclin 1: Autophagic BH3-only Protein

Anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xL can suppress autophagy by binding to beclin 1 (Atg6), a protein involved in autophagosome formation. As illustrated in the figure, the BH3 domain of beclin 1 (upper panel) interacts with Bcl-xL in a similar fashion to that seen for other BH3-only proteins such as Bim (lower panel). Functional outcomes are similar in that beclin 1-Bcl-xL interaction sequesters beclin 1 from interacting with other Atg proteins, thereby inhibiting autophagosome formation. In a similar manner, interaction between Bim and Bcl-xL functions to prevent Bim from activating Bax for MOMP induction.
Adapted from Oberstein et al., J Biol Chem, 2007
to form the autophagosome nucleation complex; thus preventing the initiation of autophagy. Bcl-2/Bcl-xL-beclin 1 interaction can be altered by both phosphorylation of critical serine residues on beclin 1 and Bcl-2 (Wei et al., 2008; Zalckvar et al., 2009a; Zalckvar et al., 2009b); and disruption via other BH3-only proteins such as Bad or small molecule BH3 mimetics such as ABT-737 (Maiuri et al., 2007a). Thus in addition to regulating MOMP, Bcl-2 family proteins also play a role in inhibiting autophagy.

1.2.5 REGULATION OF EXECUTIONER CASPASES

1.2.5.1 BIOCHEMISTRY OF CASPASES

The primary function of Bcl-2 family proteins is to regulate the release of PCD second messengers such as cytochrome c. Ultimately this release influences the activation of downstream caspases. Caspases, cysteine-aspartic acid proteases, are cysteine proteases which cleave at the carboxyl terminal side of aspartic acid residues within their recognition motifs (Shi, 2004) (Figure 19). In addition to their pivotal roles in cellular destruction, a subgroup of caspases regulate cytokine activation (caspase-1, -4, -5 and -11) and skin cell development (caspase-14) (Lamkanfi et al., 2007). Caspases which function to regulate PCD can be functionally divided into either initiator (caspase-2, -8, -9 and -10) or executioner caspases (caspase-3, -6 and -7) (Shi, 2004) (Figure 20). As indicated above, initiator caspases are activated in the context of initiating activation platforms (caspase-8 – DISC, caspase-9 – apoptosome) in response to apoptotic stimuli. Following activation of the pro-enzyme, caspases cleave crucial cellular substrates ultimately resulting in cell death. In healthy cells, the majority of caspases activity is kept inactive as zymogens (Bao and Shi, 2007). Structurally, initiator caspases have a larger
**Figure 19. Caspase Substrate Specificity**

(A) As shown in this figure, residues of caspase substrate within active sites is named from the residue on the amino-terminal of the scissile bond (lightning bolt) starting with P1 and the one on the carboxyl-terminal as P1'. Other residues within the recognition sequences are numbered P1 and P1'. A number of these residues occupy pockets on the enzyme and are named SX where X corresponds to the numbering associated with the particular residue which occupies the pocket. (B) Studies using synthetic peptides and peptide libraries have demonstrated preferred substrate specificity at each residue bound within the active sites of various caspases. Note that P1 position is exclusively aspartic acid. Other residue preferences include glutamine at position P3, and P1' where charged residues are not well-tolerated. As such these positions consist mostly of glycine, alaine, threonine, serine, or asparagine. Residues at position P2 are highly variable and thus specified as X. Typically, substrate specificity involves the recognition of a tetrapeptide (P1 to P4), however caspase-2 possesses additional recognition specificity for position P5 which can enhance catalytic rates by 30-fold. (C) A previous study identified the potential of many caspase substrates to gain IBM-like motifs following processing by caspases. These newly generated IBM-like motifs can theoretically inhibit inhibitor of apoptosis proteins using a similar mechanism as molecules like Smac/DIABLO and Omi/HtrA2.
Adapted from
Hell et al., Cell Death Differ, 2003
Timmer and Salvesen, Cell Death Differ, 2007
Figure 20. Caspase Members Involved in PCD Regulation

Unlike in *C. elegans* which only possess a single caspase within its genome, both fruit flys and mammals have evolved a number of different caspases. Interestingly aside from PCD functions, some caspases are primarily associated with physiologic functions such as inflammation and keratinocyte differentiation. As shown in this figure, caspases involved in PCD regulation in mammals and fruit flies can be divided into two functional groups: initiator and effector/executioner caspases. Initiator caspases in most cases are activated by autocatalytic cleavage (black arrows) following recruitment into large protein complexes such as DISC or apoptosome. As such, they generally possess interaction domains such as DED and CARD to bind to these activating platforms. Executioner caspases, in contrast, do not possess these interaction domains as they are activated via proteolytic cleavage (black arrows) by initiator caspases. Following the initial activating intrachain cleavage events, subsequent processing (gray arrows) is often required for full caspase maturation but in many cases is not required for catalytic activity. The p20 and p10 fragments together form a single caspase monomer. The four surface loops structuring the catalytic groove are indicated as L1 to L4. The red line within the L2 loops indicate the catalytic cysteine.
Adapted from
pro-domain containing DED or CARD motifs (caspase-8/10 and caspase-9, respectively) to facilitate caspase recruitment to the appropriate activation platform (Shi, 2004) (Figure 21A). Removal of the pro-domains and dimerization are required for full activation of initiator caspases (Figure 21B). Initiator caspases in turn cleave and activate executioner caspases through removal of shorter pro-domains (Figure 22).

For caspase-3, initiator caspases mediate the cleavage between the large and small subunit of caspase-3 (p20/p12), which can subsequently undergo a further dimerization with another molecule of caspase-3 to produce the catalytically active heterotetramer complex (Mittl et al., 1997). The subsequent autocatalytic processing removes the prodomain from the large subunit, thus producing the fully matured p17/p12 of the enzyme (Figure 21B). Caspase-7 is believed to be activated via a similar mechanism by initiator caspases into its intermediate p22/p12 form (Choi et al., 2009), however it remains unclear whether it is capable of the subsequent autocatalytic conversion into the fully matured p19/p12 form, or that full maturation requires the activity of another enzyme such as caspase-3.

1.2.5.2 REGULATION OF CASPASE ACTIVITY

Given their cell-degrading activities, the regulation of caspase activity is clearly critical to the cell. As such, multiple mechanisms have evolved to regulate caspase activity. Here, the regulatory mechanisms involved in activation and activity of executioner caspases will be discussed in greater detail.

For the extrinsic pathway in which caspase-8 and caspase-10 act to cleave executioner caspases. This action is opposed by inhibitory molecules such as cFLIP
Figure 21. Mechanisms of Caspase Activation

(A) As described in Figure 20, caspase activation involves proteolytic cleavages at multiple sites. The first site is at the inter-subunit linker (between p20 and p10 subunits) and the second is between the pro-domain and p20 subunit. (B) For initiator caspses, recruitment to activating platforms such as DISC or the apoptosome promotes dimerization and autocatalytic activation by virtue of their conformational topology. In contrast, executioner caspses are first cleaved by initiator caspses in the cell cytoplasm, and subsequently removes the pro-domain through autocatalytic cleavages. Both initiator and executioner caspses exist in a dimeric form when activated. Following their activation, regulatory mechanisms are available to suppress their catalytic activities.
Adapted from Pop and Salvesen, *J Biol Chem*, 2009
**Figure 22. Structural Basis of Caspase Activation**

(A) Schematic representations of pro-caspase-7 and active caspase-7. Each caspase-7 asymmetric homodimer is comprised of two identical monomers containing large (blue) and small (gray) subunits. Flexible loops with undefined electron density are in yellow. Regions of active caspase-7 which have undergone a conformational change compared to pro-caspase-7 are in red. (B) Conformational changes during activation of caspase-7. The catalytic cysteines in pro-caspase-7 and active caspase-7 are coloured gold and red, respectively. (C) As illustrated in the figure, a catalytic groove is produced following the activation of caspase-7.
Adapted from
Chai et al., Cell, 2001
Riedl et al., Proc Natl Acad Sci U S A, 2001
which interacts with FADD or TRADD to compete with caspase-8 in the DISC, thereby preventing DISC activation (Figure 23) (Irmler et al., 1997; Scaffidi et al., 1999; Thome et al., 1997). Two additional proteins have been recently discovered to bind and negatively regulate DED-containing caspases. Termed caspase-8/10-associated RING proteins (CARPs), they are believed to regulate caspase-8/10 by promoting their proteasomal degradation via the E3 ubiquitin ligase activity of their RING domains (McDonald and El-Deiry, 2004). These proteins have also been implicated in mediating proteasomal degradation of phosphorylated and unphosphorylated p53 (Yang et al., 2007). CARP2 has also been shown to function as an ubiquitin ligase for RIPK1 and to regulate TNF-α-dependent NFκB activity (Liao et al., 2008). Recent studies have clarified the role which caspase-8 and RIPK1 play in regulating PCD and NFκB signaling, respectively, following stimulation of TNF receptors or Fas (O'Donnell et al., 2007; Wang et al., 2008). It appears that CARPs may also play a role in such processes (Yang and El-Deiry, 2007).

In contrast IAPs, especially XIAP, have been shown in a variety of studies to inhibit the activity of caspase-9, -3 and -7 (Deveraux et al., 1999; Takahashi et al., 1998). With respect to caspase-9, XIAP was shown to disrupt caspase dimerization through interaction of its BIR3 domain (Shiozaki et al., 2003) (Figure 24). In addition due to the presence of a RING domain, XIAP, cIAP-1 and cIAP-2 can inhibit caspase activity through ubiquitin-mediated proteasomal degradation (Choi et al., 2009; Schile et al., 2008) (Figure 25). This mechanism is analogous to the principal mode of inhibition utilized by DIAP1 and DIAP2 in Drosophila (Steller, 2008). Though cIAP-1 and cIAP-2 also possess CARD motifs, suggesting that they may be capable of interacting with
Figure 23. Mechanism of Caspase-8 Activation and Inhibition

The extrinsic pathway is initiated by death receptor activation and oligomerization, leading to the recruitment of other proteins such as FADD and caspase-8. This dimerization of caspase-8/10 in the DISC facilitates their activation, allowing them to further advance the PCD pathway by cleaving downstream targets such as Bid and executioner caspases. Caspase-8/10 dimerization can be regulated by competitive binding of FLIP (short and long forms denoted as FLIP$_S$ and FLIP$_L$, respectively) through their DEDs. As illustrated in the figure (left), when FLIP levels are sufficiently low, adequate amounts of pro-caspase-8 molecules are recruited to the DISC through homotypic DED interactions with FADD in order to promote autocatalytic activation. However in the presence of either FLIP$_S$ (centre) or FLIP$_L$ (right), FLIP molecules are recruited to the DISC in place of more caspase-8 molecules. Thus autocatalytic activation is prevented and no active caspase-8 is released to further activate downstream PCD pathways. Note that while structural differences exist between the two isoforms of FLIP, they can both disrupt caspase-8 activation by competitive binding to the DISC and prevent caspase-8 recruitment.
Adapted from Thome and Tschopp, *Nat Rev Immunol*, 2001
Figure 24. Mechanism of Caspase-9 Inhibition by XIAP

(A) The interaction between BIR3 domain of XIAP and caspase-9 occurs at the dimer interface of caspase-9 (top), thus preventing the initiator caspase from forming active dimers (bottom). (B) As caspase-9 is only active as a dimer, XIAP can putatively interact with apoptosome-bound caspase-9 as well as unbound caspase-9. Thus it is possible have multiple configurations of caspase-9 heterocomplexes which could be inactive.
Adapted from Shiozaki et al., Mol Cell, 2003
Figure 25. Molecular Basis of Ubiquitination and Proteasome-Mediated Degradation

The conjugation of ubiquitin typically occurs either on the -amino group of a Lys residue or to the extreme amino terminus of a polypeptide. Unlike protein phosphorylation, ubiquitination requires a much more complex sequence of events prior to the addition of a ubiquitin moiety onto the target proteins. As illustrated in the figure, three sequential steps mediated by three different classes of enzymes are required for ubiquitination. The first step, as catalyzed by E1 ubiquitin-activating enzyme, involves adenylation of the carboxyl termini of ubiquitin molecules followed by an enzymatic attack of the adenylated carboxyl terminal glycine to produce an E1-ubiquitin thioester intermediate. The newly activated ubiquitin is subsequently transferred to an E2 ubiquitin-conjugating enzyme. In conjunction with an E3 ubiquitin ligating enzyme (or often called E3 ubiquitin ligase), the activated ubiquitin is transferred to a substrate recognized by the E3 ubiquitin ligase. Repeated cycles of this process can result in the construction of polyubiquitin chains on a target protein. Deubiquitinating enzymes or deubiquitinase (DUBs) can remove ubiquitin molecules from a target protein, just as protein phosphatases can remove phosphate groups conjugated onto a substrate previously phosphorylated by protein kinases. Traditionally, polyubiquitin chains were believed to be created by the lysine side chain of one ubiquitin molecule conjugating to the carboxyl terminus of another ubiquitin molecule, and generally occurred at lysine-48 or lysine-63 in ubiquitin. However, more recent studies have demonstrated that linkages can also occur at lysine-6, -11, -27, -29, and -33. Furthermore, evidence for linear chains of ubiquitin molecules joined in a head-to-tail manner has recently been shown. Proteins
containing RING domains are E3 ubiquitin ligase, thus cIAP-1, cIAP-2, and XIAP are considered to be E3 ubiquitin ligases. Interestingly, BRUCE/Apollon has been demonstrated to possess both E2 and E3 activities, and therefore functions as an E2/E3 chimeric protein.
Adapted from Dikic et al., Nat Rev Mol Cell Biol, 2009
CARD-containing caspases like caspase-9 to date, only the IBM-BIR3 interaction between caspase-9 and IAPs have been shown to be critical (Shi, 2004; Sun et al., 2000).

As indicated above, IAP members can function as inhibitors of caspase-3 and -7 activity. In contrast to cIAP-1 (and likely cIAP-2), XIAP is the only IAP family member demonstrated to have a direct inhibitory effect on caspase-3 and -7 (Deveraux et al., 1997; Eckelman and Salvesen, 2006; Scott et al., 2005). This distinct inhibitory function is mediated by both the BIR2 domain and its amino terminal linker (Huang et al., 2001; Scott et al., 2005; Suzuki et al., 2001b). X-ray crystal structures of XIAP fragments in complex with caspase-3 or caspase-7 have demonstrated the BIR2 domain interacts with the caspase IBMs while the linker region inserts into the catalytic site of the caspase, thereby blocking substrate recognition (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001) (Figure 26). cIAPs were originally believed to be direct inhibitors of executioner caspases as well; however subsequent studies have indicated that they possess no direct inhibitory activity (Eckelman and Salvesen, 2006; Roy et al., 1997). Thus the principal mode of inhibition of executioner caspases by cIAPs is believed to be through proteasomal degradation. To this end, it has been shown both in vitro and in vivo that levels of activated executioner caspases can be modulated by cIAP-1/-2 and XIAP. Importantly, mutations within the RING domain render them non-functional with respect to inhibition of the executioner caspases (Schile et al., 2008). Interestingly despite their high level of sequence homology, a recent study demonstrated that caspase-3 and caspase-7 are differentially regulated by cIAP-1 (Choi et al., 2009). It appears that in vitro, cIAP-1 ubiquitinates these two executioner caspases at different stages of enzymatic maturation. Whereas cIAP-1 was shown to promote degradation of the
Figure 26. Mechanism of Caspase-3/7 Inhibition by XIAP

(A) The structural backbone of caspase-3 and caspase-7 is highly similar. As illustrated in the figure, critical residues of XIAP (D148 is shown) inserts directly into the catalytic site of caspase-3 and caspase-7 (marked by the tetrapeptide DVAD caspase substrate, P1 aspartic acid in yellow; catalytic cysteine of caspases are coloured green). (B) Unlike normal caspase substrates (bottom), XIAP residues inserts into the catalytic site in the reverse orientation from that of caspase substrates. (C) The modes of interaction involved in the binding of caspase-3 (depicted in figures) and caspase-7 to the BIR2 domain and linker region of XIAP. Two sites of interactions are apparent both in the ribbon diagram of the crystal structure (left) and in the schematic illustration (right). The linker region on the amino-terminus of BIR2 is positioned into the catalytic site of caspase-3/7 to directly inhibit the catalytic activity as indicated above. Interestingly, this amino terminus of BIR2 is typically flexible in solution; thus it is likely that binding to caspases-3/7 can induce conformation stabilization of this region. Furthermore, a second site of interaction between XIAP and executioner caspase is situated between the IBM located within BIR2 (XIAP) and the amino-termimins of the small subunit from the other caspase in the active dimer. Thus it is proposed that these interaction sites contribute to the binding affinity for caspase-3/7 by XIAP. In addition, despite possessing very similar backbone structures, caspase-3 and caspase-7 may be differentially regulated by XIAP due to subtle differences at these interaction interfaces.
Adapted from
Chai et al., Cell, 2001
Riedl et al., Cell, 2001
Scott et al., EMBO J, 2005
intermediate p20/p12 form of caspase-3, it is the fully matured form of caspase-7 which is targeted for ubiquitination and degradation by cIAP-1. The authors demonstrated via mutational analyses of the IBMs within both caspase-3 and caspase-7 that these differential effects were the result of subtle differences in their respective IBMs. Furthermore it was determined that cIAP-1 preferentially utilized different E2 ubiquitin-conjugating enzymes for ubiquitination of caspase-3 and caspase-7, respectively; highlighting potential differences in their three-dimensional structures (Choi et al., 2009).

1.2.5.3 INHIBITOR OF APOPTOSIS PROTEINS

Natural caspase inhibitors were discovered as a consequence of the inhibition of cell death initiated by baculoviral replication in insect cells (Clem et al., 1991; Crook et al., 1993). This subsequently led to the identification of p35 and iap. Later identification in both Drosophila and mammals revealed a group of related proteins which now constitute the IAP family (Eckelman et al., 2006; Hay et al., 1995; Liston et al., 1996). IAPs are classified on the basis of their baculoviral IAP repeat (BIR) domains, a highly homologous sequence of approximately 70 amino acids. Each member of the IAP family contains at least one BIR domain, with several members possessing three tandem BIR domains (Eckelman et al., 2006) (Figure 27). Presently, there are seven and eight members identified within the murine and human genomes, respectively. Although many IAPs function in the context of PCD regulation, IAPs have other identified roles in cell cyto-architecture and spindle formation. In terms of PCD-related functions, most work to date has focussed upon XIAP, cIAP-1 and cIAP-2.
Figure 27. Inhibitor of Apoptosis Proteins

The human and mouse genomes encode eight and seven distinct inhibitor of apoptosis proteins, respectively (mice lacks ILP2). The minimal structural commonality between these proteins is the baculovirus IAP repeat (BIR) domain. In addition to BIR domains, multiple IAPs also possess CARD, Really Interesting New Gene (RING), and ubiquitin-associated (UBA) domains while some have specific domains such as NACHT (domain found in NAIP, CIITA, HET-E, and TP-1), ubiquitin-conjugation (UBC), and leucine-rich repeats (LRR) domains. Though NAIP, cIAP-1, cIAP-2, and XIAP all possess some sequence conservation in the linker region at the amino terminal of their respective BIR2 domains believed to be crucial for inhibition of caspase-3 and caspase-7, only XIAP has been shown to directly inhibit the enzymatic activity of these executioner caspases. Conversely, caspase-9 inhibition has been primarily associated with BIR3 domains and has been demonstrated for cIAP-1, cIAP-2, XIAP, and BRUCE/Apollon. Evidence for the inhibition of caspase-9 and executioner caspases by other IAPs are lacking at present, and their role in PCD regulation, if any, is likely to be their effects on the expression of other IAP family members.
Adapted from
Eckelman et al., EMBO Reports, 2006
Several IAP family members also possess a RING (really interesting new gene) domain (Figure 27) (Eckelman et al., 2006). The RING domain confers E3 ubiquitin ligase activity (present on XIAP, cIAP-1, cIAP-2, livin and ILP2 in humans) (Vaux and Silke, 2005). These IAPs can thus act to recognize and ubiquitinate substrates, and in some cases can auto-ubiquitinate themselves. In addition, BRUCE/Apollon had been shown to possess E2 ubiquitin-conjugating activity via its ubiquitin conjugation (UBC) domain, making it a unique chimeric E2/E3 ubiquitin ligase among IAP family members (Bartke et al., 2004). Recently, an evolutionarily conserved ubiquitin-associated (UBA) domain was identified in XIAP, cIAP-1 and cIAP-2 (Blankenship et al., 2009; Gyrd-Hansen et al., 2008). These UBA domains can interact with lysine 63 (K63)-linked, and in some cases K48-linked, polyubiquitin chains. Whereas the exact function of the UBA is as yet unclear, with some studies finding it crucial for NFκB signaling (Gyrd-Hansen et al., 2008); while others suggest it plays a role in IAP recruitment for protein degradation (Blankenship et al., 2009).

In Drosophila, DIAP1 and DIAP2 bind and inactivate caspases such as Dronc, drICE and Dcp-1 through ubiquitin-mediated proteasomal degradation (Herman-Bachinsky et al., 2007; Lisi et al., 2000; Wilson et al., 2002). However recent evidence has also identified a non-degradative aspect of drICE ubiquitination by DIAP1 (Ditzel et al., 2008). Specifically, ubiquitination sterically disrupts substrate binding and reduces proteolytic activity. However, this non-degradative ubiquitination of executioner caspase regulation has yet to be reported in mammals.
Ubiquitination of IAPs is also used to regulate expression levels in both normal healthy and dying cells. The interaction between survivin and XIAP has been shown to promote XIAP stability (Dohi et al., 2004a; Dohi et al., 2004b; Dohi et al., 2007). This IAP-IAP interaction was observed to prevent the binding of ubiquitin-conjugating enzyme UbcH5 to XIAP, thereby preventing its ubiquitination and degradation. Similarly, cIAP-1 has been shown to regulate the expression levels of other IAP members (Cheung et al., 2008). TRAF2 was also recently shown to enhance the stability of cIAP-1, and to a lesser extent, cIAP-2 while having no effect on XIAP (Csomos et al., 2009a; Csomos et al., 2009b). As it was found that both cIAPs can perform auto-ubiquitination and thus promote their own degradation, it was suggested that their interactions with TRAF2 limited RING domain-dependent auto-ubiquitination and therefore resulted in stabilized cIAP levels (Csomos et al., 2009a).

1.2.5.5 OTHER FUNCTIONS OF INHIBITOR OF APOPTOSIS PROTEINS

Though termed “inhibitor of apoptosis proteins”, numerous non-PCD functions have now been described to various IAP members (O'Riordan et al., 2008; Srinivasula and Ashwell, 2008). These functions range from NFκB signaling to copper homeostasis. XIAP, the strongest endogenous caspase inhibitor identified to date, has been shown to interact with and regulate COMMD1/MURR1 (Burstein et al., 2004; Mufti et al., 2006) (Figure 28). COMMD1/MURR1 is a protein involved in copper homeostasis and is negatively regulated by XIAP through ubiquitin-mediated proteasomal degradation (Burstein et al., 2004). Excessive intracellular copper binds directly to XIAP, causing a reversible conformational change which renders XIAP more prone to degradation (Mufti
Non-PCD functions have been implicated for a growing number of IAP family members. The name “IAP” may in fact be a misnomer as PCD regulation is not a principal function for some IAP family members. Instead it is currently recognized that IAPs are proteins imparted with modular functions and thus can serve in a wide variety of signaling pathways. As illustrated in the upper panel of this figure, each domain in XIAP can participate in distinct set of interactions, thus linking XIAP to various functions (lower panel).
Adapted from
Galbán and Duckett, *Cell Death Differ*, 2010
et al., 2006). Consequently, the reduced levels of XIAP results in higher levels of
COMMD1/MURR1 expression in response to rising intracellular copper. The baculoviral
IAP, Op-IAP, undergoes a similar conformational changes upon binding to copper,
suggesting that copper binding may be an evolutionarily conserved property among IAP
members (Mufti et al., 2006).

cIAP-1 was demonstrated recently to promote tumorigenesis due to its
degradation of Mad1, a negative regulator of Myc (Xu et al., 2007). The oncogene Myc is
well-known for its promotion of both transformation and anchorage-independent cell
growth (Luscher and Larsson, 1999). Myc transcriptional activity is regulated by both
Max and Mad1 in that Myc/Mad1 heterodimers recruit repressor complexes to suppress
the transcription of Myc, actions opposed by Max (Grandori et al., 2000).

Survivin, another IAP member, has been shown to act as a crucial regulator of
mitosis. Along with proteins such as Aurora B, inner centromere protein (INCENP),
Dasra B/Borealin/Csc1 and Dasra A, survivin is involved in the formation of the
chromosomal passenger complex (CPC) (Ruchaud et al., 2007). This complex is critical
for correction of misaligned chromosomes, proper formation of the spindle and
completion of cytokinesis. The interactions between these proteins are also required for
proper localization to the kinetochores. These survivin-mediated functions are thought to
be evolutionarily conserved as similar activities have been seen in C. elegans and yeast
homologues of survivin (BIR-1 and Bir1p, respectively) (Fraser et al., 1999; Li et al.,
2000a; Uren et al., 1999). Survivin is localized to microtubules where it is believed to
regulate microtubule dynamics (Ruchaud et al., 2007). Both in vitro and in vivo findings
following genetic deletion of survivin support a role for it in stabilizing microtubules (Uren et al., 2000).

At present whether additional IAP members like BRUCE/Apollon, Livin and NAIP possess non-PCD functions remain to be examined.

1.2.5.6 ROLE OF IBM-CONTAINING PROTEINS

As indicated previously, Smac/DIABLO is an apoptogenic protein released during MOMP. Smac/DIABLO belongs to a group of evolutionarily conserved proteins sharing sequence homology in only a small region termed the IAP-binding motif (IBM) (Silke et al., 2000). These proteins include Smac/DIABLO and Omi/HtrA2 in mammals, Reaper, Head Involution Defective (HID), Grim, etc. in Drosophila (also termed RHG proteins) (Steller, 2008). The IAP-binding motifs of these proteins enable them to interact with IAP BIR domains (Figure 29A). Such interactions are believed to serve two principal functions: (1) suppress the IAP-mediated inhibition of caspases through competitive binding, thus releasing free caspases to promote cellular destruction (Figure 29D); and (2) promotion of IAP degradation by inducing auto-ubiquitination. Interestingly, RHG proteins in fly are primarily regulated via transcription (Brodsky et al., 2000; Robinow et al., 1997; White et al., 1994); whereas their mammalian counterparts are principally sequestered in the mitochondria until their release into the cytosol (Du et al., 2000; Suzuki et al., 2001a; Verhagen et al., 2000).
Figure 29. Suppression of IAPs by Smac/DIABLO and RHG Proteins

(A) Schematic representation of the Smac/DIABLO-XIAP BIR3 structure illustrating the interaction between the amino terminus of Smac and XIAP BIR3 domain. In addition, a second interaction site exists between helix H2 on Smac/DIABLO and helix α2 of a second XIAP BIR3 domain. The two distinct modes of interaction between Smac/DIABLO and XIAP BIR3 domain illustrate why monomeric mutants do not promote caspase activity as readily as the wild-type protein, as the activity of Smac/DIABLO depends heavily upon its dimeric state. Biophysical studies demonstrate that the Smac/DIABLO dimer is extremely stable (zeptomolar range – 10^{-21} M dissociation constant), and thus is unlikely to dissociate once the protein is expressed and dimerize (Goncalves et al., 2008). (B) Sequence alignment at the IAP-binding motif (IBM) located in Smac/DIABLO, Omi/HtrA2, RHG proteins, and cleaved caspase-9. The highly conserved tetrapeptide sequence is highlighted in conjunction with the exposed amino group following proteolytic cleavage of these proteins are critical for interactions with IAPs. (C) The mechanism of interaction between IAP and IBM-containing proteins is conserved from fruit flies to mammals. Clear structural similarities exist between the XIAP BIR3 domain and the BIR2 domain of DIAP1; and the Drosophila RHG proteins Hid and Grim compared with Smac/DIABLO (left panel). The principal site of interaction between Smac/DIABLO amino-terminal tetrapeptide and XIAP BIR3 domain (shown by electrostatic potential). The tetrapeptide binds to a groove on XIAP BIR3 domain, with the amino-terminal alanine extending multiple hydrogen bonds with the corresponding negatively charged surface on XIAP BIR3 domain (right panel). (D) Proposed mode of inhibition for XIAP on caspase-7. Two XIAP BIR2 domains are
modelled for interactions with a Smac/DIABLO (based on previous work on XIAP BIR3-Smac/DIABLO crystal structure). In this model, the binding of Smac/DIABLO to XIAP BIR2 domain would make the XIAP BIR2 domain unavailable for binding to caspase-7 as Smac/DIABLO and caspase-7 share an approximate interaction interface as shown in the figure.
Adapted from
Chai et al., Cell, 2001
Shi, Mol Cell, 2002
Wu et al., Nature 2000
1.2.5.7 PROPERTIES OF IBM-CONTAINING PROTEINS

As indicated above, IBM-containing proteins share sequence homology in the amino-termini of the matured proteins (Figure 29B). Generally composed of an exposed amino group and a AXPX sequence, these IAP-binding motifs are critical to the proper functioning of these proteins as mutational analyses have shown that alterations in these sequences result in a significant reduction in activity (Figure 29B-C) (Chai et al., 2000; Liu et al., 2000; Wu et al., 2000). Aside from the IBM tetrapeptide, homodimerization is also crucial for their activity as alterations in the dimer interface can diminish their abilities to interact with IAPs (Chai et al., 2000). In contrast to their *Drosophila* homologues which have been shown to be critically involved in PCD signaling *in vivo* as their ablations result in substantial reductions in PCD, the precise contributions to PCD signaling by Smac/DIABLO and Omi/HtrA2 *in vivo* have yet to be elucidated (Martins et al., 2004; Okada et al., 2002).

As nuclear-encoded proteins, Smac/DIABLO and Omi/HtrA2 are produced as precursors possessing mitochondrial targeting sequences which are cleaved following import. Cleavage to the mature form generates the amino-terminal IBM sequences (Du et al., 2000; Seong et al., 2004; Suzuki et al., 2001a; Verhagen et al., 2000). In homologous RHG proteins, the initial methionine is enzymatically removed to expose the IBM sequence.

RHG proteins function to suppress the inhibitory effects of DIAP1 and DIAP2 (Yoo et al., 2002), thereby promoting the activity of *Drosophila* caspases such as DRONC and drICE (Chai et al., 2003; Yan et al., 2004). A similar mechanism operates in mammals in which Smac/DIABLO and Omi/HtrA2 inhibit IAPs to enhanced caspase
activity (Du et al., 2000; van Loo et al., 2002; Verhagen et al., 2000). In addition to its IAP antagonistic properties, Omi/HtrA2 also possesses a serine protease activity which is thought to be the primary cause of the neurodegenerative effects observed in mice lacking Htra2 (Martins et al., 2004). Functional mapping has demonstrated that the mnd2 mutation is within the Htra2 locus, which exhibits muscle wasting, neurodegeneration, involution of the spleen and thymus and premature death (Jones et al., 2003b).

Omi/HtrA2 has also been shown to interact with proteins associated with PTEN-induced putative kinase 1 (PINK1) and amyloid precursor protein (APP) (Huttunen et al., 2007; Park et al., 2006; Plun-Favreau et al., 2007). Despite this, questions still remain regarding mechanisms of Omi/HtrA2 action. Given its evolutionary relationship with bacterial chaperonins DegP, DegS and DegQ (Vande Walle et al., 2008), it has been proposed that Omi/HtrA2 may function as a mitochondrial chaperonin to aid in the removal of misfolded proteins in the mitochondria (Huttunen et al., 2007; Liu et al., 2009; Radke et al., 2008).

Recent analysis of an alternatively spliced variant of Smac/DIABLO (Smac β) suggests that a second pro-apoptotic functional domain may be encoded on the carboxyl-terminus of Smac/DIABLO as overexpression of this (IBM lacking) transcript promotes PCD (Roberts et al., 2001). Although the precise mechanism of this novel pro-apoptotic activity has yet to be identified, it has been suggested that Smac/DIABLO may interact with the p75NTR-associated cell death executor (NADE) (Yoon et al., 2004). Coexpression of Smac/DIABLO and NADE were found to potentiate TRAIL-mediated cell death. Based upon XIAP co-immunoprecipitation and ubiquitination status in the
presence and absence of NADE, the study concluded that NADE disrupts Smac/DIABLO binding to XIAP, thereby preventing Smac/DIABLO degradation.

1.2.5.8 OTHER IAP ANTAGONISTS

Two additional proteins have been identified to date which function as IAP antagonists despite a lack of sequence homology to either Smac/DIABLO or Omi/HtrA2. XIAP associated factor 1 (XAF1) was identified on the basis of its specific interaction with XIAP and its ability to redistribute XIAP to the nucleus from the cytosol (Liston et al., 2001). While XAF1 is expressed ubiquitiously, it is dramatically downregulated in many cancer cell lines (Fong et al., 2000). Like Smac/DIABLO, overexpression of XAF1 enhances caspase activity (Arora et al., 2007). XAF1 does not possess any IBM and thus interacts with XIAP through some alternative mechanisms.

In addition to XAF1, an alternatively spliced variant of the *Sept4* gene was discovered to encode for a protein termed apoptosis-related protein in the TGF-beta signalling pathway (ARTS) which can interact with and inhibit XIAP to promote caspase activity (Gottfried et al., 2004). ARTS is normally located in the mitochondria and translocates to the nucleus during PCD (Larisch et al., 2000). Its interaction with XIAP promotes the ubiquitin-mediated proteasomal degradation of XIAP (Gottfried et al., 2004). Normally expression of ARTS is low in healthy cells due to proteasomal degradation. However during PCD, ARTS level rises to promote caspase activity (Lotan et al., 2005). Elevated levels of ARTS appear capable of reducing levels of Bcl-2 and Bcl-xL via a caspase-independent mechanism. This action facilitates the release of cytochrome c into the cell cytosol triggering caspase activation (Gottfried et al., 2004).
Although the precise mode of interaction between ARTS and XIAP is currently unknown, it has been demonstrated that a carboxyl-terminal deletion mutant of ARTS could not bind to XIAP (Larisch et al., 2000), in contrast to IAP antagonists like Smac/DIABLO and RHG proteins which require an amino-terminal alanine and IBM for XIAP binding. It will be interesting to determine whether other IAP members can be regulated by XAF1 and ARTS.

1.2.6 SUMMARY

The regulation of life and death has traditionally been focused upon “life” and the processes of cellular proliferation and differentiation. However beginning with the morphological and subsequent molecular description of programmed cell death we have come to appreciate that “death” too is also an actively regulated process. PCD dysregulation has now been implicated in numerous human diseases ranging from cancers to neurodegeneration. Thus one major goal of basic research in the past two decades has been a greater understanding of the regulation of PCD. Our current understanding of PCD is no longer bounded by the number of regulatory and effector proteins involved, but rather limited by our appreciation of how they might function together in complex protein-protein networks to control the crucial decision between the life and death of a cell. Extensive research in the past two decades has demonstrated the critical involvement of the mitochondria in regulating of mammalian PCD. Though we now possess a general understanding of how mitochondrial signaling contributes to PCD regulation, much is left to understand regarding how those proteins control distinct spatial and temporal regulatory networks. Executioner caspase activity is similarly regulated via
complex networks of protein-protein interactions heavily influenced by mitochondrial-derived signals. Hence, a greater in-depth understanding of these regulatory networks will give us insight into the critical regulatory mechanism of apoptosis. This is a necessary first step to developing novel means of therapeutic interventions into the various pathologic conditions in which PCD dysregulation has been implicated.
1.3 THESIS AIMS & HYPOTHESES

The thesis aims to address several key issues associated with the PCD regulatory checkpoint at the level of the mitochondria: (1) the *in vivo* role of Bcl-2 protein family interactions in developmental and injury-induced PCD in specific subpopulations of motor neurons; (2) the physiologic role of endogenous Smac/DIABLO in the regulation of executioner caspases. Specifically, the two principal thesis hypotheses are:

1. Bcl-2-mediated interactions differentially regulate the survival of distinct motor neuron subpopulations *in vivo* in developmental PCD and can be modulated via pharmacologic means to enhance neuronal survival following injury-induced PCD.

2. The *in vivo* physiologic regulation of executioner caspases by endogenous Smac/DIABLO is solely mediated via its inhibition of XIAP.
SECTION 2: MATERIALS AND METHODS
Experimental Animals

Experiments were performed and verified on two independent Bcl2 gene targeting events ("Bcl2DL" B13 and "Bcl2SK" Z3 – The Jackson Laboratory) (Nakayama et al., 1994; Veis et al., 1993). Mice were obtained from appropriate heterozygous intercrosses. In order to assess the potential contributions of genetic background on any effects observed in Bcl2 null mice, two sublines of the initial “Bcl2DL” (mixed 129Sv/Cp, C57BL/6J background) stock were established through back-crossing onto either 129Sv/Cp (four generations) or C57BL/6J (five generations) to generate sublines “Bcl2DL-129” and “Bcl2DL-C57”. The results described were obtained from comparative groups of wild-type, heterozygous or Bcl2 null mice did not vary significantly from either of the sublines or comparative Bcl2SK mice. The data shown represents data from Bcl2 homozygous or heterozygous littermates derived from C57BL/6J intercrosses (Bcl2DL-C57 mice).

Calcineurin A alpha (Ppp3ca) null mice and controls were generated from intercrosses of (Ppp3ca^+/^- Ppp3ca^-/-, or Ppp3ca^+/- x Ppp3ca^+/-) stock (Zhang et al., 1996). Results from Ppp3ca heterozygous mice were found to be equivalent to those of wild-type Ppp3ca^+/+, thus only Ppp3ca heterozygotes data are shown. For experiments involving drug treatment in vivo, wild-type mice (CD-1) generated from colony stocks were utilized. Results from CD-1 mice are shown with the exception of Ppp3ca mice which were produced and bred on a 129/SvImJ background. Analyses of facial motor neuron survival, with drug or vehicle treatments were performed in both 129/SvImJ and CD-1 backgrounds, and demonstrated comparable results.
Casp3 and Diablo null animals have been previously described and were generously donated by the laboratory of Tak Mak (Okada et al., 2002; Woo et al., 1998). The effects of deleting a single copy of Diablo from Casp3+/− and Casp3−/− animals were first examined by performing Casp3+/−, Diablo+/+ × Casp3−/−, Diablo+/− and Casp3+/−, Diablo+/+ × Casp3−/−, Diablo−/− intercrosses. Double knockout animals were generated from matings of Casp3+/−, Diablo−/− × Casp3−/−, Diablo−/− animals. All of the procedures were in accordance with the Canadian Council on Animal Care and approved by the University of Toronto Faculty Advisory Committee on Animal Services. Animal genotypes were confirmed by PCR using genomic tail DNA as described previously (Nakayama et al., 1994; Okada et al., 2002; Woo et al., 1998; Zhang et al., 1996). PCR primers and parameters used for genotyping for listed in Tables 1A and 1B, respectively.

Facial Nerve Axotomy

Postnatal day (PND) 3 or 8 pups were anesthetized by hypothermia and the left facial nerve exposed, freed of surrounding vascular and connective tissue and transected just distal to the stylomastoid foramen. A one-millimetre segment of the distal nerve was then removed to prevent re-innervation of the nerve stump. Animals were sutured with 6.0 silk and placed in a water-heated platform at 30°C for 20 minutes to allow recovery prior to return to their dams. Pups were sacrificed 4 or 7 days following axotomy (PND 7 or PND 10) and the brainstems removed for subsequent analysis.

Total motor neuron counts, counts of facial motor neurons expressing activated caspase-3 and immunofluorescent labeling of activated astrocytes and microglia were performed using tissues from distinct sets of experimental animals.
### Table 1A: PCR Genotyping Primers

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<td>Reverse: 5′-ACAGCCTGCAGCTTTGCATGACATC-3′</td>
</tr>
<tr>
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<tr>
<td></td>
<td>Reverse: 5′-CCTGCAGCCAATATGGCA-3′</td>
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<td></td>
<td>Reverse: 5′-CCATACATGGGAGCAAAGTCAG-3′</td>
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### Table 1B: PCR Parameters

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<td>60°C / 60 s</td>
<td>72°C / 90 s</td>
<td>30</td>
</tr>
<tr>
<td>Casp3</td>
<td>95°C / 30 s</td>
<td>60°C / 30 s</td>
<td>72°C / 30 s</td>
<td>35</td>
</tr>
<tr>
<td>Diablo</td>
<td>94°C / 60 s</td>
<td>60°C / 60 s</td>
<td>72°C / 60 s</td>
<td>35</td>
</tr>
<tr>
<td>Ppp3ca</td>
<td>94°C / 60 s</td>
<td>60°C / 60 s</td>
<td>72°C / 90 s</td>
<td>30</td>
</tr>
</tbody>
</table>
**Facial Motor Neuron Counts**

For facial motor neuron counts, mice were sacrificed by decapitation and the brains carefully removed. Brainstems were isolated and immersion-fixed in 4% paraformaldehyde (PFA) in 100 mM phosphate-buffered saline (PBS; 0.9% NaCl, pH 7.4) at 4°C overnight. Following fixation, tissue samples were processed for paraffin sectioning. Paraffin-embedded tissue samples were serially sectioned at a thickness of 7 µm through the full extent of the facial nucleus using a microtome (Leica Microsystems, Inc., 2125RT). Sections were stained with 0.1% thionin and motor neuron counts obtained for every 9th section. Facial motor neurons were counted if they contained a clear nucleus and nucleoli within the facial nucleus; counts were not corrected for split nucleoli. Motor neuron number was determined by the method of physical dissector using frame interval of sixty-three micrometres (Coggeshall and Lekan, 1996).

**Retrograde Nerve Tracing**

Postnatal day 30 animals were anesthetized by intraperitoneal injection of 2.5% Avertin (20 µl/g body weight). Following the removal of overlying hair, a surgical incision was made in the skin and underlying fascia, and the desired nerve isolated. Following isolation of the nerve from surrounding tissue, a 5% solution of Rhodamine Green dextran (Invitrogen Corp., D-7163) or Fast Dil (Invitrogen Corp., D-3899) was pressure injected into the nerve indicated (250 nl) over a period of 3 minutes. For sciatic nerves, the injection placement was three millimetres distal to the obdurator tendon. Following injection, incisions were closed with 6.0 sutures and animals were allowed to
recover for 48 hours, at which time they were sacrificed and the appropriate tissues processed.

*Spinal Cord Preparation and Analysis*

Animals were anesthetized with an overdose of 2.5% Avertin. Following an examination for a lack of deep tendon responsiveness, animals were perfused transcardially with 15 ml of PBS, followed immediately by 50 ml of freshly prepared 4% PFA in PBS at 4°C. A two-millimetre segment of the medial facial nerve (MFN) was dissected and prepared as indicated below, as were muscles of the upper (flexor carpi ulnaris) and lower limbs (soleus and medial gastrocnemius). Whole brains were removed and the brainstem isolated from each animal. The spinal column corresponding to segments T12 to L4 was exposed and isolated. Tissue samples were subsequently post-fixed in 4% PFA in PBS at 4°C overnight. At this point, the T12-L3 segment of the spinal cord, and the L4 dorsal and ventral roots were dissected out from each tissue sample. Spinal cords were processed for either frozen or paraffin wax sectioning according to standard procedures (Culling, 1974). Spinal roots and muscle were processed as indicated below. Following processing, each sample block was given a coded identification number so that data derived could be analyzed in a blinded manner.

For stereotactic analyses, the entirety of the T13-L2 segments of the lumbar spinal cord were processed as serial sets of 7 µm paraffin sections. Following de-waxing, sections were stained with 0.1% thionin, dehydrated and mounted according to standard procedures. For counts of spinal cord choline acetyl transferase (ChAT)-positive neurons and Nissl-stained series, the T13-L2 levels of spinal cords were isolated and cut as serial
sets of 30 µm frozen sections (300 µm interval, every 9th section collected). Alternate sections in each series were stained with 0.1% thionin to compare Nissl counts of motor neuron number to those obtained using ChAT staining. For Nissl and ChAT series, no significant systematic variation in spinal motor neuron counts was observed between Nissl and ChAT, hence only counts of ChAT-positive motor neurons are described herein. For all analyses numeric counts were not corrected for split nucleoli.

Thin Sections Preparation

Nerve segments and muscle spindle samples were post-fixed in a solution of freshly prepared 2.5% glutaraldehyde in PBS at 4°C for 4 hours; and rinsed free of glutaraldehyde and fixed in 1% osmium tetroxide buffered in PBS for 1 hour. Samples were dehydrated in a series of water/ethanol and ethanol/propylene oxide baths. Following removal of propylene oxide, samples were embedded in spurr resin and baked at 50°C for 36 hours. A series of one-micrometre thick cross-sections were obtained and stained with 1% toluidine blue according to standard procedures (Culling, 1974). For spinal roots, thin sections were obtained at the point midway between the attached DRG and the spinal end of the nerve.

Analyses of Axon/Nerve Morphometry

Morphometric analyses of nerve and muscle cross-sections were performed using a Leitz-Wetzlar microscope equipped with 25×, 54× and 100× objectives, a colour video camera (JVC Canada, Inc., TK-1280U) and a 360° rotating slide platform equipped with X and Y controllers. Axon and nerve areas were measured using an image processing and
analysis program (Leica Microsystems, Inc., Quantimet Q500MC). The system was calibrated before and verified following each use using a ten-micrometre ruled calibration slide. Prior to analyzing each cross-section, a low-resolution (25× objective) map was first generated and a hardcopy printed. This was used as a reference to place each of the individual nerve (analyzed at 100x) or muscle (analyzed at 40×) sectors in a given morphometric analysis. In each case data was gathered for the nerve cross-section in its entirety. Sample data were coded and analyzed in a double-blinded manner.

Drug Preparation and Application

Sterile cyclosporin A (Sandimmune) was purchased from Novartis Pharmaceuticals. FK-506 (Tacrolimus – Prograft) was obtained from Fujisawa Pharmaceutical Co., Ltd. Drugs were removed from sealed glass ampules and diluted in 0.9% sodium chloride immediately prior to use. Cypermethrin (C5557) and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG, A6880) were purchased from LC Laboratories and dissolved in 100% ethanol at an initial concentration of 15 mg/ml. Rapamycin (Sirolimus – Rapamune) was obtained from Wyeth Pharmaceuticals.

Cyclosporin A, 17-AAG and cypermethrin were diluted in a vehicle consisting of ethanol (final concentration 33%), PEG-60 (hydrogenated castor oil, 17%) diluted in PBS. Cyclosporin A (20 mg/kg), FK-506 (3 mg/kg), cypermethrin (10 mg/kg) and rapamycin (3 mg/kg) were administered once per day via subcutaneous injection to animals receiving facial nerve axotomy. 17-AAG was administered subcutaneously twice daily at 5 mg/kg for a total dose of 10 mg/kg per day. The dosages of various pharmacologic inhibitors utilized in the study were performed in accordance with doses previously
demonstrated to exert neural effects (Ehninger et al., 2008; Kociok et al., 2007; Patel et al., 2006; Tao and Aldskogius, 1998; Waza et al., 2005).

**Derivation and Cell Culture of Mouse Embryonic Fibroblasts**

Mouse embryonic fibroblasts (MEFs) of all experimental genotypes (Casp3<sup>+/−</sup>, Diablo<sup>+/−</sup>; Casp3<sup>−/−</sup>, Diablo<sup>+/−</sup>; Casp3<sup>+/−</sup>, Diablo<sup>−/−</sup>; Casp3<sup>−/−</sup>, Diablo<sup>−/−</sup>) were derived from embryonic day 13.5 (E13.5) embryos generated from Casp3<sup>+/−</sup>, Diablo<sup>−/−</sup> × Casp3<sup>−/−</sup>, Diablo<sup>+/−</sup> breedings according to standard procedures (Serrano et al., 1997). Multiple independently derived MEF lines were generated for each experimental genotype and passage numbers for experiment were limited to ≤ 6. MEFs were maintained at 37°C in 6% CO₂ and cultured in Dulbecco's Modified Eagle Medium (DMEM, 25 mM HEPES) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corp., 12483020), 2 mM glutamine and 1% antibiotics (penicillin and streptomycin) (Invitrogen Corp., 10378016).

**Transfection of Small Interfering RNA**

Small interfering RNA (siRNA) for caspase-6 (MQ-063186), caspase-7 (MQ-057362), cIAP-1 (MQ-041990), cIAP-2 (MQ-062425), Naip1 (MU-047682), survivin (MQ-043690) and XIAP (MQ-040021) used were purchased from Dharmacon, Inc. Scrambled control siRNA was synthesized by Sigma-Genosys Canada, Ltd. All siRNA sequences used in knockdown experiments are described in Table 2A. Cells were cultured on 6-, 24- and 96-well microplates and transfected at a confluency of 70-75% with a concentration of 50 nM individual or pooled siRNAs using transfection reagent.
<table>
<thead>
<tr>
<th>Genes</th>
<th>siRNA Sequences</th>
</tr>
</thead>
</table>
| Birc2 (cIAP-1) | 5′-GAUAUUGUCUCAGUACUUU-3′  
5′-GAAUGGCCUCUUUCAACA-3′  
5′-GGAGUUUGUGAUGAGAUU-3′  
5′-GCAAGAGAGCUAAUUGACA-3′ |
| Birc3 (cIAP-2) | 5′-GGAAAUUGACCCUCGUGUA-3′  
5′-GAACCGAGCCUUAUGCC-3′  
5′-CGAAGAGGAGCAUAGAAA-3′  
5′-GAAUGCCGGAGCUGCCU-3′ |
| Birc5 (survivin) | 5′-GAACUAACCGUCAGUGAAU-3′  
5′-GACGAGCGCGUAGAUGG-3′  
5′-CGAAGAGCUUGGCUUGUC-3′  
5′-GAAUGCCUUAAACGACCUC-3′ |
| Casp6 (caspase-6) | 5′-GCAACCACGCUUUACGCAUA-3′  
5′-CCACAUAGAUUGCCGAUUG-3′  
5′-GACGUGACUGGCUUGUUC-3′  
5′-GAAUGCCUUAAACGACCUC-3′ |
| Casp7 (caspase-7) | 5′-CCACUUAUCUGUACCAGCAUA-3′  
5′-GGGUAAUUGCAUCAUAUA-3′  
5′-CGACAAAGCGACAGGUUAUG-3′  
5′-CAACGCAUUGCACCCUUA-3′ |
| Naip1 (Naip1) | 5′-GCUGUUAUCUCUGUGAUAUU-3′  
5′-GUUCAUAGCUGUCUAUAUA-3′  
5′-GAGUUAGAUGCUUCGAUA-3′  
5′-GAGACCAUAAGAGAUAUA-3′ |
| Xiap (XIAP) | 5′-GCAAGAGACUACAGCGAAGG-3′  
5′-GAGACACUUUCGCAAUG-3′  
5′-CAACAAACUCUCCAGA-3′  
5′-GGGCAAGACGAGCGGGAAG-3′ |

Table 2B: siRNA Transfection Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (96-well)</th>
<th>Volume (24-well)</th>
<th>Volume (6-well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA (20 µM)</td>
<td>0.25 µl</td>
<td>1.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>serum-free cell culture media</td>
<td>0.25 µl</td>
<td>1.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>DharmaFECT 1</td>
<td>0.3 µl</td>
<td>2 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>serum-free cell culture media</td>
<td>9.7 µl</td>
<td>48 µl</td>
<td>197 µl</td>
</tr>
<tr>
<td>antibiotics-free cell culture media</td>
<td>89.5 µl</td>
<td>447.5 µl</td>
<td>795 µl</td>
</tr>
</tbody>
</table>
DharmaFECT 1 (Dharmacon, Inc., T-2001) according to the manufacturer’s protocol. Briefly, siRNA and DharmaFECT 1 were diluted separately in serum-free cell culture media and incubated at room temperature for 5 minutes prior to mixing. Mixtures of siRNA and DharmaFECT 1 were incubated at room temperature for 30 minutes, prior to addition to antibiotic-free cell culture media and applied to cells. Specific volumes of each reagent are listed in Table 2B. siRNA-containing media was exchanged for fresh cell culture media at 24 hours, and all experiments were initiated 72 hours after siRNA treatment. Knockdown efficiencies were analysed using either immunoblotting or quantitative PCR.

*Immunohistochemical and Immunofluorescence Analyses*

Following fixation samples were processed as either frozen or paraffin sections. During processing, each sample was given a coded identification number so that the sections derived from each block could be analyzed in a blinded manner. Paraffin-embedded tissue samples were sectioned at 7 µm. For frozen sections, tissue samples were first cryoprotected in 30% sucrose in PBS at 4°C overnight, then frozen in OCT compound (Electron Microscopy Sciences, 62550). Frozen sections were obtained at a thickness of 30 µm at –22°C using a cryostat from Leica Microsystems, Inc. (CM 3050s). For serial sets of sections, frozen sections were collected at intervals of either 120 µm (facial motor neurons) or 300 µm (spinal motor neurons).

Tissue sections used for horse radish peroxidase (HRP) immunohistochemistry were first placed in a solution of 3% H₂O₂/methanol for 30 minutes at room temperature, followed by washing in PBS (3 × 5 minutes) to destroy endogenous peroxidase activity.
For both immunohistochemistry and immunofluorescence analyses, non-specific antibody binding was blocked through pre-incubation in 5% goat serum, 0.3% Tween-20 in PBS (blocking solution) at 4°C for 30 minutes. Following pre-incubation, primary antisera were diluted in blocking solution and incubated at 4°C for 16 hours. Primary antisera used are listed in Table 3. Following incubation and washing with blocking buffer (3 × 5 minutes), tissue sections were incubated with fluorescent or biotinylated secondary antibody (anti-goat IgG – 1:200, Vector Laboratories, Inc., BA-9500; anti-rabbit IgG – 1:200, Vector Laboratories, Inc., BA-1400) for 2 hours. Biotinylated reagents were further incubated with HRP-conjugated streptavidin (1:100, Vector Laboratories, Inc., PK-6100) for 1 hour and visualized using 3,3-diaminobenzidine (DAB; Vector Laboratories, Inc., SK-4100) or Texas Red Avidin D (Vector Laboratories, Inc., A-2006). Immunofluorescent secondary antibodies utilized were anti-rabbit IgG AlexaFluor-488 (1:200, Invitrogen Corp., A11008), anti-rabbit AlexaFluor-546 (1:200, Invitrogen Corp., A11010) and anti-mouse AlexaFlour-594 (1:200, Invitrogen Corp., A11032).

**Analysis of Bad Phosphorylation Status**

Levels of Bad serine-112 phosphorylation were determined by immunofluorescence using a phosphorylated Bad S112 antibody. Signal distribution were determined for individual facial motor neurons (≥100 per facial nuclei examined) for both injured and uninjured facial nuclei for each treatment group (vehicle, cypermethin, FK-506). A minimum of two independent replicates of each experimental series was performed for every group. For imaging, image collection parameters were held constant.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
<th>Species</th>
<th>Dilution / Concentration</th>
<th>Epitope specificity</th>
<th>Application(s) Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-AIF</td>
<td>Millipore Corp. (AB16501)</td>
<td>rabbit</td>
<td>1:200</td>
<td>apoptosis inducing factor</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-β-actin</td>
<td>Sigma-Aldrich Co. (A3854)</td>
<td>mouse</td>
<td>1:100,000</td>
<td>beta-actin</td>
<td>IB</td>
</tr>
<tr>
<td>Anti-phosphorylated Bad S112</td>
<td>Cell Signaling Technology, Inc. (5284)</td>
<td>rabbit</td>
<td>1:200</td>
<td>Bad, phosphorylated serine 112</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-Bcl-2</td>
<td>DAKO Canada Inc. (M0887)</td>
<td>mouse</td>
<td>1:20</td>
<td>Bcl-2</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-activated caspase-3</td>
<td>Cell Signaling Technology, Inc. (9661)</td>
<td>rabbit</td>
<td>1:300 (IB) 1:200 (IHC)</td>
<td>exposed epitope of caspase-3 upon proteolytic activation (Asp175), p20 subunit</td>
<td>IB, IHC</td>
</tr>
<tr>
<td>Anti-activated caspase-7</td>
<td>Cell Signaling Technology, Inc. (9491)</td>
<td>rabbit</td>
<td>1:300</td>
<td>exposed epitope of caspase-7 upon proteolytic activation (Asp198), p20 subunit</td>
<td>IB</td>
</tr>
<tr>
<td>Anti-caspase-3</td>
<td>BD Transduction Laboratories (610322)</td>
<td>mouse</td>
<td>1:250</td>
<td>caspase-3</td>
<td>IB</td>
</tr>
<tr>
<td>Anti-caspase-6</td>
<td>Cell Signaling Technology, Inc. (9762)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>caspase-6</td>
<td>IB</td>
</tr>
<tr>
<td>Anti-caspase-7</td>
<td>Assay Designs, Inc. (AAM-127)</td>
<td>mouse</td>
<td>1:300</td>
<td>caspase-7</td>
<td>IB</td>
</tr>
<tr>
<td>Anti-ChAT</td>
<td>Millipore Corp. (AB144)</td>
<td>goat</td>
<td>1:200</td>
<td>choline acetyltransferase</td>
<td>IHC</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>BD Transduction Laboratories (610198)</td>
<td>mouse</td>
<td>1 µg/ml</td>
<td>Fas (CD95)</td>
<td>Fas activation</td>
</tr>
<tr>
<td>Antibody</td>
<td>Vendor</td>
<td>Species</td>
<td>Dilution</td>
<td>Target</td>
<td>Staining</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>---------------------------------------------</td>
<td>---------</td>
<td>----------</td>
<td>-----------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>DAKO Canada Inc. (Z0334)</td>
<td>rabbit</td>
<td>1:800</td>
<td>glial filbrillary acidic protein</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-Islet-1/2</td>
<td>Jessell Laboratory (K5)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>islet-1/2</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-PARP</td>
<td>Cell Signaling Technology, Inc. (9532)</td>
<td>rabbit</td>
<td>1:500</td>
<td>poly (ADP-ribose) polymerase</td>
<td>IB</td>
</tr>
<tr>
<td>Tomato lectin (Lycopersicon esculentum agglutinin)</td>
<td>Sigma-Aldrich Co. (L0651) (biotinylated)</td>
<td>N/A</td>
<td>2.5 µg/ml</td>
<td>N-acetyl-β-D-glucosamine oligomers</td>
<td>IF</td>
</tr>
</tbody>
</table>
Inclusion parameters used for motor neuron imaging were identical to those utilized to perform stereoscopic counts. Collected images were analyzed in terms of 256 gray levels using ImageJ. Average Bad S112 signal intensities within the non-nuclear component of each motor neuron. For each injured/uninjured facial nucleus pair, signal intensity was normalized as a function of background signal. Treatment with secondary antibody alone resulted in no detectable signal for each group, and correlation analyses of motor neurons area among the different treatment groups showed no systematic differences. Results are presented for injured facial motor neurons as the percentage relative to that seen in uninjured facial motor neurons.

Cell Viability Analysis

MEF lines from each of the experimental genotypes were examined for their resistance to cell death induced by either staurosporine (LC Laboratories, S9300), tunicamycin (Sigma-Aldrich Co., T7765), Fas-activating antibody (Jo2, BD Pharmingen, 554254), or UVC irradiation. Cell viability was measured by trypan blue exclusion assay (Invitrogen Corp., 15250061). Briefly, 2.5×10^4 cells were plated onto each well of a 24-well plate and subjected to the treatments indicated. At specified time points cells were lifted using 0.25% trypsin in saline/EDTA and combined with the original cell culture supernatant for that well (containing potential floating dead cells) followed by centrifugation to retrieve the cell pellet. Cell pellets were resuspended in cell culture media and a sample was diluted 1:1 with 0.4% trypan blue. Cell counts were performed using a hemocytometer. Trypan blue-stained cells were counted as dead cells while those unstained by this treatment were considered viable. Cell viability was calculated as the
percentage of live cells divided by total number of cells observed. Treatment with other small molecule inhibitors were performed using embelin (Sigma-Aldrich Co., E1406) and necrostatin-1 (Sigma-Aldrich Co., N9037).

**AIF Nuclear Translocation Analysis**

The subcellular localization of AIF was examined by immunofluorescence using standard protocols. Briefly, 2.5×10⁴ cells were plated onto poly-L-lysine (Sigma-Aldrich Co., P1399) coated glass coverslips placed into wells of a 24-well plate. Cells were then treated with UVC irradiation (20 J/m²) and incubated for the indicated times prior to analysis. Cells were then fixed with 4% PFA for 16 hours. Following fixation, cells were incubated in blocking buffer (5% goat serum and 0.3% Tween-20 in PBS) for 30 minutes prior to the addition of primary AIF antisera (1:200, Millipore Corp., AB16501) in blocking buffer. Cells were incubated with at 4°C for 16 hours and washed with blocking buffer (3 × 5 minutes) before the addition of fluorescent secondary antibody (AlexaFluor-488, 1:200, Invitrogen Corp., A11008). Following two hours of incubation, cells were stained with Hoechst-33258 (2 µg/ml, Sigma-Aldrich Co., B2883) for 5 minutes and washed with PBS (3 × 5 minutes) prior to analysis. Counts were conducted to determine what percentage of cells showed significant AIF immunofluorescence in the nuclear compartment. Experiments were performed using three independently derived MEF lines for each genotype and repeated in triplicates, with at least 100 cells counted for each replicate.
**Immunoblot analysis**

For immunoblot analyses, 3.5×10^5 cells were plated in each well of a 6-well plate and subjected to the indicated treatment. At the specified time points, cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton-X, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% mammalian protease inhibitor cocktail (Sigma-Aldrich Co., P8340) at 37°C for 1 hour. Cell lysates were then centrifuged, and the supernatants collected and stored at 4°C. Protein concentrations of cell lysates were determined using bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Inc., 23227) with a bovine serum albumin standard. Assays were performed on 96-well plates with the detector set at 562 nm (Molecular Devices, Inc., SpectraMaxPlus 384). Forty micrograms of total protein were then separated by SDS-PAGE using 12% polyacrylamide gels (BioShop Canada, Inc., ACR005), transferred to nitrocellulose membrane (Pall Corp., 66485) by using a wet blotting apparatus. Following protein transfer, individual proteins were detected by immunoblotting using the primary antibodies listed in Table 2. Primary antibodies were diluted in 50 mM Tris-buffered saline, Tween 20 (TBS-T; 150 mM NaCl, 0.2% Tween 20, pH 8.0) containing 5% casein or bovine serum albumin. Following incubation with primary antisera and washing with TBS-T (6 × 3 minutes), membranes were incubated with secondary antisera diluted in TBS-T with 5% casein for 2 hours. Secondary antibodies were purchased from Bio-Rad Laboratories, Inc. and included anti-rabbit HRP conjugate (1:6000, 170-5046) and anti-mouse HRP-conjugate (1:3000, 170-6461). Protein-antibody complexes were visualized by SuperSignal WestPico enhanced chemiluminescence substrate (Thermo Fisher
Scientific, Inc., 34080) using film (Intersciences, Inc., CLMR810). Band densitometry were quantified using an imaging system (FluorChem, Alpha Innotech Corp.).

Analysis of Executioner Caspase Activity

Executioner caspase (DEVDase) activity was measured using SensoLyte Homogeneous Rh110 Caspase-3/7 Assay Kit (Anaspec, Inc., 71114) according to the manufacturer’s protocol. Briefly, 1.0×10^4 cells were plated into each well of a 96-well plate and subjected to the indicated treatments. At the specified time points, 33 µl of a freshly solution containing fluorogenic caspase substrate (DEVD-Rho110) was added to each well (containing 100 µl cell culture media) and incubated at room temperature for 18 hours. Following this period fluorescent measurements were recorded using a fluorescence microplate reader (excitation wavelength = 496 nm, emission wavelength = 520 nm; Molecular Devices, Inc., SpectraMax M2).

Quantitative PCR

Quantitative PCR was performed to determine relative knockdown efficiencies achieved for specific siRNA. Total cellular RNA was first isolated using standard methods. For these experiments, 3.5×10^5 cells were plated in each well of a 6-well plate and subjected to the siRNA treatments described. Following 72 hours of siRNA treatment, 1 ml of TRI-reagent (BioShop Canada, Inc., TRI118) was added to each well following aspiration of cell culture media. Following addition of the TRI-reagent, cells were incubated at room temperature for 5 minutes with shaking in order to detach cells. This solution was then collected and 200 µl of chloroform was added for a 5-minute
incubation. Samples were then centrifugated to separate aqueous and organic phases. The aqueous phase was transferred to 500 µl of ice-cold isopropanol and stored at -20°C overnight to facilitate RNA precipitation. RNA pellets were collected by centrifugation at 16,100 × g for 30 minutes and washed with RNase-free 70% ethanol before resuspension in 20 µl of DEPC-treated water. RNA concentrations were determined using by spectrophotometry (Thermo Fisher Scientific, Inc., NanoDrop 2000).

cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., 4368814) according to the manufacturer’s protocol. A 2 µg aliquot of each RNA sample was treated with 2 units of DNase I (Fermentas Canada, Inc., EN0521) in a 20 µl reaction at 37°C for 30 minutes prior to cDNA synthesis. Following enzyme inactivation at 75°C for 10 minutes, cDNA synthesis was performed by adding the appropriate volumes of reaction buffer, deoxynucleotides, random hexamer primers and reverse transcriptase, and incubating at 25°C for 10 minutes, 42°C for 50 minutes and 72°C for 10 minutes. Following cDNA synthesis, reaction volumes were diluted through addition of 160 µl of DEPC-treated water and stored at -20°C until use.

Quantitative PCR was performed using SYBR Green dye (Applied Biosystems, Inc., 4309155) in conjunction with real-time PCR (Applied Biosystems, Inc., 7900HT) using 384-well plates. Primer validation was performed on all primers prior to utilization in experiments with 25, 5, 1, 0.2 0.04 and 0.008 ng of prepared universal mouse cDNA (Clontech Laboratories, Inc., 636657). Quantitative PCR was performed using 25 ng of sample total cDNA and 150 nM of each primer in a 10-µl reaction volume. The qPCR standard utilized was cyclophilin B. All qPCR reactions were conducted in triplicates.
Parameters for qPCR are as follows: 50°C × 2 minutes, 95°C × 10 minutes, 40 cycles of 95°C × 15 seconds and 60°C × 1 minute, 95°C × 15 seconds, 60°C × 15 seconds and 95°C × 15 seconds. Primers used for quantitative PCR are listed in Table 4. Quantitative PCD data were analyzed via the delta-delta Ct method.

**Statistical methods**

Statistical analyses were performed using Microsoft Excel 2008. Assessment of differences between means was determined by unpaired, two-tailed parametric Student’s t test with assumption of unequal variance. Null hypotheses were rejected at the 0.05 level. Results are expressed as mean ± S.E.M. and determined to be significant if $p < 0.05$.

**Image Acquisition and manipulation**

Fluorescent and bright field images were captured using a motorized microscope (Nikon Corp., Eclipse E-1000) equipped for epifluorescence with 1×, 4×, 10×, 20×, 40× and 100× lens. Images were captured using a Hamamatsu ORCA-285 camera (fluorescent imaging) or a Nikon DS-Fi1 camera (bright field imaging). Image capture software was Simple PCI (Compix, Inc., Imaging Systems) and NIS Elements F (Nikon Corp.), respectively. Images were captured and exported as TIFF files and figures created using Adobe Photoshop 11 and Illustrator 14. No manipulations other than contrast and brightness adjustments were performed on captured images.
Table 4: Quantitative PCR Reagents

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birc2</strong></td>
<td>Forward: 5’-AGAACACGCAATGGTTTC-3’</td>
</tr>
<tr>
<td>(cIAP-1)</td>
<td>Reverse: 5’-CTCCTGACCCTCATCCGTA-3’</td>
</tr>
<tr>
<td><strong>Birc3</strong></td>
<td>Forward: 5’-TGGCGTTCAAGCCTAGGAA-3’</td>
</tr>
<tr>
<td>(cIAP-2)</td>
<td>Reverse: 5’-CATGGCTTCTGGTCGGTTT-3’</td>
</tr>
<tr>
<td><strong>Naip1</strong></td>
<td>Forward: 5’-ACGACAGCGTCTTCGCTAAT-3’</td>
</tr>
<tr>
<td>(Naip1)</td>
<td>Reverse: 5’-CAAAAGCATAAACAGCCACAG-3’</td>
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<tr>
<td><strong>Ppib</strong></td>
<td>Forward: 5’-GGAGATGGCAAGGAGGAA-3’</td>
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<td>(cyclophilin B)</td>
<td>Reverse: 5’-GCCGTAGTGCTTAGCTTT-3’</td>
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<td><strong>Xiap</strong></td>
<td>Forward: 5’-TAGTGGGCACACATGTGA-3’</td>
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<td>(XIAP)</td>
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SECTION 3: STUDIES
STUDY 1: DIFFERENTIAL SENSITIVITY OF SKELETAL AND FUSIMOTOR NEURONS TO BCL2 MEDIATED APOPTOSIS DURING NEUROMUSCULAR DEVELOPMENT

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3.1.1 ABSTRACT

Proper development of the nervous system requires that a carefully controlled balance be maintained between both proliferation and survival. The process of programmed cell death is believed to play a key role in regulating ultimate levels of neuronal survival, in large part through the action of anti-apoptotic proteins such as Bcl-2. Consistent with this Bcl-2 has been shown to be a critical regulator of apoptotic signaling in post-mitotic neurons. However we still know remarkably little regarding the differential role which Bcl-2 plays in regulating the survival of motor neuron subpopulations. In the present study I have examined the developmental PCD of somatic motor neurons of the lumbar spinal cord together with branchiomotor neurons of the facial nucleus in Bcl2 null mice. Examination of neuronal and axon number, axonal area and the distribution of axonal loss in Bcl2 null mice demonstrates that, in contrast to the great majority of alpha motor neurons, gamma motor neurons exhibit a unique dependence upon Bcl-2 for survival. These results demonstrate for the first time the connection between Bcl-2 expression, motor neuron survival and the establishment of different motor populations.
3.1.2 INTRODUCTION

While much information regarding the regulatory interactions between Bcl-2 family proteins involved in PCD signalling has been derived from in vitro cell-free or cellular studies, much remains unknown about the role of specific Bcl-2 family proteins possess with respect to specific cell populations in vivo during development. This is especially of interest within the central nervous system, as more post-mitotic neurons are produced during development than are ultimately incorporated into the mature synaptic network (Oppenheim, 1991). In the motor system, this is believed to in part reflect competition among developing neurons for limited levels of neurotrophic support (Greenlund et al., 1995; Oppenheim, 1991). Neurons which fail to achieve optimal innervation are in turn eliminated during the latter embryonic and early postnatal periods (Oppenheim, 1991). Numerous studies have demonstrated the critical role which programmed cell death (PCD) plays in this process (Akhtar et al., 2004). Previous knockout and overexpression studies in vivo have identified Bcl-2 as a key PCD regulator within both developing and postnatal neurons in mammals (Akhtar et al., 2004).

During development, Bcl-2 and Bcl-xL in particular are widely expressed within the central nervous system both during and following neurogenesis (Abe-Dohmae et al., 1993; Gonzalez-Garcia et al., 1995; Merry et al., 1994). Consistent with this, both proteins are thought to play a role in regulating naturally-occurring cell death (NOCD). The expression pattern of these anti-apoptotic proteins differ however, with respect to latter development. Bcl-xL continues to be widely expressed in many regions of the postnatal CNS, whereas Bcl-2 expression declines in many CNS regions postnatally (Abe-Dohmae et al., 1993; Krajewska et al., 2002; Merry et al., 1994; Yachnis et al.,
1998). However, some neuronal populations do maintain Bcl-2 expression postnatally. These include neurons of the sympathetic and sensory ganglia (Merry et al., 1994; Michaelidis et al., 1996; Pinon et al., 1997), granule cells of the cerebellum and dentate gyrus (Abe-Dohmae et al., 1993), branchiomotor neurons (Michaelidis et al., 1996) and motor and interneurons of the spinal cord (Martinou et al., 1994; Merry et al., 1994; Michaelidis et al., 1996; Yachnis et al., 1998). With respect to motor neurons, a previous study has suggested that differential sensitivity to Bcl-2 inhibition may exist within branchiomotor neurons of the facial nucleus (Michaelidis et al., 1996).

Several independent lines of Bcl2 null mice have been generated previously, eliminating either the Bcl2 α (Veis et al., 1993), or Bcl2 α/β (Michaelidis et al., 1996; Nakayama et al., 1994) transcripts. Each of these lines shows essentially identical phenotypes. Bcl2 null mice have been shown to exhibit a reduced growth rate, reduced lifespan (largely due to polycystic kidneys), reduced T-cell lifespan, craniofacial anomalies and hair graying during the second follicle cycle (Michaelidis et al., 1996; Nakayama et al., 1994; Veis et al., 1993). While previous knockout studies involving downstream PCD regulators have demonstrated that the elimination of these genes (Casp3, Casp9, Apaf1) in post-mitotic neurons can result in CNS developmental abnormalities and lethality, no gross developmental anomalies have been described for Bcl2 null animals. Nonetheless, Bcl2 null mice have been shown to exhibit some developmental loss of sensory, sympathetic and branchiomotor neurons during the early postnatal period (Michaelidis et al., 1996). Similarly, postnatal loss of retinal ganglion cells (RGCs) has been observed in Bcl2 null mice between PND 10 and PND 15, beyond the period of NOCD (Cellerino et al., 1999). In vitro analyses of sympathetic and
trigeminal neurons have suggested a role for Bcl-2 in NOCD (Ohga et al., 1996; Pinon et al., 1997). With respect to effects on injury-related survival, *in vivo* transection of both the optic (Dietz et al., 2001) and facial (Michaelidis et al., 1996) nerves show no difference in survival in *Bcl2* null mice versus controls. Similarly, *Bcl2* null mice exhibit no difference compared to controls in either neurotrophin-mediated survival *in vitro* (Ohga et al., 1996), or following acute injury (Michaelidis et al., 1996).

In order to determine the effects of *Bcl2* ablation on various motor neuron populations *in vivo*, I have examined the properties of lumbar motor neurons of the spinal cord with branchiomotor neurons of the facial nucleus in several strains of *Bcl2* null mice. The results demonstrate for the first time that Bcl-2 plays a unique and critical role in the survival of gamma motor neurons generated during the latter part of neurogenesis.
3.1.3 RESULTS

3.1.3.1 Bcl-2 Differentially Regulates the Survival of Spinal Motor Neurons

In order to determine the effect of Bcl-2 deficiency on lumbar spinal motor neurons, total counts of motor neurons were performed on ChAT and Nissl-stained serial sections. To first determine those spinal levels which contribute motor axons to the sciatic nerve in *Bcl2* null mice and controls, retrograde tracing of sciatic nerves was performed for each genotype. As shown in Figure 30A-B, *Bcl2* null mice exhibit a comparable distribution of lumbar spinal motor neurons with respect to both rostral-caudal and intra-laminar distribution compared to control littermates. As shown in Figure 30C, comparisons of ChAT-positive motor neurons from sciatic levels T13-L2 demonstrates that *Bcl2* null mice exhibit a significant reduction (26±3%) in total motor neuron number at postnatal day 30 compared to control littermates. Counts of spinal motor neuron number performed on additional (alternate) Nissl-stained sections demonstrated comparable levels of motor neuron loss, indicating that *Bcl2* ablation does not affect ChAT expression. These results demonstrate that Bcl-2 deficiency results in the loss of a subpopulation of sciatic motor neurons.

To examine the pattern of Bcl-2 expression within spinal motor neurons, spinal cord sections through the sciatic motor pool were stained for Bcl-2 in the early postnatal period. As shown in Figure 31, a minor population of motor neurons exhibited persistent expression of Bcl-2 postnatally. Stereotactic analysis of wild-type mice within sciatic spinal levels indicated that 32±8% of motor neurons within this region expressed Bcl-2 postnatally.
Figure 30. Properties of Sciatic Motor Pool in Bcl2 Null Mice

Distribution of motor neurons comprising the sciatic pool was examined in Bcl2 null mice and controls. (A) Summary of rostral-caudal distribution of labelled motor neurons from sciatic nerve with respect to spinal level in Bcl2 wild-type, heterozygous and null mice (n = 5 animals per genotype). Spinal segments are indicated for reference. (B) Distribution of sciatic motor neurons in Bcl2 null mice. Left panel shows a typical pattern of motor neuron labelling observed 48 hours following the application of rhodamine dextran to sciatic nerve stumps transected 3 mm distal to the obdurator tendon in Bcl2-/- mice (spinal level = L1). Right panel shows a thionin-stained section at similar level highlighting Nissl-stained neurons of the sciatic motor pool. (C) Histogram of choline acetyl transferase-positive neurons with spinal levels contributing to sciatic motor pools (T13-L2) in Bcl2+/− and Bcl2−/− mice.
**Figure 31. Persistent Expression of Bcl-2 in a Subpopulation of Spinal Motor Neurons**

Pattern of immunofluorescent staining for Bcl-2 (red) and Islet-1/2 (green) was examined in wild-type mice at postnatal day 1. Islet-1/2 staining was used as an independent marker to delineate motor neurons within lamina IX and X at postnatal day 1. (A) Pattern of Bcl-2 staining, (B) Islet-1/2 staining, (C) Bcl-2 and Islet-1/2 overlay. Insets show enlargement of region indicated, showing double-labeled motor neurons expressing Bcl-2. Scale bar represents a distance of 50 µm. Note that only a sub-population of lumbar motor neurons expresses Bcl-2 at this stage.
To better understand the nature of the motor neuron loss seen in Bcl2 null mice, one-micrometre thick thin sections were cut through the L4 ventral roots of Bcl2+/+, Bcl2+/− and Bcl2−/− mice at postnatal day 30. As shown in Figure 32A-B, within the L4 ventral root, total axon counts demonstrated that Bcl2 null mice exhibited a small but significant reduction in axon number compared to littermate controls, despite no significant reduction in total nerve area. Interestingly, the percent reduction in axon number (24±2%) seen in Bcl2 null mice was similar to that observed for spinal motor neurons expressing Bcl-2 postnatally.

To examine this relationship in greater detail, the distribution of axon cross-sectional areas were determined in whole cross-sections of L4 ventral roots. Individual axon cross-sectional areas were determined rather than the standard practice of measuring axon diameter, due to its inherently greater reliability. As shown in Figure 32C-D, Bcl2 null mice exhibit a preferential loss of small caliber axons (≤3 μm²). The percent loss of small caliber axons was 61±2% compared to a 12±3% reduction in larger (>3 μm²) caliber axons in Bcl2 null mice. Analysis of L4 ventral roots, as shown in Figure 32E-F, demonstrated that Bcl2+/− mice exhibit a dramatic reduction in small caliber axons compared to heterozygous Bcl2 littermates. The extent of this axonal reduction (24±2%) strongly correlated with the magnitude of the reduction in ChAT-positive neurons (26±3%) seen in sciatic spinal levels and the population of motor neurons expressing Bcl-2 postnatally (32±8%). These data indicate that the loss of motor axons seen in Bcl2 null mice occur as a consequence of motor neuron death.

Previous studies have suggested that the neuronal loss resulting from the ablation of Bcl2 occurs during the late embryonic/early postnatal periods, with neuronal
Figure 32. Analysis of Motor Axon Populations in Bcl2 Null Mice

Shown are the properties of motor axons in the L4 ventral root in Bcl2 null mice and controls. (A) Total cross-sectional area of the L4 ventral roots in Bcl2 null mice and controls (n = 7 and 6 for Bcl2+/− and Bcl2−/− groups, respectively). Areas are indicated in µm². Note that with respect to total L4 ventral root area, groups did not significantly differ from one another (p = 0.14). (B) Total axon numbers within the L4 ventral root (n = 7 and 6 for Bcl2+/− and Bcl2−/− groups, respectively). Asterisk denotes significant difference from control group at p < 0.01. (C) Histogram showing the distribution of axon areas within L4 ventral roots of Bcl2+/− mice. Histogram represents the axon areas observed in complete L4 cross-sections (n animal ≥ 5, n axon ≥ 4300). (D) Histogram distribution for Bcl2 null mice (n animal ≥ 5, n axons ≥ 3300). For both (C) and (D) approximately ≥95% of all axon areas fall within the range indicated on the graph, with <5% exhibiting areas greater than the indicated range. (E-F) Photomicrographs of L4 ventral root of Bcl2+/− and Bcl2−/− L4 ventral roots, respectively, at postnatal day 30. Scale bar represents a distance of 50 µm.
reductions stabilizing around postnatal day 9 (P9) (Michaelidis et al., 1996). To
determine whether this pattern was also observed in our lineages of Bcl2 mice, Bcl2 null
mice and controls were examined at postnatal day 10. Counts of L4 ventral roots at PND
10 indicated an 18±2% reduction in Bcl2 null mice compared to controls (axon means:
694±11 versus 848±9 for controls, respectively).

3.1.3.2 Reduction of Muscle Spindle Innervation and Sensory Losses in Bcl2 Null Mice

The loss of small caliber motor axons seen in Bcl2 null mice indicated that
gamma motor neurons are uniquely sensitive to developmental ablation of Bcl2. As a
result, I have next examined the fusimotor innervation of muscle spindles. As this
structure also receives sensory innervation, sensory axons were also examined in Bcl2
null mice. As shown in Figure 33A-B, both nerve area and axon number were reduced
within the dorsal root by 28±9% and 27±2% respectively; suggesting that Bcl2−/− mice
undergo a significant loss of sensory neurons within the dorsal root ganglion by PND 30.
Consistent with this, prior analysis of the L3 dorsal root ganglion in the Bcl2−/− mice has
demonstrated significant neuronal loss at postnatal day 9 (Michaelidis et al., 1996).

In order to more clearly define the effects of sensory and motor axon reduction on
muscle spindles, the number and morphology of muscle spindles were examined in Bcl2
null mice. Total numbers of muscle spindles were determined in serial cross-sections of
both forelimb (flexor carpi ulnaris) and hindlimb (soleus) muscles. At both sites, spindle
number did not differ significantly between genotypes. Similarly, the cross-sectional area
of intrafusal (bag2) fibers, or adjacent extrafusal (skeletal) muscle fibers of Bcl2+/- and
Bcl2−/− mice did not differ significantly (Table 5). To determine the level of fusimotor
Figure 33. Loss of Bcl2 Alters Sensory and Muscle Spindles Innervation

The extent of reduction in sensory and muscle spindle innervation was determined for
Bcl2 null mice. (A) Total cross-sectional area of the L4 dorsal roots in Bcl2<sup>+</sup>/− and Bcl2<sup>−/−</sup>
mice at PND 30. (B) Total axon counts of L4 dorsal root of Bcl2<sup>+</sup>/− and Bcl2<sup>−/−</sup> mice at
PND 30 (n = 7 and 6 for Bcl2<sup>+</sup>/− and Bcl2<sup>−/−</sup> groups, respectively). Asterisk indicates
significant difference from the control group at <i>p</i> < 0.01). (C) Example of soleus muscle
spindle in Bcl2<sup>+</sup>/− mouse. Note that the spindle receives a large Ia sensory axon, two
smaller secondary sensory axons (arrowheads), and three small fusimotor axons (arrows).
Intrafusal fibres within the spindle are denoted by IF, which are surrounded by spindle
capsule (CP). Extrafusal skeletal muscle fibres surround the spindle capsule and neural
axons. (D) Example of soleus muscle spindles in Bcl2<sup>−/−</sup> mouse. Note that Bcl2<sup>−/−</sup> spindle
receives an Ia axon (arrowhead), and a single apparent fusimotor axon (arrow) with no
secondary sensory axons. For (C) and (D), scale bar denotes 50 µm.
Table 5. *Bcl2* Deletion Does Not Alter the Cross-Sectional Area of Muscle Spindles

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<tr>
<th>Genotype</th>
<th><em>Bcl2</em>&lt;sup&gt;+/-&lt;/sup&gt;</th>
<th><em>Bcl2</em>&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td><strong>Intrafusal</strong></td>
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<td></td>
</tr>
<tr>
<td><em>(n = 40 fibers/genotype)</em></td>
<td>22±4 µm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>28±3 µm&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Extrafusal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>(n = 80 fibers/genotype)</em></td>
<td>225±22 µm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>219±17 µm&lt;sup&gt;2&lt;/sup&gt;</td>
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innervation of muscle spindles in $Bcl2^{-/-}$ mice, soleus muscle spindles were cut through both the central (capsular) and terminal regions. As shown in Figure 32C, soleus muscle spindles from $Bcl2$ heterozygous and wild-type mice receive innervation from multiple fusimotor axons; while spindles from $Bcl2^{+/-}$ mice are typically innervated by only a single apparent motor axon (Figure 33D). Counts of total fusimotor fibers within the soleus muscle indicate a significant decrease in fusimotor innervation in $Bcl2^{-/-}$ mice versus controls (Table 6). Thus while $Bcl2$ null mice exhibit a substantial loss of fusimotor innervation, some residual motor innervation to muscle spindles remained in these animals. Consistent with this, esterase activity is observed within at least some muscle spindles in $Bcl2$ null mice (Table 6). This innervation may represent the influence of either a small population of surviving fusimotor fibers, or collateral sprouting from existing alpha motor fibers (beta innervation). This residual motor innervation, may explain the absence of gross dystonia or ataxia seen in $Bcl2$ null mice. Examination of soleus muscle termini in $Bcl2$ null mice also demonstrated the presence of normal golgi tendon organs.

3.1.3.3 Role of $Bcl2$ in Programmed Cell Death in the Facial Nucleus

In order to compare the results observed in spinal motor neurons, branchiomotor neurons of the facial nucleus were examined in $Bcl2$ null mice. This population was chosen for analyses both because it lacks significant gamma motor innervation (Bowden and Mahran, 1956), and has been examined previously in lines of $Bcl2$ null mice (Michaelidis et al., 1996). In addition, I had wished to examine motor neuron survival within lateral components of the facial nucleus, as these have previously been suggested
Table 6. Properties of Muscle Spindles (Soleus) of *Bcl2* null mice (PND 25-30)

<table>
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<tr>
<th>Genotype</th>
<th><em>Bcl2</em>&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th><em>Bcl2</em>&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Fusimotor fibres</td>
<td>80±5</td>
<td>15±5*</td>
</tr>
<tr>
<td>Sensory innervation</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>NT-3 expression</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Muscle spindles</td>
<td>10±2</td>
<td>9±2</td>
</tr>
<tr>
<td>Esterase staining</td>
<td>Present</td>
<td>Present</td>
</tr>
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* *p* < 0.05
to undergo preferential degeneration in Bcl2 null mice. To analyze the lateral (nasolabial) component of the facial nucleus, a retrograde tracer was injected into the medial facial nerve (MFN), below the bifurcation of cervical and mandibular facial branches (Figure 34A) to examine this subpopulation within the facial nucleus proper (overview, Figure 34B) for each genotype. As shown in Figure 34C-D, motor neurons which innervate the MFN reside within the lateral portion of the facial nucleus. Retrograde labeling of the MFN in Bcl2 null mice demonstrated no significant difference in motor neuron distribution compared to control littermates (n = 5 animals per genotype, labelled facial motor neurons ≥1220 neurons per genotype, control labelling confined to lateral 35% of the facial nucleus). It should be noted however, that a very small group of motor neurons at the extreme lateral extent of the facial nucleus were not labelled following MFN injection in any of the genotypes examined.

In order to determine the overall impact of Bcl2 deletion on motor neurons of the facial nucleus, total counts of motor neurons within the facial nucleus were performed in P30 Bcl2 null mice and controls. As shown in Figure 34E, our lineages of Bcl2 null mice exhibited a reduction of 21±2% in motor neurons within the facial nucleus compared to controls. Interestingly, it has previously been suggested that Bcl2 null mice exhibit an enhanced degeneration of motor neurons within the lateral component of the facial nucleus at P9 (Michaelidis et al., 1996). To investigate this in our lines of Bcl2 null mice, cross-sectional area, total axon number and distribution of axon calibers within the MFN were determined for Bcl2 null mice and controls. As shown in Figure 35A-B, Bcl2 null mice exhibited a reduction in both total axon number (15±3%) and mean cross-sectional area (39±6%) compared to controls at postnatal day 30. In addition, the distribution of
**Figure 34. Effects of Bcl2 in Facial Motor Neurons**

Total and sub-populations of the facial nucleus analyzed in Bcl2 null mice and controls.  
(A) Overview of medial branches of the facial nerve showing retrograde tracing site, and point of segment collection for the medial facial nerve (MFN) (boxed region) used for analysis of axon morphometry (med - medial branch of the facial nerve, cerv - cervical branch of the facial nerve, mnd - mandibular branch of the facial nerve). (B) Photomicrograph showing coronal section of the brainstem delineating motor neurons within the facial nucleus (dotted zone). (C-D) Photomicrographs showing the pattern of retrograde labelling within the facial nucleus, following tracer injection to the MFN. Shown are typical distribution patterns for Bcl2<sup>+/−</sup> (C) and Bcl2<sup>−/−</sup> (D) mice 48 hours following injection of tracer (n = 5 animals per genotype, labelled facial motor neurons ≥ 1220 neurons per genotype, control labelling confined to lateral 35% of the facial nucleus). (E) Counts of total motor neuron number within the facial nucleus of Bcl2<sup>+/−</sup> and Bcl2<sup>−/−</sup> mice (n = 7 and 6 for Bcl2<sup>+/−</sup> and Bcl2<sup>−/−</sup> groups, respectively). Asterisk indicates significance at p < 0.01.
Figure 35. Analysis of Axon/Nerve Morphology within the Medial Facial Nerve in Bcl2 Null Mice

The morphology of medial facial nerves was examined to assess the influence of Bcl2 upon this subpopulation of facial motor neurons. (A) Total axon numbers within the MFN (n = 8 and 6 for Bcl2\textsuperscript{+/−} and Bcl2\textsuperscript{−/−} groups, respectively. Asterisk indicates significance at $p < 0.01$. (B) Total cross-sectional area of the MFN. Areas are indicated in $\mu$m\textsuperscript{2} (n = 10 and 8 for Bcl2\textsuperscript{+/−} and Bcl2\textsuperscript{−/−} groups, respectively). Asterisk indicates significant difference from control group at $p < 0.01$. (C) Histogram showing distribution of axon calibre within the MFN in Bcl2\textsuperscript{+/−} mice. Histogram represents the axon areas observed in complete cross-sections of Bcl2\textsuperscript{+/−} animals (n animal $\geq$ 5, n axon $\geq$ 4120). (D) Distribution of axon calibre within MFN of Bcl2 null mice (n animal $\geq$ 5, n axons $\geq$ 3520). For histograms (C) and (D), $\geq$95% of all axon areas fall within the ranges indicated, with <5% of axons exhibiting areas greater than the indicated range.
axon calibers was shifted toward smaller axon sizes in Bcl2 null mice compared to controls (Figure 35C-D). Axons within the MFN having cross-sectional areas ≤3 µm² represent 49±6% of the total axon population in Bcl2 null mice, whereas this group represents only 21±11% of the total axon population in Bcl2 heterozygous or wild-type littermates. Given that the facial motor neurons exhibited a total reduction of 21±2%, and counts of retrogradely labeled (MFN) lateral facial neurons showed a reduction of only 17±3% (1359±42 versus 1643±73 for controls, n = 5 animals per genotype) in Bcl2 null mice versus controls at PND 30, these data suggest that motor neurons with the lateral facial nucleus do not exhibit a differential sensitivity to Bcl2 ablation; in contrast to previous findings at PND 28 (Michaelidis et al., 1996).
3.1.4 DISCUSSION

To determine the effect of Bcl2 deletion on motor neuron survival, I have examined two motor neuron populations which differ significantly with respect to their fusimotor representation; branchiomotor neurons of the facial nucleus and somatic motor neurons of the lumbar spinal cord. Analyses of choline acetyl transferase-positive neurons within the sciatic motor pool and total axon numbers within the L4 ventral root indicated that Bcl2^−/− mice exhibit significant motor neuron loss (24-26%) compared to control littermates by postnatal day 30. Retrograde tracings of the sciatic nerve to determine sciatic motor pool populations in Bcl2^{+/+}, Bcl2^{+/-} and Bcl2^{−/−} mice demonstrate equivalent motor representations for each genotype. Analysis of total axon cross-sectional areas within L4 ventral roots, demonstrate that the axonal losses observed in Bcl2^{−/−} animals are not evenly distributed with respect to axon caliber, but rather disproportionately affect small caliber (≤3µm²) axons; indicating that small (gamma) motor neurons are selectively dependent upon Bcl-2 expression. Indeed in terms of axon classes affected, 68% of the reduction observed in Bcl2 null mice affects axons of areas ≤3µm². The predominant loss in small caliber axons seen in Bcl2 null mice corresponds to an extensive reduction in motor innervation (approximately 80%) within muscle spindles as demonstrated from analysis of the soleus muscle. Residual motor input to muscle spindles may represent innervation from either a small number of surviving fusimotor fibers, or the result of induced collateral input from adjacent alpha motor neurons (beta innervation).

In addition to spinal motor neurons, similar to a previous report (Michaelidis et al., 1996) I observed losses of both branchiomotor and sensory neurons in Bcl2 null mice.
However, the degree of neuronal loss I have observed in our lineages of Bcl2 null mice on two different genetic backgrounds is somewhat lower than that previously reported (21±2% PND 30 versus 32% PND 28 for facial motor neurons; 27±2% PND 30 versus 44% PND 44 for sensory neurons). In addition, my analysis of retrograde tracing and axonal counts of neurons innervating the medial branch of the facial nerve (nasolabial musculature) suggest that this population of facial motor neurons are not differentially sensitive to Bcl2 ablation; as previously reported (Michaelidis et al., 1996). While nature of this discrepancy remains unclear, it may be related to genetic differences in the murine backgrounds employed. Such differences are unlikely to be related to minor differences in the age of the animals employed (PND 30 versus PND 44), as both studies observed little increase in motor neuron due to Bcl2 deletion death beyond postnatal day 9.

Within sciatic motor pools, previous analyses (Hashizume et al., 1988) and my own studies, place the fusimotor representation at approximately 30% of the total motor neuron population. Consistent with these findings, I have observed that the population of lumbar spinal motor neurons which strongly express Bcl-2 postnatally is comparable in number to both the degree of motor neuron loss seen in Bcl2 null mice at postnatal day 30 and the fusimotor representation in this population. Alpha and gamma motor neurons represent two closely related lineages derived from a common neural precursor (Dutton et al., 1999). Initially, both neural types make similar innervations to primary myofibers (Kucera et al., 1989). A primary difference between these two motor classes relates to the temporal pattern of their differentiation (Ringstedt et al., 1998). The majority of motor neurons differentiate during the early phase of spinal cord development (E10-E11 in rodents), and primary motor neurogenesis is essentially complete by E14 with peak motor
numbers achieved by E13.5 (Lance-Jones, 1982). Alpha motor neurons (and interestingly group Ia sensory neurons which provide innervation to muscle spindles) differentiate and become established early in this process. By contrast, completion of gamma motor neuron (and group II sensory neuron) differentiation appears to occur subsequent to this process; as the earliest known antigenic markers of these populations are observed beginning at E16.5-E17 (Ringstedt et al., 1998). Thus it appears (at least within somatic motor pools) that the latter differentiation events of gamma motor neurons set the stage for enhanced dependence upon Bcl-2 for survival; in contrast to earlier differentiation for alpha motor neurons. Thus, the differentiation of motor neurons into distinct classes occurs over the same developmental period that motor neuron numbers are principally adjusted through the process of programmed cell death, from the peak levels of genesis, to functional levels seen postnatally (E14-E18). My results in Bcl2 null mice with spinal cord motor neurons demonstrate that these two processes can be mechanistically linked, guiding the development specific motor populations in mammals in vivo.

It is interesting to note that ablation of Bcl2 not only influences gamma motor neurons, but result in the loss of a subpopulation of alpha motor neurons in both lumbar spinal (12±3% based upon loss of axons >3 µm²) and branchiomotor (21±2% based upon total facial nucleus counts) neurons. It is tempting to speculate that these Bcl-2-dependent alpha motor neurons may represent latter-differentiating members of the population. The concept of a temporal pattern for Bcl-2 in regulating neuronal survival is consistent with my observation that neither muscle spindle number (regulated by Ia sensory innervation), nor levels of group Ia innervation within muscle spindles were significantly altered in Bcl2 null compared to controls. Thus the loss of sensory neurons seen within the dorsal
root ganglion of Bcl2 null mice reflects Bcl-2-dependent cell death in a class (or classes) of sensory neurons; perhaps group II neurons which differentiate subsequently to group Ia neurons during embryonic development (Lawson and Biscoe, 1979).

The development of muscle spindles is initiated upon the innervation of primary myotubes by proprioceptive (Ia) afferents (Kucera and Waldro, 1990). Arrival of sensory fibers is sufficient to trigger the differentiation of primary myofibers to early intrafusal (bag2) fibers (Wang et al., 1997). It has been shown, at least for muscles of the hindlimb, that limiting concentrations of NT-3 produced within the developing myofibers regulates the outgrowth of these proprioceptive axons from the dorsal root ganglia to muscle spindles (Wright et al., 1997). The differentiation of muscle spindles occurs around E15.5 following contact with group Ia afferents with developing myotubes (Tourtellotte et al., 2001). This process has been shown to depend in part upon the transcription factor Egr3 (Tourtellotte et al., 2001). In the absence of Egr3, myotubes contacted by Ia afferents do not undergo proper differentiation into muscle spindles and degenerate after birth. In addition, neurotrophic factor 3 (NT-3) has been shown to be necessary for proper maintenance of muscle spindles and gamma fusimotor efferents (Kucera et al., 1995; Ringstedt et al., 1998; Ringstedt et al., 1997; Wright et al., 1997). Consistent with this ectopic injection of NT-3 into the hind limbs of Egr3-/- mice was able to maintain functional circuitry between the group Ia afferents and muscle spindles (Chen et al., 2002). Interestingly as development proceeds, expression of NT-3 becomes restricted to intrafusal fibers within the developing spindle. Motor innervation of the muscle spindle occurs subsequent to the arrival of sensory fibers and the differentiation of the myotube. Consistent with this, survival of fusimotor neurons has been shown to be dependent upon
the presence of Ia afferents and/or muscle spindles; as fusimotor neurons are not observed in mice lacking muscle spindles (Ringstedt et al., 1998). By contrast, several studies have demonstrated that the near or total absence of motor innervation to muscle spindles does not prevent their differentiation or maintenance (Kucera and Walro, 1992; Ringstedt et al., 1998). This model is supported by my present work in Bcl2 null mice, in which the loss of a substantial portion of fusimotor innervation did not significantly alter the number of muscle spindles within the hindlimb.

Gamma fusimotor neurons have also been shown to be dependent upon neurotrophic support derived from muscle spindles (principally in the form of glial-derived neurotrophic factor/GDNF and NT-3) (Buss et al., 2006; Gould et al., 2008; Shneider et al., 2009; Whitehead et al., 2005). Interestingly loss of gamma fusimotor neurons due to deficiencies in GDNF, Ret or GFRα1 (components of the GDNF receptor) did not occur when such losses occurred after PND5, revealing that GDNF-mediated trophic support is required only during the early (postnatal) period of fusimotor circuit development (Gould et al., 2008). Previous studies in Bcl2 null mice demonstrate that the neuronal losses observed in these animals occur during late embryonic development and early postnatal period (Michaelidis et al., 1996). This period coincides with the time in which gamma fusimotor neurons are dependent upon GDNF trophic support from their innervation targets.

In support of the model that Bcl-2 differentially regulates the survival of gamma motor neurons, examinations of several lines of genetically modified mice which lack or overexpress factors known to act through Bcl-2 (Bax and Ntf3 null mutants, Gdnf heterozygous mutants, Myo-GDNF transgenic mice) exhibit significant differences in
their gamma population (Buss et al., 2006; White et al., 1998; Whitehead et al., 2005). That gamma motor neurons require GDNF for survival derived from muscle spindles is suggested from studies in which significant losses of small diameter motor axons are observed in Gdnf heterozygous animals. Similarly, transgenic ectopically expressing GDNF within skeletal muscle exhibit a substantial increase in small diameter motor axons within the L4 ventral root (Buss et al., 2006; Whitehead et al., 2005); though the precise nature of the motor neurons contributing these axons is unclear. Analysis of the L4 ventral root in Bax null mice reveals a significant increase in total numbers of myelinated axons, which is contributed exclusively by small diameter axons believed to be derived from gamma motor neurons (Buss et al., 2006; White et al., 1998). The greater relative increase in motor axons in Bax null mice (71%) compared to the reductions seen in Bcl2 null mice (24%), likely reflects the greater potential for functional compensation by Bcl-2 homologues (Bcl-xL, Mcl-1, Bcl-w, etc.) compared to Bax homologues (Bak, Bok, etc.) for motor neuron survival. Interestingly it was also demonstrated that a substantial population of unmyelinated axons was rescued by Bax deletion, and believed to represent the population of atrophied alpha motor neurons described previously (Buss et al., 2006; Sun and Oppenheim, 2003). While the ventral roots of adult wild-type mice normally contain very low numbers of unmyelinated axons, this is also true for our lineages of Bcl2 null mice (Figure 36). Given the model proposed for Bcl-2, and the nature of interactions between Bcl-2 and Bax, this finding is not surprising and paints a consistent picture of Bcl-2’s action in relation to previous studies on interacting signaling proteins. Based upon the results in Bcl2 null mice, the mechanisms by which neurotrophic agents such as NT-3 and GDNF exert control over the development of
Figure 36. Electron Photomicrographs of L4 Ventral Root of Bcl2 Null Mice and Controls

Representative views are shown of regions between motor axons within L4 ventral root of Bcl2 null mice and controls at postnatal day 30. Arrowheads denote myelinated axons. SC denotes Schwann cells, n denotes corresponding nuclei. Scale bars represent a distance of 2 µm. (A-B) Bcl2 heterozygotes. (C-D) Bcl2 null mice. EM analysis of Bcl2 null animals indicates that their ventral roots are comparable to heterozygous and wild-type control littermates and exhibit very low levels of unmyelinated axons.
specific motor populations such as fusimotor neurons (and potentially alpha subgroups),
can be clearly framed in a broader understanding of their influence by Bcl-2/Bax
interactions to regulate the process of programmed cell death.

Interestingly, in addition to their critical roles in promoting the survival of various
neuronal subpopulations during development, neurotrophic factors such as BDNF, CNTF
and GDNF have all been demonstrated to be powerful agents in protecting injured
neurons (Henderson et al., 1994a; Sendtner et al., 1990; Yan et al., 1994). However,
despite their compelling neuroprotective abilities, their potentials for clinical application
were never fulfilled as delivery to the site of injury at therapeutic concentrations was
difficult (e.g., blood-brain barrier, proteolytic degradation, etc.) and systemic
administration (CNTF) proved to have devastating side effects (Henderson et al., 1996;
Henderson et al., 1994b). Thus, while other experimental approaches such as direct cell-
mediated delivery of neurotrophic factors or viral-mediated gene therapy are currently
under intense investigation (Hottinger et al., 2000; Kurozumi et al., 2004; Ruitenberg et
al., 2004; Sasaki et al., 2009), pharmacologic modulation of PCD by low molecular
weight compounds remain as the only proven therapeutic option at the present time. To
this end, numerous drug candidates, including minocycline, methylprednisolone and
BAPTA, have been proposed to possess substantial neuroprotective abilities (Bracken et
al., 1990; Tymianski et al., 1993; Yrjanheikki et al., 1999). However, these compounds
have ultimately all failed to display any significant neuroprotection when tested in
clinical trials. A particular cause for their failures may be that neural injuries are often
very complex in nature, and the associated neuronal cell death could be initiated by
multiple independent pathways such that any attempt to modulate a particular pathway,
no matter how central it may be, can typically be circumvented via other minor pathways. However, as multiple molecular pathways converge on PCD regulation to induce neuronal cell death following neural injuries, it is therefore unsurprising that PCD modulation via genetic manipulation has reveal it to be an optimal therapeutic target for neuroprotection (Deckwerth et al., 1996; Dubois-Dauphin et al., 1994; Farlie et al., 1995; Kanungo et al., 2008; Kostic et al., 1997; Parsadanian et al., 1998; Sun and Oppenheim, 2003; Yin et al., 2002).
STUDY 2: MOLECULAR MECHANISM OF IMMUNOPHILIN LIGAND-MEDIATED NEUROPROTECTION IN INJURED MOTOR NEURONS

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3.2.1 ABSTRACT

The immunosuppressive agents cyclosporin A and FK-506 have previously been shown to exhibit neurotrophic and neuroprotective properties in vivo. Given that significant clinical expertise exists for both drugs, they represent an attractive starting point for treatment of acute neural injuries. One putative mechanism for neuroprotection by these drugs relates to inhibition of calcineurin activity. However each drug-immunophilin complex can potentially influence additional signal transduction pathways. Furthermore, several non-immunosuppressive immunophilin ligands have been described as possessing neuroprotective properties, suggesting that neuroprotection may be separable from calcineurin inhibition. In the present study, I have examined the mechanism of this neuroprotection in facial motor neurons following axotomy-induced injury. Similar to previous studies in rat, cyclosporin A and FK-506 enhanced motor neuron survival in mice following acute injury. To examine the mechanism responsible for neuroprotection by these agents, pharmacologic inhibitors of several potential alternate signaling pathways (17-(allylamino)-17-demethoxygeldanamycin, rapamycin, cypermethrin) were evaluated with respect to neuroprotection. Of these, only cypermethrin, a direct calcineurin inhibitor not previously associated with neuronal survival properties, was observed to significantly enhance motor neuron survival following injury. The results demonstrate for the first time that direct inhibition of calcineurin is neuroprotective in vivo. These data support a model in which calcineurin inhibition promotes neuronal survival, distinct from effects upon neurite outgrowth.
3.2.2 INTRODUCTION

A major shortcoming of traditional drug therapy is the promiscuity for drugs to have multiple targets within the cell across multiple tissues within a patient, in that the specific therapeutic effect is often accompanied by undesirable side effects. However, often a deeper molecular understanding of the mechanism of action for these drugs can typically lead to a molecular dissection of the desired therapeutic targets and undesired off-targets, thus leading to optimized therapy. As described in this chapter, a class of immunosuppressive agents called immunophilin ligands was previously demonstrated to possess neuroprotective properties in a wide number of neural injury paradigms. A longstanding controversy followed regarding their mechanisms of neuroprotection, involving putative inhibitory action on the mitochondrial permeability transition pore, immunosuppressive target calcineurin and other immunophilin-mediated functions (Dawson et al., 1994; Gold et al., 2005; Gold et al., 1997; Matsumoto et al., 1999; Snyder et al., 1998a; Steiner et al., 1997a; Steiner et al., 1992). In order to address this controversy and clearly identify the molecular mechanism of neuroprotection associated with these agents I have systemically examined these putative pathways by pharmacologic means and genetic manipulation, and established the critical involvement of calcineurin and mitochondrial signaling in neuroprotection mediated by this class of immunophilin ligands.

Immunosuppressants such as cyclosporin A (CsA) and FK-506 (Tarcolimus) are currently used clinically to treat graft-versus-host rejection following organ transplantations (Fung and Starzl, 1994; Morris, 1991). The cellular receptors for these molecules are collectively termed immunophilins. Cyclosporin A binds to proteins of the
cyclophilin family such as cyclophilin A and D, while FK-506 interacts with FK-506 binding proteins (FKBPs) (Snyder et al., 1998b). A number of studies have demonstrated that the immunosuppressive actions of CsA and FK-506 are mediated through a gain-of-function action induced by the drug-immunophilin complex; resulting in the inhibition of calcineurin phosphatase activity (Liu et al., 1991). Notably, studies have demonstrated that expression of some immunophilins is 50 times greater in the CNS than immune cells (Steiner et al., 1992). In addition, in vivo applications of CsA or FK-506 have both been shown to exert neuroprotective and neurotrophic effects in specific neuronal populations (Dawson et al., 1993; Snyder et al., 1998a). Administration of FK-506 in rats has been shown to increase neuronal survival and improve functional recovery following facial and sciatic nerve axotomy, photo-thrombotic spinal cord injury and transection of the medial forebrain bundle (Gold et al., 1995; Madsen et al., 1998; Winter et al., 2000). FK-506 has also been shown to enhance nerve growth factor (NGF)-dependent neuritic outgrowth in PC-12 cells and primary cultures of dorsal root ganglion (sensory) neurons (Lyons et al., 1994). Similarly, CsA has been shown to protect dopaminergic neurons from 6-hydroxydopamine toxicity and to reduce cerebral infarct volumes in experimental models of stroke (Kuroda et al., 1999; Matsumoto et al., 1999; Steiner et al., 1997b). These studies demonstrate that both CsA and FK-506 can reduce the level of neuronal cell loss under a variety of injury states. However, questions remain regarding the mechanism by which these agents promote neuronal survival following injury.

Some evidence suggests that CsA exerts its survival promoting effects through inhibition of cyclophilin D, which comprises part of the mitochondrial permeability transition pore (mPTP) (Matsumoto et al., 1999). Opening of the pore results in loss of
mitochondrial membrane potential ($\Delta \Psi$) and mitochondrial swelling, which ultimately manifest in rupture of the mitochondrial outer membrane. Formation of the mPTP is linked to the release of pro-apoptotic factors present in the mitochondrial intermembranous space such as holo-cytochrome c, apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO), into the cytoplasm where they are involved in downstream PCD pathways (Newmeyer and Ferguson-Miller, 2003). While CsA has been shown to reduce infarct size following middle cerebral artery occlusion (Matsumoto et al., 1999), FK-506, a drug which lacks effect on the mPTP, also exhibits similar survival promoting properties (Butcher et al., 1997). While it is possible that these agents may exert their effects through unrelated mechanisms, their commonality with respect to immune function (inhibition of calcineurin signaling) suggests a potential mechanism (Liu et al., 1991).

Interestingly, immunophilin and calcineurin expression are strongly correlated within the CNS, suggesting a functional connection (Dawson et al., 1994; Steiner et al., 1992). A linkage between calcineurin inhibition and neuronal survival is suggested from studies which shown that calcineurin mediates dephosphorylation of Bad; a pro-apoptotic Bcl-2 family protein (Springer et al., 2000; Wang et al., 1999; Yang et al., 2004). Bad has previously shown to influence the release of cytochrome c and other apoptogenic proteins from the mitochondria intermembraneous space following stimulation of programmed cell death (Springer et al., 2000; Wang et al., 1999; Yang et al., 2004). The phosphorylation status of Bad has been implicated as the primary regulatory mechanism governing this BH3-only protein, since phosphorylation of serine residues S112, S136
and S155 enhance the interaction of Bad with 14-3-3, which prevents it from translocating to the mitochondria (S112 and S136), or disrupts its inhibition of anti-apoptotic Bcl-xL (S155) (Datta et al., 2000; Datta et al., 2002; Tan et al., 2000; Zha et al., 1996).

In the present study I have shown that CsA and FK-506 enhanced neuronal survival following axotomy-induced facial motor neuron injury in mice, similar to previous work in rats (Tao and Aldskogius, 1998). I have further demonstrated that a direct inhibition of calcineurin by cypermethrin (which acts independently of immunophilins) also promotes motor neuron survival following axotomy. In contrast, other signaling pathways related to immunophilin functions did not alter motor neuron survival. These data indicate that the survival promoting effects of CsA and FK-506 on motor neurons following injury are a direct consequence of their ability to inhibit the phosphatase activity of calcineurin and its subsequent influences on mitochondrial signaling.
3.2.3 RESULTS

3.2.3.1 Motor Neuron Survival is Enhanced by Cyclosporin A and FK-506 Treatment following Facial Axotomy

I first sought to determine whether the survival promoting effects previously observed in rat, could be reproduced in murine strains. For both rats and mice, neonatal axotomy of the facial nerve results in a well-characterized pattern of motor neuron loss through the process of programmed cell death (PCD) (de Bilbao and Dubois-Dauphin, 1996; Kou et al., 1995). While strain variations exist, this lesion paradigm typically results in a loss of >80% of facial motor neurons by one week post-axotomy (Kou et al., 1995). In the current study, mice were subjected to unilateral facial nerve axotomy on postnatal day 3 (PND 3), and given either CsA or FK-506 once daily for seven days following injury until the time of sacrifice. As shown in Figure 37, an enhancement of motor neuron survival was observed in mice administered either CsA (20 mg/kg) (C-D) or FK-506 (3 mg/kg) (E-F) compared to vehicle-treated controls (A-B) within injured facial nuclei (dotted regions). Stereologic serial counts of surviving motor neurons were performed through the full extent of the facial nucleus for nuclei on both contralateral (uninjured) and ipsilateral (injured) to the axotomized facial nerve, in order to quantify the effect of drug treatment on axotomized motor neurons. As shown in Figure 37G (left panel), CsA treatment improved motor neuron survival from 15±1% (vehicle) to 27±3%, while FK-506 administration (Figure 37G, centre panel) resulted in 40±3% motor neuron survival compared to control (16±2%). To determine whether the survival promoting effects of CsA and FK-506 acted through similar or disparate mechanisms, mice were treated with both agents following facial nerve axotomy.
**Figure 37. Survival Characteristics of Injured Facial Motor Neurons Following Cyclosporin A or FK-506 Treatment**

Thionin-stained cross-sections taken through the facial nucleus at 7 days following axotomy are shown for each treatment. Sections shown are through comparable levels of the facial nucleus from representative individuals of each treatment group. Panels (A), (C), (E) depict coronal cross-sections through uninjured facial nuclei (contralateral nucleus – con.). Panels (B), (D), (F) show coronal cross-sections taken through injured nuclei (ipsilateral nucleus – ips.). Scale bars represent a distance of 250 µm. (G)

Summary of stereologic counts of facial motor neurons in axotomized and uninjured facial nuclei following cyclosporin A or FK-506 treatment. Administration of CsA and FK-506 under the conditions indicated resulted in the survival of 27±3% and 40±3% total facial motor neurons, respectively, compared to 15±1% and 16±2% for respective vehicle-treated controls. [* indicates statistical significance between treatment group (i.e., CsA and controls) at $p < 0.05$; ** indicates statistical significance between treatment groups (i.e., FK-506 and CsA + FK-506 co-treatment versus CsA) at $p < 0.01$]
Stereologic analysis of facial nuclei from these animals demonstrated levels of motor neuron survival comparable to that observed using FK-506 alone (Figure 37G, right panel), indicating that the effects of these agents are not additive with respect to enhancement in neuronal survival, thus suggesting they act through similar targets.

3.2.3.2 Cyclosporin A and FK-506 Protect Motor Neuron following Injury by Reducing Caspase-3 Activation

To better understand the mechanism by which CsA and FK-506 treatments enhance the survival of injured facial motor neurons, I have investigated the downstream pattern of PCD progression by examining executioner caspase activation within the facial nucleus. Such caspases have been shown to be activated within injured facial motor neurons following neonatal axotomy (de Bilbao et al., 1999; Guarin et al., 1999; Kanungo et al., 2008). Inhibition of this response can therefore be taken as evidence of inhibition of PCD in facial motor neurons at a point upstream of executioner caspase activation. For these experiments, levels of caspase-3 activation were examined through the full extent of facial nuclei by stereologic counts at 20 hours following injury (a peak period of injury-induced motor neuron death). Significant elevation in levels of activated caspase-3 was observed in injured as compared to uninjured facial nuclei in vehicle-treated controls (Figure 38A-B). Administration of CsA (Figure 38C-D) and FK-506 (Figure 38E-F) significantly reduced the levels of activated caspase-3 in injured facial nuclei relative to vehicle treatment. Stereologic counts of motor neurons expressing activated caspase-3 throughout the facial nucleus demonstrated that both CsA and FK-506 significantly reduced caspase-3 activation (16±2% and 36±3% reduction compared
Figure 38. Cyclosporin A and FK-506 Reduces Levels of Activated Caspase-3 in Facial Motor Neurons Following Facial Nerve Axotomy

Coronal cross-sections through the facial nucleus were examined for activated caspase-3 at 20 hours following axotomy for drug treatment groups and vehicle-treated controls. Panels (A), (C), and (E) show sections through uninjured facial nuclei (contralateral – con.). Panels (B), (D), and (F) show sections through injured facial nuclei (ipsilateral – ips.). Scale bars represent a distance of 250 µm. (G) Stereologic counts of activated caspase-3 positive neurons through the facial nucleus show that both CsA and FK-506 treatments significantly reduced the number of motor neurons positive for activated caspase-3 compared to vehicle treatment (** indicates statistical significance at $p < 0.01$ between treatment groups and vehicle controls). Notably, CsA treatment was observed to increase the total number of motor neurons with activated caspase-3 in the uninjured facial nuclei compared to vehicle controls (# indicates statistical significance at $p < 0.01$ between CsA and vehicle treatments) while FK-506 did not appear to have any effects on uninjured facial motor neurons.
to vehicle, respectively) (Figure 38G). Interestingly, facial motor neurons expressing activated caspase-3 were significantly increased in uninjured facial nuclei of CsA-treated animals relative to vehicle-treated controls (95±3%) (Figure 38C, G). In contrast, FK-506 administration did not appear to alter the levels of naturally-occurring programmed cell death in uninjured facial nuclei during the same period (Figure 38E, G).

3.2.3.3 Cyclosporin A and FK-506 Do Not Alter Levels of Microglial Infiltration or Reactive Gliosis Following Injury

Microglial activation and reactive gliosis are frequently observed following many forms of neural injury, and have been suggested to contribute to levels of neuronal death (Giulian et al., 1993; Sargsyan et al., 2005). To determine the effect of CsA and FK-506 treatments on these secondary cellular responses, the degree and pattern of reactive gliosis and microglial infiltration were examined in control and treated facial nuclei at four days post-injury (Figures 39-40). As indicated in the figure, levels of activated microglia present within injured facial nuclei were not altered by either drug treatment (Figure 39). Similarly, numbers of reactive (GFAP-positive) astrocytes within injured facial nuclei were also observed to be comparable between treated and controls groups (Figure 40). Together, these data support a model that CsA and FK-506 act in a cell autonomous manner to promote neuronal survival of injured facial motor neurons.
Figure 39. Cyclosporin A and FK-506 Do Not Suppress Levels of Activated Microglia Following Facial Nerve Axotomy

Cryostat sections through facial nuclei were labeled with tomato lectin to examine numbers of activated microglia at 4 days following motor neuron injury. Panels (A), (D), and (G) show sections of unoperated (contralateral – con.) facial nuclei. Panels (B), (E), and (H) show sections through operated (ipsilateral – ips.) facial nuclei. Scale bars indicate a distance of 500 µm. Panels (C), (F), and (I) show higher magnification views of that shown in (B), (E), and (H), respectively. Scale bars represent a distance of 250 µm. Note that similar levels of microglial activation were observed between vehicle and CsA/FK-506 treatment groups.
Figure 40. Cyclosporin A and FK-506 Do Not Suppress Levels of Reactive Gliosis Following Facial Nerve Axotomy

Sections were labeled with GFAP to examine numbers of reactive astrocytes at 4 days following facial axotomy. Panels (A), (D), and (G) show sections through unoperated facial nuclei (contralateral – con.). Panels (B), (E), and (H) show sections through operated facial nuclei (ipsilateral – ips.). Scale bars represent 500 µm. Panels (C), (F), and (I) show injured facial nuclei at higher magnification. Scale bars represent 250 µm. Note that similar levels of reactive gliosis were observed between vehicle and CsA/FK-506 treatment groups.
3.2.3.4 Enhancement in Neuronal Survival Is Not Mediated via Calcineurin-Independent Signaling Pathways

Due to the array of potential cellular interaction targets for both CsA and FK-506, several possible mechanisms of neuronal survival effects exist beyond that of calcineurin inhibition. Recently, several calcineurin-independent signaling pathways have been suggested as possible means by which CsA and FK-506 exert their neuroprotective effects using non-immunosuppressive (calcineurin-independent) immunophilin ligands such as GPI-1046 and V-10367 (Gold et al., 2005; Gold et al., 1997; Guo et al., 2001; Snyder et al., 1998b; Steiner et al., 1997b). In addition, an alternative calcineurin-independent model of FK-506-mediated neuritic outgrowth has recently been proposed (Gold et al., 1999; Gold and Zhong, 2004). In this model, FK-506 promotes neuritic outgrowth via binding with FKBP-52 (Gold et al., 1999). The resulting drug-immunophilin complex inhibits formation of the Hsp-90/steroid receptor complex, which in turn releases p23 to activate downstream ERK pathways (Gold and Zhong, 2004). To determine whether such calcineurin-independent signaling pathways are implicated in survival enhancements observed in injured facial motor neurons as a result of treatments with CsA or FK-506, several additional pharmacologic inhibitors were examined for their ability to enhance motor neuron survival following axotomy. 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a cyclophilin/FKBP/calcineurin-independent inhibitor of Hsp-90 and rapamycin, a FKBP-dependent, calcineurin-independent inhibitor of mammalian target of rapamycin (mTOR), were administered daily to animals following axotomy until the time of sacrifice at doses previously demonstrated to produced neural effects in vivo (Ehninger et al., 2008; Kociok et al., 2007; Waza et al.,
2005). As shown in Figure 41, motor neuron counts performed on the ipsilateral (injured) and contralateral (uninjured) facial nuclei demonstrated that neither 17-AAG (10 mg/kg) (Figure 41A-B) nor rapamycin (3 mg/kg) (Figure 41C-D) had any effects on neuronal survival following injury compared to vehicle (17-AAG versus vehicle controls: 22±1% and 25±2%, respectively, 4 days post-injury; rapamycin versus vehicle controls: 17±2% and 13±1%, respectively). These results demonstrate that such calcineurin-independent pathways do not contribute to the survival enhancement of injured facial motor neurons observed using CsA or FK-506.

3.2.3.5 Role of Calcineurin Inhibition in Cyclosporin A- and FK-506-Mediated Facial Motor Neuron Survival

The absence of survival promoting effects observed following the administration of several calcineurin-independent agents prompted us to investigate the contribution calcineurin inhibition has with respect to neuronal survival in greater detail. To perform these studies, we examined inhibitors of calcineurin for which inhibition occurs through a manner disparate to that of CsA and FK-506. Cypermethrin is a type II synthetic pyrethroid insecticide which inhibits calcineurin through direct binding, thus functions as a cyclophilin/FKBP-independent calcineurin inhibitor (Enan and Matsumura, 1992). To examine the potential of cypermethrin to enhance neuronal survival following injury, cypermethrin (10 mg/kg) was administered to animals on a daily basis following axotomy until the time of sacrifice. Total counts of facial motor neurons demonstrated that cypermethrin treatment significantly enhanced motor neuron survival following axotomy compared to vehicle controls (32±3% versus 12±1%, respectively) (Figure 42A-B). This
Figure 41. 17-AAG and Rapamycin Do Not Enhance Facial Motor Neuron Survival Following Injury

(A) Histogram of stereologic counts of facial motor neurons from mice treated with 17-AAG (10 mg/kg) or vehicle performed at 4 days post-axotomy. Treatment with 17-AAG did not enhance levels of motor neuron survival compared to controls (22±1% versus 25±2% for 17-AAG and vehicle-treated groups, respectively). (B) depicts a typical injured facial nucleus from an animal treated daily with 17-AAG following axotomy. (C) Histogram of facial motor neuron survival following rapamycin treatment. Mice receiving daily rapamycin administration (3 mg/kg) following injury until time of sacrifice showed no significant difference in motor neuron survival compared to vehicle-treated controls (17±2% versus 13±1%, respectively), indicating these respective immunophilin-related pathways are not involved in neuroprotection. Shown in (D) is a typical injured facial nucleus from rapamycin-treated animals.
Figure 42. Inhibition of Calcineurin Results in Enhanced Facial Motor Neuron Neuronal Survival Following Facial Nerve Axotomy, CNA isoform α Plays a Dispensable Role in Regulating Neuronal Survival

(A) Daily administration of cypermethrin (10 mg/kg) demonstrated enhanced facial motor neuron survival from 12±1% in vehicle-treated animals to 32±3% in cypermethrin-treated mice, thus suggesting a prominent role for calcineurin inhibition in promoting neuronal survival (** indicates statistical significance between cypermethrin treatment and controls at $p < 0.01$). (B) shows an increased number of motor neurons in whole facial nuclei of cypermethrin-treated animals compared to controls. To further examine the role of calcineurin inhibition in enhancing neuronal survival following injury, axotomy-induced injury was performed in $Ppp3ca$ null mice and heterozygous controls. (C) Histogram of facial motor neuron survival in $Ppp3ca$ null mice. Mice homozygous for a targeted deletion of the dominant isoform of CNA in the CNS did not exhibit enhanced motor neuron survival compared to heterozygous littermates (19±1% versus 21±3%, respectively). (D) A typical injured facial nucleus from $Ppp3ca$ null animals following axotomy, with no observable enhancement in motor neuron survival compared to heterozygous controls.
A

![Bar chart showing the number of motor neurons with vehicle and cypermethrin (10 mg/kg) treatments.](Image)

- Vehicle: n = 6, 12%
- Cypermethrin: n = 5, 32% with *p < 0.05*

B

![Image of cypermethrin-related tissue damage.](Image)

C

![Bar chart comparing motor neurons in Ppp3ca (+/-) and (-/-).](Image)

- Ppp3ca (+/-): n = 4, 21%
- Ppp3ca (-/-): n = 5, 15% with **p < 0.01**

D

![Image of Ppp3ca (-/-) tissue.](Image)
represents the first demonstration that cypermethrin and, by extension, direct calcineurin inhibition enhance motor neuron survival following injury in vivo.

3.2.3.6 Calcineurin Subunit A Isoform Alpha Is Dispensable for Regulating Motor Neuron Survival Following Injury

To further investigate the potential role of calcineurin inhibition in enhancing motor neuron survival, neonatal facial axotomies was performed in mice lacking the calcineurin A alpha (CNAα) isoform. However, genetic deletion of this calcineurin isoform alone was not observed to enhance motor neuron survival (Ppp3ca+/− versus Ppp3ca−/−: 21±3% and 19±1%, respectively) (Figure 42C-D). Similarly, deletion of CNAα was not observed to reduce developmental motor neuron PCD, as indicated by the comparable numbers of facial motor neurons seen in Ppp3ca+/− and Ppp3ca−/− animals (Figure 42C). Thus, CNAα appears to be dispensable both for developmental and injury-mediated loss of facial motor neurons. Thus the motor neuron survival enhancement observed using CsA, FK-506 and cypermethrin appear to reflect an inhibition which extends beyond CNAα alone, and most probably due to the inhibition of β and γ isoforms in facial motor neurons as well.

3.2.3.7 Inhibition of Calcineurin-Mediated Bad Dephosphorylation by Cypermethrin and FK-506 Following Injury

A previous in vitro study had demonstrated an inhibition of calcineurin-mediated Bad dephosphorylation by FK-506 following glutamate stimulation was correlated with enhanced survival of the stimulated neurons, thus suggesting that inhibition of
calcineurin-mediated Bad dephosphorylation is, at least partially, responsible for enhanced survival of facial motor neurons following administration of CsA, FK-506 and cypermethrin (Wang et al., 1999). To determine whether Bad serine 112 (S112) dephosphorylation occurs within facial motor neurons following axotomy, and whether this process is regulated through calcineurin, I have examined levels of Bad S112 phosphorylation by immunofluorescence at 20 hours following facial nerve axotomy in vehicle, FK-506 and cypermethrin-treated animals. In vehicle-treated animals, Bad S112 phosphorylation was on average reduced in injured facial motor neurons to 55±4% of that observed in the contralateral (uninjured) facial nuclei (Figure 43). In contrast, FK-506 and cypermethrin-treated animals exhibited substantially reduced levels of Bad S112 dephosphorylation compared to vehicle-treated controls, demonstrating that both treatments inhibited calcineurin-mediated Bad dephosphorylation at serine 112. While cypermethrin treatment enhanced levels of Bad S112 phosphorylation to 87±11% of that seen in uninjured facial motor neurons, treatment with FK-506 appeared to have completely restored levels of Bad S112 phosphorylation to that observed in the contralateral facial nuclei (100±12%) (Figure 43). These results support the notion that regulation of Bad phosphorylation status may be a critical PCD signaling event downstream of calcineurin inhibition. The results further suggest that this can occur through either indirect inhibition by the drug-immunophilin complex, or directly through inhibition of calcineurin.
Figure 43. Inhibition of Calcineurin-Mediated Bad Dephosphorylation by Cypermethrin and FK-506

Coronal cross-sections through the facial nucleus were examined for Bad S112 phosphorylation at 20 hours following axotomy for drug treatment groups and vehicle-treated controls. Panels (A), (C), and (E) show sections through uninjured facial nuclei (contralateral – con.). Panels (B), (D), and (F) show sections through injured facial nuclei (ipsilateral – ips.). Scale bars represent a distance of 250 µm. (G) and (H) show sections through injured facial nuclei at higher magnification from vehicle and cypermethrin-treated animals, respectively. Phosphorylated Bad S112 is denoted in the green signal while facial motor neurons are marked as NeuN-positive cells labeled in red. Scale bars represent a distance of 100 µm. (I) Bad S112 phosphorylation in the injured facial nuclei was significantly reduced following axotomy to 55±4% compared to the uninjured facial nuclei ($p < 0.01$). Both cypermethrin and FK-506 treatments inhibited Bad S112 dephosphorylation by calcineurin and restored phosphorylation levels in the injured facial nuclei to 87±11% (* indicates statistical significance at $p < 0.05$ between vehicle and cypermethrin treatment) and 100±12% (** indicates statistical significance at $p < 0.01$ between vehicle and FK-506 treatment) of the uninjured facial nuclei, respectively.
3.2.4 DISCUSSION

I have demonstrated that three agents whose sole commonality is the inhibition of calcineurin to enhance the survival of motor neurons following acute injury. Two of these agents (CsA and FK-506) have previously been shown to enhance neuronal survival \textit{in vivo}, while the third (cypermethrin) has not been previously described with respect to neuronal survival effects \textit{in vivo}. By contrast examination of reagents representing alternative signaling pathways (17-AAG) or a non-calcineurin inhibiting FKBP-dependent immunosuppressant (rapamycin) failed to enhance the survival of motor neurons following axotomy. With respect to neuronal survival, FK-506 and to a lesser extent CsA, at daily doses of 3 mg/kg and 20 mg/kg, respectively, significantly enhanced motor neuron survival following axotomy. We have sought to determine the effects of these ligands in mice due to several experimental advantages. Previous studies have shown that administration of CsA or FK-506 enhanced motor neuron survival following neonatal facial axotomy in rat (Tao and Aldskogius, 1998). While rats exhibit several advantages as a mammalian model system including size and detailed physiologic knowledge, this system poses several obstacles to complete mechanistic studies including genetic heterogeneity, difficulty in performing homologous gene targeting and incomplete genome sequencing status. By contrast, several strains of mice including 129/SvImJ are well-characterized inbred strains well-suited to gene deletion and targeting studies and whose genomes have been fully sequenced (Simpson et al., 1997). Compared to previous findings in rat, application of CsA in mice demonstrated lower levels of motor neuron rescue following injury (rat – 34%, mouse – 27% at doses of 17.5 mg/kg and 20 mg/kg, respectively). While administration of FK-506 resulted in comparable
levels of motor neuron survival between these species (rat – 35%, mouse – 40% survival), the dosage required to obtain this level of neuronal survival was greater for mice (rat – 1 mg/kg, mouse – 3 mg/kg). These effects may reflect differences in drug metabolism between the two species, as mice exhibit higher rates of metabolism for a number of known drugs (Komura and Iwaki, 2007). They are unlikely to be due to differential access through the blood brain barrier, as this structure is not yet fully established in rodents over the period of examination (PND 3-10) (Moos and Mollgard, 1993). In mice, elevation of the applied dose of CsA or FK-506 beyond the above levels did not enhance motor neuron survival further, suggesting that the dosages applied are at or above optimal levels. Similarly, combined application of CsA and FK-506 did not further enhance motor neuron survival following injury, suggesting that these agents work through similar signaling pathways.

Given the immunosuppressive nature of CsA and FK-506, it might be postulated that the observed enhancement in motor neuron survival mediated by these agents is related to their suppression of immunologic phenomena retarding the rate of destruction of facial motor neurons. This is unlikely for several reasons. (1) FK-506 and CsA act to protect motor neurons from cell death over a time period which is incompatible with immunosuppression (Borel et al., 1977), given that suppression of caspase-3 activity is seen in the facial nucleus by 20 hours post-injury (earliest time point examined). (2) In addition, if CsA and FK-506 mediated motor neuron survival through suppression of inflammatory responses within the facial nucleus, one would expect cellular processes such as reactive gliosis and infiltration of activated microglia to be suppressed in the presence of these drugs; both of which were not observed in the present study. (3)
Finally, if direct or immune-stimulated cell-cell interactions were a significant cause of motor neuron death within the facial nucleus, dying motor neurons would not exhibit the apoptotic morphologic and biochemical features which have previous been well-characterized for this lesion paradigm (de Bilbao and Dubois-Dauphin, 1996; Kanungo et al., 2008). Rather, the data demonstrate that the neuronal survival effects observed are the result of a cell autonomous reduction in PCD with facial motor neurons.

If the immunophilin ligands CsA and FK-506 act in a cell intrinsic manner to reduce PCD in injured motor neurons, what is the mechanism of these effects? Neuroprotection by cyclosporin A has long been rationalized in terms of an inhibitory effect upon mPTP formation through interaction with cyclophilin D (Matsumoto et al., 1999; Snyder et al., 1998b). However, with respect to enhancing facial motor neuron survival, this appears unlikely to be the principal mechanism given that: (1) combined application of CsA and FK-506 does not result in elevated levels of motor neuron survival above that seen with FK-506 alone, suggesting that these agents act through similar signaling mechanisms; (2) FK-506 does not interfere with mPTP formation in the manner that CsA does; and (3) the maximum rescue effect of FK-506 is significantly greater than that seen with CsA. These effects suggest that the survival promoting effects of CsA lie beyond a mPTP effect.

An alternative mechanism recently proposed to explain the neurotrophic/neuroprotective effects of FK-506 and CsA, highlights the ability of both of these agents to disrupt binding between components of steroid hormone receptor complexes (Hsp-90 and p23) (Gold et al., 1999; Gold and Zhong, 2004). In this model, drug-immunophilin complexes [FK-506 with FKBP-52 and CsA potentially with
cyclophilin-40 (Carrello et al., 1999; Owens-Grillo et al., 1995; Ratajczak and Carrello, 1996)] disrupts the interaction of Hsp-90 with other components of the receptor complex, thereby allowing p23 to dissociate and initiate ERK signaling pathways (Gold et al., 1999; Gold and Zhong, 2004). As FKBP-52 and Cyp40 share a similar binding site on Hsp-90 (Owens-Grillo et al., 1995; Ratajczak and Carrello, 1996); this pathway provides a common putative signaling mechanism for FK-506 and CsA, respectively. To examine whether the inhibition of Hsp-90 plays a role in regulating the neuronal survival observed with FK-506 and CsA treatments, Hsp-90 was pharmacologically inhibited using 17-AAG. Using several different dosing regimens at 17-AAG concentrations previously shown to inhibit Hsp-90 in mice in vivo (Kociok et al., 2007; Waza et al., 2005), no enhancement of motor neuron survival was observed following axotomy. Thus with respect to acute motor neuron injury (in contrast to neuritic outgrowth) (Gold et al., 1999), Hsp-90-mediated signaling does not appear to regulate the survival promoting effects of CsA and FK-506.

The immunosuppressive drug rapamycin principally forms a drug-protein complex with FKBP-12 and/or FKBP-52, destabilizing the mTOR complex 1 (mTORC1) thus eliminating its kinase activity (Liu et al., 1991). For the experiments described in the present studies performed using rapamycin, the dose utilized was not observed to significantly enhance levels of neuroprotection. Potentially this might reflect a lack of pharmacologic inhibition by this agent toward the desired target (calcineurin). However the dose utilized has previously been shown in previous of studies to be appropriate for the in vivo studies performed (Ehninger et al., 2008). Consistent with this, while controversial findings exist regarding the neuroprotective potential of mTOR inhibition
by rapamycin (Erlich et al., 2007; Hu et al., 2010; Malagelada et al., 2010), there is currently little evidence to support the notion that the neuroprotective effects mediated by cyclosporin A and FK-506 involve mTOR inhibition; as neither of these drugs inhibits mTOR function (Yip et al., 2010).

The results in facial motor neurons suggest that it is the ability of CsA and FK-506 to inhibit calcineurin phosphatase activity which is the principal cause of the observed effects. Such a possibility has previously been proposed to explain the neurotrophic/neuroprotective actions of these agents (Dawson et al., 1993; Snyder et al., 1998a). However in recent years, attention has shifted away from calcineurin as a neural target as new non-immunosuppressive (i.e., calcineurin-independent) FK-506 derivatives have been suggested to possess neurotrophic/neuroprotective activities in several neural injury paradigms (Guo et al., 2001; Powers et al., 2004; Steiner et al., 1997b). To determine the role which calcineurin inhibition plays in regulating the survival promoting effects of CsA and FK-506, I examined the ability of cypermethrin, a type II synthetic pyrethroid insecticide which is a cell permeable, immunophilin-independent inhibitor of calcineurin to promote neuronal survival. The results demonstrate for the first time, the ability of cypermethrin to promote motor neuron survival following injury in vivo. Given the alternative mechanistic modes of cypermethrin, CsA and FK-506 action, these data indicate that it is the common inhibition of calcineurin activity which mediates the survival promoting effects observed in facial motor neurons. To further distinguish the lack of immunophilin binding as a requirement in mediating these effects, rapamycin [an immunophilin ligand which exerts its immunosuppressive effects via FKBP binding but independent of calcineurin inhibition (Kuo et al., 1992)] was examined with respect to
facial motor neuron survival, and found to be without effect. Thus, FKBP binding alone by rapamycin (or other immunophilin ligands) is insufficient for neuroprotection in the absence of calcineurin inhibition.

The concept that the inhibition of calcineurin activity can enhance motor neuron survival is attractive in that this phosphatase is highly expressed within CNS tissue, and is known to dephosphorylate pro-apoptotic Bcl-2 protein Bad at residues critical to the promotion of its pro-apoptotic activity (Datta et al., 2000; Datta et al., 2002; Springer et al., 2000; Tan et al., 2000; Wang et al., 1999; Yang et al., 2004; Zha et al., 1996). In this study, I have shown that Bad S112 phosphorylation is reduced significantly in facial motor neurons following axotomy, and that the inhibition of calcineurin by FK-506 or cypermethrin can largely reverse this effect. This is the first demonstration that cypermethrin or similar agents in its class have been shown to enhance neuronal survival \textit{in vivo} following injury. Together with the results seen in 17-AAG, rapamycin, cyclosporin A and FK-506-treated animals, these data clearly demonstrate the critical role which calcineurin and its influence on mitochondrial signaling has in regulating the survival of motor neurons following injury \textit{in vivo}.

While the current study provides evidence to connect calcineurin inhibition, Bad phosphorylation status and enhanced survival of facial motor neurons following injury, parallel mechanisms also exist with respect to calcineurin-dependent, Bad–independent pathways which may also play a role in eliciting components of the neuroprotective effects observed using cyclosporin A, FK-506 and cypermethrin. One candidate in this regard is the calcineurin-mediated dephosphorylation/activation of NFAT. Such modification results in exposure of the NFAT nuclear localization signal (NLS). NFAT
activation has previously been shown to enhance neuronal survival following injury through neurotrophic factors such as BDNF. In support of this, several studies have demonstrated a requirement for NFAT activation in promoting transcriptional enhancement of BDNF (Groth and Mermelstein, 2003; Vashishta et al., 2009). However studies have also reported that activated NFAT may amplify neuronal cell death signals through the promotion of Fas ligand levels in injured neurons (Gomez-Sintes and Lucas, 2010; Jayanthi et al., 2005; Luoma and Zirpel, 2008). NFAT activation has also been implicated in the recruitment of activated astrocytes to the site of neural injury (Jones et al., 2003a). Thus there is evidence to suggest that calcineurin-dependent NFAT activation may induce both protective and detrimental effects with respect to the survival of injured neurons (Fernandez et al., 2007; Sama et al., 2008). Thus in contrast to the Bad-mediated calcineurin mechanisms described, the net effect of NFAT activation on the neuronal injury paradigms examined is unclear.

In addition to the calcineurin-mediated effects described, it should be noted that pro-apoptotic activity of Bad could also be regulated in a calcineurin-independent manner. Four phosphorylation sites have presently been mapped on Bad (S112, S128, S136, S155) which have been demonstrated to influence its pro-apoptotic functions (Zha et al., 1996). Calcineurin-mediated effects have been principally associated with dephosphorylation of serine residues 112 and 155. Dephosphorylation at S112 reduces the affinity for Bad for the cytosolic scaffolding protein 14-3-3 (Springer et al., 2000; Wang et al., 1999) thereby inhibiting Bad function and promoting cell survival. Similarly dephosphorylation at S155 within the core BH3 motif of Bad removes a negative charge, ultimately decreases the affinity of Bad for anti-apoptotic factors such as Bcl-xL, thereby
promoting cell death (Tan et al., 2000; Yang et al., 2004). Calcineurin however is not the only phosphatase know to target Bad. Protein phosphatase 2A (PP2A) (Chiang et al., 2001; Chiang et al., 2003), PP1 (Ayllon et al., 2001; Klumpp et al., 2004) and PP2C (Klumpp et al., 2003) also target this protein. Counteracting the effects of calcineurin, Bad can be phosphorylated by a number of kinases including protein kinase A (Harada et al., 1999), JNK (e-Jun N-terminal kinase) (Kamada et al., 2007; Wang et al., 2007), Akt (Datta et al., 1997), p70 S6 kinase (Harada et al., 2001; Pastor et al., 2009) and Raf-1 (Alejandro and Johnson, 2008; Wang et al., 1996).

Interestingly in addition to its neuroprotective effects, cyclosporin A was observed to measurably enhance levels of caspase-3 activation in uninjured facial motor neurons at PND3. This finding was only observed only in cyclosporin A-treated animals, and not observed in the FK-506 treatment group. As such these effects appear unrelated to the common calcineurin-mediated effects observed for both drugs, but rather tied to cyclophilin-dependent events. In terms of mechanistic explanations for these effects, several possibilities exist. Among these, inhibition of mPTP by cyclosporin A may have disrupted normal mitochondrial calcium oscillations linked to mitochondrial respiration and other aspects of cellular metabolism (Chance, 1965; Duchen, 1999; Gunter et al., 1994; Hajnoczky et al., 1995; Kupzig et al., 2005; McCormack and Denton, 1993). Consistent with this, it was recently demonstrated in the heart muscles of Ppif knockout mice (encoding cyclophilin D) exhibit a substantial cardiac hypertrophy and fibrosis due to loss of metabolic flexibility resulting from elevation in mitochondrial matrix calcium levels (Elrod et al., 2010). Alterations of such cellular functions in neurons in my study could have provided a stress signal which neurons could respond to by inducing caspase-
3 activation. Alternatively, given that cyclophilins are known for their peptidyl-prolyl cis-trans isomerase (PPIase) activity, inhibition of this function may result in an increase in mitochondrial protein misfolding, thus acting as an additional cellular stressor (Baum et al., 2009; He and Lemasters, 2002; Rassow et al., 1995). However it is important to note that though cyclosporin A treatment significantly enhanced levels of caspase-3 activation, it did not statistically enhance levels of neuronal cell death in uninjured facial motor neurons beyond levels seen in vehicle treated controls.

To examine the role which specific CNA isoforms might play in regulating motor neuron PCD, I have examined survival of facial motor neurons following genetic deletion of the CNAα gene (Ppp3ca), the dominant isoform of calcineurin A in the CNS, after facial nerve axotomy. Analysis of these mice demonstrates that inhibition of CNAα activity is dispensable with respect to survival of injured facial motor neurons. In this regard, it is notable that two additional calcineurin A isoforms exist within the CNS (Ppp3cb and Ppp3cc) (Eastwood et al., 2005; Kuno et al., 1992); and these lower abundance isoforms may serve either a direct or compensatory role with respect to motor neuron injury. Thus, the CNA response of motor neurons may be similar to that seen in T cells. Despite the fact that calcineurin is the known physiologic target of CsA and FK-506 in T cells, T cells which lack Ppp3ca remain sensitive to these agents, thus demonstrating the functional redundancy which exists among calcineurin A isoforms (Zhang et al., 1996).

A variety of signaling mechanisms have previously been postulated to explain the neurotrophic/neuroprotective effects seen for CsA and FK-506. Using a variety of agents and approaches as summarized in Figure 44, I have demonstrated that it is the inhibition
of calcineurin activity which promotes neuronal survival observed in facial motor neurons following axotomy in vivo. Given this, how does one rationalize these findings with studies which demonstrate an enhancement in neural regeneration using non-immunosuppressive immunophilin ligands? A possible explanation lies in clearly distinguishing effects upon neural regeneration – neuritic outgrowth in response to an injury stimulus distal to the neuronal cell soma, and effects upon neuronal survival. This latter form occurs when the injury stimulus is proximal to the cell soma. Programmed cell death, autophagy and necrosis represent different forms of response to this type of cellular injury. In contrast, a sizeable body of experimental data suggests that more distal forms of neuronal injury trigger a distinct set of cellular response through signaling pathways such as the c-Jun/JNK pathway (Herdegen et al., 1997; Kenney and Kocsis, 1998; Villegas-Perez et al., 1993), therefore the neural regenerative effects seen with calcineurin-dependent/independent inhibitors may reflect actions on disparate components of the injury response in the CNS.

In contrast to previous studies utilizing CsA, FK-506 and related non-immunosuppressive immunophilin ligands to enhance neuronal survival following CNS injury, my data support a model in which it is the inhibition of calcineurin activity which enhances neuronal survival. As such, calcineurin inhibition is an attractive therapeutic target with significant clinical potential with respect to acute motor neuron injury for several reasons. Prior studies have demonstrated that neurotrophic factors such as GDNF, BDNF and CNTF are particularly effective in promoting neuronal survival following injury. The principal mechanism by which these agents promote neuroprotection is through the influence of Bcl-2 family proteins at the mitochondrial outer membrane; a
Figure 44. Model of Neuroprotection by Cyclosporin A and FK-506

Based upon results in facial motor neurons, the following model is proposed to explain the effects observed using CsA and FK-506. As indicated in this model, data from this study suggest that the observed survival promoting and neurotrophic effects of these immunophilin ligands are mediated through distinct sets of molecular interactions. Evidence for exclusion of specific pathway is as indicated below. (1) CsA-mediated MPTP blockade is likely not the principle route of CsA-mediated neural rescue given that CsA and FK-506 treatment exhibit several similar mechanistic features, and co-administration of CsA and FK-506 did not enhance motor neuron survival over FK-506 treatment alone. (2) Administration of CsA or FK-506 did not act to reduce levels of reactive gliosis or infiltration of activated microglia; thus the enhanced survival of facial motor neurons was not due to repression of secondary injury responses. (3) Cypermethrin, an immunophilin-independent inhibitor of calcineurin (denoted by CN), significantly enhanced motor neuron survival, hence highlighting calcineurin inhibition as a principal mechanism of regulating neuronal survival mediated by immunophilin ligands. (4) FK-506 and rapamycin both interact with FKBP-12, but only FK-506/FKBP-12 complexes inhibit calcineurin signaling. The failure of rapamycin to enhance motor neuron survival, following injury implicates calcineurin in the signaling pathway of motor neuron injury, and rules out mTOR-mediated effects. (5) Application of 17-AAG (a pharmacological inhibitor of Hsp-90) failed to exhibit enhance motor neuron survival following injury, indicating that FK-506 and CsA-mediated enhancement in neuronal survival is distinct from the documented disruption in steroid receptor complex formation involved in enhancing neuritic outgrowth. (6) Inhibition of calcineurin-mediated Bad
S112 dephosphorylation was observed at 20 hours following axotomy for treatments with a single dose of either cypermethrin or FK-506, demonstrating a common mechanism by which cypermethrin, FK-506, and possibly CsA can enhance neuronal survival following injury.
similar mechanism to that which I have now demonstrated for the calcineurin inhibitors, FK-506, CsA and cypermethrin. With respect to injury-induced motor neuron PCD, an advantage of therapeutic interventions aimed at the level of Bcl-2 family proteins is that they exhibit a significant post-injury treatment window (Putcha et al., 1999; Springer et al., 2000). Consistent with effects seen using neurotrophic factors, we have observed that neuroprotection by immunophilin ligands occur even when administration is delayed by 3 hours following injury. These effects on motor neuron PCD are unlikely to be primarily the result of effects on calcium entry, since neuronal rescue seen using calcium chelators such as BAPTA (Tymianski et al., 1993), has been shown to be efficacious only when given as a pre-treatment. In contrast to neurotrophic factors however, substantial clinical experience with FK-506 and CsA demonstrates that application of these agents in humans does not result in the serious side effects which halted human clinical trials of neurotrophic factors for motor neuron injury (Henderson et al., 1996; Henderson et al., 1994b). Also, small molecule inhibitors of calcineurin circumvent other drawbacks seen with respect to clinical application of neurotrophic factors, such as their poor CNS distribution and blood-brain barrier permeability and the difficulties associated with manufacture and certification of such agents.

Despite their long history, the mechanism by which agents such as CsA and FK-506 act to promote neuroprotection remains controversial. In the present study, I have demonstrated that with respect to their ability to promote neuronal survival in vivo following acute motor neuron injury, these agents act through the inhibition of calcineurin, and excluded several other potential actions of these agents as causes for the observed effects. While the current study had examined the effects of calcineurin
inhibitors on motor neuron survival following injury, it is important to note that numerous studies have demonstrated that CsA and FK-506 are capable of protecting a variety of neuronal targets in a number of *in vivo* injury paradigms, ranging from stroke, to Parkinson’s disease, to methamphetamine-induced neurotoxicity (Butcher et al., 1997; Furuichi et al., 2003; Guo et al., 2001; Koike et al., 2005). Hence the clinical potential of small molecule calcineurin inhibitors to promote neuronal survival may extend well beyond that of acute motor neuron injury.

Finally, it has been suggested that several non-immunosuppressive (calcineurin-independent) immunophilin ligands may exhibit neuroprotective effects. While a clear picture has not yet emerged regarding the mechanism of these effects, it will be interesting to see if the distinction we observe between neuroprotective versus neuritic outgrowth pathways in motor neurons is maintained in other neural injury paradigms. Such findings will allow us to gain greater insight into the mechanism by which different neuronal populations within the mammalian CNS act to regulate the various aspects of neural injury.
STUDY 3: CASPASE-3 DEFICIENCY REVEALS A PHYSIOLOGIC ROLE FOR
SMAC/DIABLO IN REGULATING PROGRAMMED CELL DEATH

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3.3.1 ABSTRACT

IAP-binding proteins such as Grim, Reaper and HID possess a critical role in regulating caspase activity in *D. Melanogaster*. However a comparable role for their mammalian homologue Smac/DIABLO has yet to be clearly established *in vivo*. Despite recent interests in Smac mimetics to enhance chemotherapeutic potency, our understanding of the true physiologic nature of Smac/DIABLO in regulating programmed cell death (PCD) remains elusive. In order to critically evaluate the role of Smac/DIABLO in PCD regulation, caspase-3 deficiency was used as a sensitizing mutation to reduce total executioner caspase activity. We observe that combinatorial deletion of *Diablo* and *Casp3* results in perinatal lethality, and examination of both intrinsic and extrinsic PCD in murine embryonic fibroblasts demonstrate that loss of Smac/DIABLO alters both caspase-dependent and caspase-independent PCD. Knockdown of other executioner caspases and relevant IAP members supports a model in which Smac/DIABLO acts to enhance executioner caspase activity through modulating inhibitory interactions between specific IAP family members and executioner caspase-3 and -7.
3.3.2 INTRODUCTION

As shown in the previous sections, via pharmacologic and genetic means I have examined the role of mitochondria-dependent PCD signaling with respect to both developmental and injury-induced apoptosis for different neural populations. However, the mechanisms by which negative regulation of this pathway is controlled remain elusive. Extensive studies of Bcl-2 family members have demonstrated that the interactions of pro- and anti-apoptotic family members along the outer mitochondrial membrane can facilitate the release of pro-apoptotic proteins such as holo-cytochrome c, second mitochondrion-derived activator of apoptosis/direct IAP-binding protein with low pl (Smac/DIABLO), apoptosis inducing factor (AIF) and endonuclease G (EndoG) from the intermembranous space to the cell cytoplasm. While the pro-apoptotic functions of AIF and EndoG are largely confined to regulating nuclear changes during PCD (Li et al., 2001; Parrish et al., 2001; Susin et al., 1996), holo-cytochrome c and Smac/DIABLO appear to regulate PCD signaling through the initiation and promotion of caspase activity, respectively (Du et al., 2000; Liu et al., 1996; Verhagen et al., 2000). Upon release into the cytoplasm holo-cytochrome c interacts with Apaf-1 to promote dATP/ATP-dependent oligomerization (Acehan et al., 2002; Reubold et al., 2009; Riedl et al., 2005; Yu et al., 2005). This hetero-oligomeric complex known as the apoptosome functions as a template to promote the catalytic activation of caspase-9, which subsequently cleaves downstream executioner caspases such as caspase-3 (Rodriguez and Lazebnik, 1999; Yin et al., 2006).

By contrast, the principal function identified to date for mammalian Smac/DIABLO is based upon *in vitro* biochemical studies of its (inhibitory) interactions with a group of proteins known as inhibitor of apoptosis proteins (IAPs). As exemplified
by XIAP, these proteins act primarily to inhibit caspases during PCD (Du et al., 2000; Ekert et al., 2001; Goping et al., 2003; Sutton et al., 2003; Verhagen et al., 2000). The interaction of Smac/DIABLO with IAPs is mediated through an IAP binding motif (IBM). Such IBMs have been identified in Smac/DIABLO and Omi/HtrA2 in mammals, as well as Grim, Reaper, HID (Head Involution Defective) and Sickle in D. melanogaster (RHG proteins) (Riedl and Shi, 2004; Yan and Shi, 2005). The IBM motif contains a conserved amino-terminus in combination with a hydrophobic tetrapeptide sequence beginning with alanine. This sequence mediates interaction to cognate BIR (baculoviral IAP repeat) domains present on IAPs (Shi, 2002). Known mammalian IAP proteins consist of Naip1 (neuronal apoptosis inhibitory protein 1), cIAP-1 and 2 (cellular inhibitor of apoptosis proteins), XIAP (X-linked inhibitor of apoptosis), survivin, BRUCE (BIR repeat-containing ubiquitin-conjugating enzyme)/Apollon and livin. Each of these contains at least one BIR domain, allowing them to potentially interact with both IBM containing proteins and caspases-3, -6, -7 and -9. Largely through biochemical studies, IAPs such as XIAP have been shown to inhibit caspases through either direct binding, or as a consequence of accelerating their rate of proteasomal degradation. Indeed, cIAP-1, -2, XIAP, BRUCE/Apollon and livin have each been shown to possess E3 ubiquitin ligase activity. Despite this, genetic deletion of several IAP members (XIAP, cIAP-1, cIAP-2) result in only nominal in vivo phenotypes, likely due to potential functional redundancies among family members (Conte et al., 2006; Conze et al., 2005; Harlin et al., 2001; Olayioye et al., 2005). A notable exception to this is survivin and BRUCE/Apollon, due to their roles in microtubule organization/mitotic spindle assembly and p53 regulation, respectively (Conway et al., 2002; Ren et al., 2005).
Our laboratory has previously demonstrated the critical role of cytochrome c and its activation of executioner caspases in regulating several forms of neuronal cell death following acute neural injury (Kanungo et al., 2008). In contrast, the true physiologic role of Smac/DIABLO is far less clear. Despite its postulated role in promoting caspase activity during PCD, similarly to many IAP family members, mice which lack Diablo do not exhibit an overt phenotype, and Diablo null MEFs, lymphocytes and hepatocytes exhibit wild-type responses to all PCD stimuli thus far examined (Okada et al., 2002). Though functional redundancy between Smac/DIABLO and Omi/Htra2 was initially suggested as a plausible explanation for these effects, Diablo/Htra2 double null mutants do not exhibit any exacerbation compared to parental phenotypes alone (Martins et al., 2004). Htra2 null mice do exhibit a neurodegenerative phenotype; however this has been shown to be the result of a loss in serine protease activity independent of the IBM motif. Thus while in vitro overexpression and biochemical studies suggest that Smac/DIABLO may be capable of enhancing executioner caspase activity, the physiologic evidence for such a role remains elusive. This role is all the more important given the tremendous focus in recent years upon Smac mimetics as potential anti-cancer agents due to Smac/DIABLO’s putative role in inhibiting the inhibitory effects of IAPs on caspase activity.

Given that both intrinsic and extrinsic PCD pathways activate a common set of downstream effector caspases, such targets represent an important site of coordinated control with respect to the regulation of PCD signaling. Biochemical and genetic deletion analyses of executioner caspases have demonstrated that for many systems caspase-3 comprises the dominant executioner caspase activity owing to its greater comparative
expression and activity compared to caspase-6 and -7 (Slee et al., 2001; Walsh et al., 2008). Loss of Casp3 results in embryonic (129S1/SvImJ) or perinatal lethality (C57BL/6J) depending on the genetic background (Kuida et al., 1996; Woo et al., 1998). The observed differences in survival seen in these Casp3 null strains correlate with differences in levels of caspase-7 expression within the central nervous system; highlighting apparent functional redundancies between these two caspases (Houde et al., 2004). Recent studies of Casp3/Casp7 combinatorial knockouts support this interpretation as double mutants exhibit embryonic lethality even on a C57BL/6J background (Lakhani et al., 2006). Consistent with this, MEFs derived from Casp3/Casp7 null mutants exhibit enhanced resistance to a variety of PCD stimuli compared to that observed for of the parental mutants. Such findings highlight the importance of maintaining overall levels of executioner caspase activity within specific limits.

In the present study we have examined the physiologic role of Smac/DIABLO in regulating various forms of programmed cell death. Through genetic ablation of Casp3, we have reduced the total level of executioner caspase activity to reveal the relative inhibitory influences of Smac/DIABLO on IAP family members. Using this approach we demonstrate for the first time a physiologic role for Smac/DIABLO in regulating several distinct forms of PCD. Examination of the nature of these interactions in primary mouse embryonic fibroblasts indicates that Smac/DIABLO functions to fine-tune total levels of executioner caspase activity within the cell by modulating inhibitory interactions of several IAP family members in vivo.
3.3.3 RESULTS

3.3.3.1 Combinatorial Deletion of Casp3 and Diablo Results in Perinatal Lethality

Prior and our own investigations demonstrate that Diablo null mice do not exhibit overt differences at either macroscopic or cellular levels compared to littermate controls (Martins et al., 2004; Okada et al., 2002). With respect to injury-induced PCD, our analysis of developmental and axotomy-induced motor neuron death in Diablo null mice also demonstrated no differences compared to controls (Figure 45). In order to enhance the sensitivity of our analyses of the effects of Smac/DIABLO on the IAP-caspase signaling axis, we sought to reduce total levels of executioner caspase activity. As caspase-3 is the dominant source of executioner caspase activity for the PCD models we have examined, we investigated the effect of Diablo deletion in the context of reduced caspase-3 activity. In this context loss of caspase-3 acts as a form of sensitizing mutation, enhancing the influence of IAPs. As shown in Table 7, several test crosses were set up to examine the progeny of single and combinatorial Casp3/Diablo deletion. Consistent with previous observations on a C57BL/6J background, Casp3 null mice were born at a lower than expected Mendelian ratio with slightly less than half surviving to adulthood (Woo et al., 1998). Despite normal ratios of Diablo null births, surprisingly no viable Casp3/Diablo null mice were ever observed postnatally. To determine the temporal nature of these effects, we extended our analysis to prenatal time points. As shown in Table 7, a series of timed breeding demonstrated that Casp3/Diablo null embryos could still be recovered at the expected Mendelian frequency up to embryonic day 18.5. These data demonstrate that combinatorial loss of Casp3/Diablo results in perinatal lethality. The synergistic effect of combinatorial deletion of Casp3 and Diablo clearly
Figure 45. Diablo Deletion Alone Does Not Alter Levels of Motor Neuron Survival Following Axotomy

Using the well-characterized and highly reproducible facial nerve axotomy injury paradigm, the genetic ablation of Diablo did not affect the survival of facial motor neurons following axotomy-induced neuronal death. (A-B) At seven days following facial nerve axotomy, paraffin wax sections were prepared through the entire extent of the facial nuclei. In wild-type animals, this injury paradigm typically results in a substantial loss of facial motor neurons (typically 80-85% death) at the time of analysis. (C-D) The absence of Smac/DIABLO was not able to rescue any facial motor neurons from axotomy-induced death. (E) Total facial motor neuron counts were conducted in the contralateral and ipsilateral facial nuclei for homozygous null animals and heterozygous controls. No significant difference was observed between homozygous null animals (15±5%) and heterozygous controls (15±5%).
Facial nerve axotomy

<table>
<thead>
<tr>
<th>Total facial motor neurons</th>
<th>Diablo+/-</th>
<th>Diablo-/-</th>
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</thead>
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<tr>
<td>14.8% contralater facial nuclei</td>
<td>14.6% ipsilateral facial nuclei</td>
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E
Table 7. Combinatorial Deletion of Casp3 and Diablo Results in Early Perinatal Lethality

Examination of viable pups at PND3 demonstrates that Casp3/Diablo double knockouts are not observed; in contrast to each of the parental mutants alone. Analysis of time-mated dams sacrificed at the indicated periods (E13.5, E14.5, E15.5, E18.5) demonstrates that viable Casp3/Diablo DKO embryos can be recovered in approximately normal ratios on and before E18.5, suggesting that deaths occurs perinatally.

\(^a\)As demonstrated previously, half of all Casp3 null mutants on a C57BL/6J background die perinatally; the remainder of which survive to adulthood. Thus for an expected pool of 44 Casp3\(^{-/-}\)/Diablo\(^{+/+}\) mice, 20 survived beyond PND3. Similarly for Casp3\(^{-/-}\), Diablo\(^{+/-}\) mice, 6 mice were observed at PND3 of an expected pool of 11, which survived to adulthood. However of an expected pool of 56 Casp3\(^{-/-}\), Diablo\(^{+/-}\) mice, no viable pups were observed at PND3. Observation of pregnant dams suggests that animals die on or immediately following birth.
Table 7. Combinatorial Deletion of *Casp3* and *Diablo* Results in Enhanced Perinatal Lethality

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Casp3&lt;sup&gt;+/−&lt;/sup&gt;, Diablo&lt;sup&gt;+/+&lt;/sup&gt; × Casp3&lt;sup&gt;−/−&lt;/sup&gt;, Diablo&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Casp3&lt;sup&gt;−/−&lt;/sup&gt;, Diablo&lt;sup&gt;+/+&lt;/sup&gt; × Casp3&lt;sup&gt;−/−&lt;/sup&gt;, Diablo&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Casp3&lt;sup&gt;−/−&lt;/sup&gt;, Diablo&lt;sup&gt;−/−&lt;/sup&gt; × Casp3&lt;sup&gt;−/−&lt;/sup&gt;, Diablo&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Offspring genotype</td>
<td>Casp3&lt;sup&gt;+/−&lt;/sup&gt;, Diablo&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Casp3&lt;sup&gt;−/−&lt;/sup&gt;, Diablo&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Casp3&lt;sup&gt;−/−&lt;/sup&gt;, Diablo&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>Observed viable (PND3)</td>
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<td>56</td>
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<td>% observed vs. expected</td>
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<td>82%</td>
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<tr>
<td></td>
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<td>95%</td>
<td>84%</td>
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<td></td>
<td>95%</td>
<td>0%&lt;sup&gt;b&lt;/sup&gt;</td>
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* based on strict Mendelian segregation for each gene locus
demonstrates that endogenous Smac/DIABLO plays a significant physiologic role during development.

3.3.3.2 *Casp3/Diablo* Null MEFs Exhibit Enhanced Resistance to Intrinsic but Not Extrinsic PCD Stimuli

Following the effects seen in *Casp3/Diablo* null embryos we sought to determine the nature of PCD response in these animals in detail. In order to compare the results obtained with those seen previously in other PCD signaling mutants, we derived multiple lines of mouse embryonic fibroblasts (MEFs) for each of the genotypes generated. A minimum of three independently derived MEF lines was generated for DKO (Casp3⁻/⁻, *Diablo*⁻/⁻), Caspase-3 KO (Casp3⁻/⁻, *Diablo*⁺/⁻), *Diablo* KO (Casp3⁺/⁻, *Diablo*⁻/⁻), double heterozygote (Casp3⁺/⁻, *Diablo*⁺/⁻) and wild-type (Casp3⁺/⁺, *Diablo*⁺/⁺) embryos. These were then examined for their response to a variety of PCD stimuli. As shown in Figure 46A, loss of *Diablo* alone results in a significant increase in the survival of MEFs to staurosporine at both 24 and 48 hours following treatment. Interestingly the magnitude of the resistance to PCD in these lineages is comparable to that seen in *Casp3* null MEFs. A further significant increase in MEF survival to staurosporine is seen in *Casp3/Diablo* DKO MEFs compared to single knockouts and control lineages, potentially due to Smac/DIABLO’s ability to promote executioner caspase activity contributed by caspase-6 and/or caspase-7. Treatment with tunicamycin, another intrinsic pathway PCD stimulus, induces in a similar trend in cell survival to that seen with staurosporine (Figure 46B), except that DKO MEFs exhibit significant increases in survival compared to *Casp3* and *Diablo* null MEFs only at 48
Figure 46. Casp3/Diablo DKO MEFs Are Selectively Resistant to Intrinsic Pathway PCD Stimuli

A number of different PCD stimuli were used to examine the response of Casp3<sup>+/−</sup>, Diablo<sup>+/−</sup>; Casp3<sup>+/−</sup>, Diablo<sup>/−</sup>; Casp3<sup>/−</sup>, Diablo<sup>+/−</sup>; and Casp3<sup>/−</sup>, Diablo<sup>/−</sup> MEF lines to intrinsic and extrinsic mediated PCD. (A) Response to staurosporine. MEFs were exposed to 2 µM staurosporine and examined for viability at 24 and 48 hours following treatment; (B) MEFs exposed to 10 µg/ml tunicamycin and examined as above; (C) MEFs exposed to 20 J/m<sup>2</sup> UVC irradiation and examined as above. (D-E) Vehicle (DMSO) and cycloheximide treatments did not induce a significant level of cell death in MEFs of all experimental genotypes examined. (F-G) Examination of extrinsic PCD stimuli. MEF treatment with either the Fas-activating antibody Jo2 (1 µg/ml) (F); or TNF-α (10 ng/ml) in combination with the protein synthesis inhibitor cycloheximide (1 µg/ml) (G), induced similar levels of cell death all MEFs lineages examined. (H) To determine whether nuclear translocation of AIF occurs normally in DKO MEFs, AIF subcellular localization was examined at the indicated time points following UVC irradiation. The results demonstrate that AIF nuclear translocation is significantly reduced in DKO MEFs by 6 hours post-irradiation. However when examined at 9 and 12 hours post-irradiation, similar level of AIF translocation were observed in DKO MEFs compared to controls (data not shown). The results suggest that nuclear translocation of AIF is slowed but not inhibited in Casp3/Diablo MEFs. Summaries show results generated from a minimum of three independent MEF lines per genotype, experiments performed for each line in triplicate. * indicates statistical significance of \( p < 0.05 \) between single knockout and wild-type control MEFs and # indicates statistical
significance of $p < 0.05$ between DKO and single knockout MEFs. † indicates statistical significance of $p < 0.05$ between DKO and *Diablo* null MEFs. *’ and #’ (additional apostrophe) indicates statistical significance at $p < 0.01$. 
hours post-treatment, perhaps due to the more protracted nature of PCD seen with this
agent. As shown in Figure 46C, exposure of MEFs to 20 J/m² UVC results in a pattern of
PCD resistance similar to that seen with staurosporine and tunicamycin by 12 hours post-
treatment. Thus for three widely recognized intrinsic pathway PCD stimuli, Diablo null
MEFs exhibited enhanced levels of resistance to PCD similar in magnitude that seen in
Casp3 knockouts. Combinatorial deletion of Casp3 and Diablo resulted in a further
enhancement of PCD resistance, demonstrating that the actions of Smac/DIABLO on
intrinsic pathway PCD extend beyond effects on caspase-3 alone. In contrast, when
MEFs were exposed to either the Fas-activating antibody Jo2 or TNF-α plus
cycloheximide, single knockouts and DKO MEFs exhibited no enhanced resistance to
PCD with respect to controls (Figures 46D, E), suggesting that while caspase-3 and
Smac/DIABLO play unique and significant roles in regulating intrinsic PCD, they appear
dispensable with respect to extrinsic stimuli. In these experiments no significant
contribution of cell death was observed for vehicle or with cycloheximide (Figures 46F,
G).

Previously it has been shown that Casp3/Casp7 DKO MEFs do not exhibit any
AIF nuclear translocation following UV irradiation. We performed a similar analysis in
Casp3/Diablo DKO MEFs. Interestingly, though AIF nuclear translocation was delayed
in combinatorial but not single mutants of Casp3 and Diablo (Figure 46H); however it
was observed to proceed to completion by 9-12 hours post-irradiation. Thus while loss of
Casp3 and Diablo delays AIF nuclear translocation following UVC treatment, this
process was nonetheless capable of being performed in the absence of these proteins.
3.3.3.3 Executioner Caspase Activation in Casp3/Diablo Double Knockout MEFs

In order to investigate the mechanism behind the enhanced resistance of DKO MEFs to PCD, we examined the nature and pattern of executioner caspase activation in wild-type MEFs following PCD several forms of stimulation. As shown in Figure 47A, while cleavage of caspase-3 and -7 was rapid and followed similar kinetics, caspase-6 (Figure 47B) was activated much more slowly and did not proceed to completion following staurosporine treatment. Caspase-6 thus appears to play a secondary role with respect to staurosporine-induced PCD. We next examined caspase-6 and -7 activation in genetically modified MEFs during staurosporine-induced PCD. As shown in Figure 47C, ablation of caspase-3 delayed activation of both caspase-6 and caspase-7, consistent with previous studies demonstrating caspase-3’s ability to activate other executioner caspases (Denault and Salvesen, 2003; Inoue et al., 2009; Slee et al., 1999; Srinivasula et al., 1996). Loss of Diablo however did not significantly alter the activation profile of either caspase-6 or caspase-7, and activation of these caspases did not differ significantly between DKO and Casp3 KO MEFs despite an enhanced resistance to a variety of PCD stimuli. Previous biochemical studies have indicated that Smac/DIABLO can disrupt the inhibitory interaction of XIAP (and potentially other IAP family members) on caspase-9 dimerization (Kulathila et al., 2009; Shiozaki et al., 2003). However our results using MEFs with staurosporine suggest that this interaction is not of sufficient magnitude in vivo to be physiologically significant with respect to caspase activation. To examine this process further we used embelin, a small molecule antagonist with affinity for the BIR3 domain of XIAP as an independent means of interrupting the interaction of XIAP and caspase-9 (Nikolovska-Coleska et al., 2004). Consistent with the above results, embelin
Figure 47. Activation of Executioner Caspases Is Not Significantly Altered by Deletion of *Casp3* and *Diablo*

Activation of executioner caspases was examined following chronic exposure to staurosporine. **(A)** In wild-type MEFs, active forms of caspase-3 and -7 appeared with similar kinetics. In contrast, activation of caspase-6 **(B)**, was observed to occur in a more delayed manner and at a lower levels of conversion than that seen for either caspase-3 or caspase-7. **(C)** Time course of caspase-6 and caspase-7 activation for each of the *Casp3/Diablo* genotypes. The results demonstrate that loss of *Diablo* does not further reduce rates of caspase-6 or caspase-7 activation beyond that seen in *Casp3* null MEFs. Results shown are representative of two independent experiments using two independently derived MEF lines for each genotype. **(D)** Although XIAP has been demonstrated to inhibit caspase-9 via interaction of its BIR3 domain with the dimer interface of caspase-9, thus rendering it incapable of activating downstream executioner caspases, this inhibitory effect is unlikely to be significant at a physiologic level. This was demonstrated in **(C)** as the loss of Smac/DIABLO, and consequently the resultant uninhibited activity of XIAP, did not significantly alter the activation of executioner caspases. This was further examined by using a pharmacologic inhibitor of XIAP, embelin, which interacts with its BIR3 domain to determine whether inhibition of XIAP at its BIR3 domain would potentiate staurosporine-induced cell death. It was observed that embelin co-treatment (30 µM) did not enhance cell death induced by staurosporine (2 µM) in any of the experimental genotypes examined when compared to staurosporine and vehicle co-treatment.
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<th>Time (hours)</th>
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<th>6</th>
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<tr>
<td>pro-caspase-6 (35 kDa)</td>
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<tr>
<td>β-actin (42 kDa)</td>
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</tbody>
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C  
- Casp3+/-, Diablo+/-
- Casp3+/-, Diablo-/-
- Casp3-/-, Diablo+/-
- Casp3-/-, Diablo-/-

D  
Staurosporine + Embelin

- % survival
- STS + DMSO
- STS + EMB
did not enhance staurosporine-induced cell death (Figure 47D), indicating that a
disruption of IAPs’ inhibitory actions on caspase-9, either through Smac/DIABLO or a
small molecule, is unlikely to be significant at a physiologic level.

3.3.3.4 Severe Impairment of Executioner Caspase Activity in Casp3/Diablo DKO MEFs

One potential explanation for the observed changes in cell viability seen in single
KO and DKO MEFs is that executioner caspase activity was so significantly inhibited
through combinatorial loss of caspase-3 and Smac/DIABLO such that the remaining
executioner caspase activity was insufficient to allow PCD to proceed. Levels of
executioner caspase activity were therefore examined following PCD stimulation using
two independent methods, a fluorogenic caspase substrate and poly (ADP-ribose)
polymerase (PARP) cleavage. Using the fluorogenic caspase substrate DEVD-Rho110
(Previously shown to be a target of caspase-3, -6 and -7) (Baumgartner et al., 2009;
Walsh et al., 2008), executioner caspase activity was measured in MEFs at various time
points following staurosporine treatment (Figure 48A). In the absence of caspase-3,
executioner caspase activity appeared almost absent, although trends to be lower in
Casp3/Diablo DKO MEFs than in Casp3 KO MEFs following treatment with
staurosporine. Similar trends were observed following UVC irradiation and Jo2 treatment
(Figure 48B, C), suggesting that these observations are not specific to staurosporine. In
parallel we examined cleavage of the physiologic executioner caspase substrate PARP,
which has previously been shown to be cleaved by all three executioner caspases (Slee et
al., 2001). As shown in Figure 48D, full-length PARP (116 kDa) was processed by
executioner caspases to its cleaved form (89 kDa) in wild-type, Diablo null and Casp3
Figure 48. Executioner Caspase Activity Is Significantly Reduced in MEFs by the Ablation of Casp3 and Diablo Following Staurosporine Treatment

(A) DEVD-dependent caspase activity was measured in MEF lines following staurosporine treatment using the fluorogenic substrate DEVD-Rho110. The results demonstrate that the additional loss of Smac/DIABLO does not further reduce DEVD-dependent caspase activity beyond that seen in Casp3 null MEF lines. (B-C) In order to verify that this was not a phenomenon specific to staurosporine treatment, other standard PCD stimuli such as Fas activation by the Jo2 antibody and UVC irradiation were used to induce executioner caspase activity. It was observed that similar to staurosporine treatment, no significant executioner caspase activity could be measured using this fluorogenic caspase substrate in MEFs lacking caspase-3. Data shown are representative of three independently derived MEF lines for each genotype described. (D) To examine physiologic caspase-dependent cleavage toward intracellular targets, PARP cleavage was monitored as a function of staurosporine treatment. The results demonstrate that Casp3/Diablo DKO MEFs exhibited a substantial reduction in PARP cleavage compared to Diablo null and Casp3 null MEFs. Data are representative of two independent experiments using two independently derived MEF lines for each genotype described.
null MEFs within 6 hours following staurosporine exposure. However PARP cleavage does not occur in Casp3/Diablo DKO MEFs (at least within detectable limits), indicating that PARP cleavage is inhibited in MEFs only in the absence of both caspase-3 and Smac/DIABLO (Figure 48D, panel 4). These findings are consistent with the observation that the combinatorial deletion of Casp3 and Diablo in MEFs results in enhanced resistance against a wide range of PCD stimuli, and argues that insufficient executioner caspase activity alone is the cause of the enhanced PCD resistance observed.

### 3.3.3.5 Caspase-7 but Not Caspase-6 Is Responsible for PARP Cleavage Observed in the Absence of Caspase-3

Given that PARP cleavage was severely impaired only in DKO MEFs, it was of interest to identify the source of the executioner caspase activity mediating PARP cleavage in the absence of caspase-3 as this represented a target of Smac/DIABLO action. To examine this, we utilized caspase-6 and caspase-7-specific siRNAs to knockdown levels of these proteases in wild-type and mutant MEFs. By comparing levels of PARP cleavage following staurosporine exposure in MEFs treated with either scrambled control (Figure 49A), caspase-6 (Figure 49B), or caspase-7 (Figure 49C) -specific siRNA, the influence of each of these caspases could be determined. In comparison to controls (Figure 49A), caspase-6 siRNA knockdown did not alter the pattern of PARP cleavage for any of the genotypes examined (Figure 49B). However knockdown of caspase-7 resulted in a significant inhibition of PARP cleavage even in Casp3 KO MEFs following treatment with staurosporine (Figure 49C, panel 3), indicating that it is caspase-7 and not caspase-6, which acts in a redundant manner with
Figure 49. Caspase-7 but Not Caspase-6 Functions in a Redundant Manner with Caspase-3 to Cleave PARP

PARP cleavage was examined following staurosporine exposure in MEFs for each genotype described following treatment with (A) scrambled control siRNA, (B) caspase-6 or (C) caspase-7 knockdown. The results demonstrate that caspase-6 knockdown did not significantly alter the nature or extent of PARP cleavage, while inhibition of caspase-7 reduced levels of PARP cleavage in Casp3 KO MEFs to levels seen in Casp3/Diablo DKO lineages. (D) Cell viability as determined at 24 hours following staurosporine treatment in the presence of either caspase-6 or caspase-7 siRNA treatment. In agreement with previous studies, caspase-3 appears to be the dominant contributor of executioner caspase activity. Knockdown of caspase-7 did not alter cell viability in response to staurosporine treatment in cells expressing caspase-3. However knockdown caspase-7 activity did enhance resistance to staurosporine-induced death in both Casp3 and Casp3/Diablo null MEFs. Conversely siRNA knockdown of caspase-6 enhanced cell viability to a less extent compared to caspase-7, consistent with the relatively lower level of caspase-6 activation observed (Figure 2B). (E) Representative blots to demonstrate the knockdown efficiency against caspase-6 and caspase-7 in MEFs following treatment with specific siRNAs. Data shown represent the results of two independent experiments using two independently derived MEF lines for each of the genotypes described. * and # indicate statistical significance at $p < 0.05$ and $p < 0.01$ compared to scrambled siRNA controls.
A  scrambled siRNA

Time (hours)

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B  Caspase-6 knockdown

Time (hours)

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C  Caspase-7 knockdown

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<td>β-actin (42 kDa)</td>
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D  Staurosporine-induced cell death (Caspase-6 and -7 knockdown)

% survival

E  caspase-6 (35 kDa)

β-actin (42 kDa)

Caspase-7 (37 kDa)

β-actin (42 kDa)
caspase-3 to regulate PARP cleavage. This is confirmed by the failure of caspase-7 siRNA knockdown to alter patterns of PARP cleavage in either wild-type or Diablo null MEFs. Thus even though the majority of executioner caspase activity observed in intrinsic pathway-mediated PCD is contributed by caspase-3, the activity of functionally redundant caspase-7 is sufficient to promote PCD even in the total absence of caspase-3. In contrast these functions cannot be ascribed to caspase-6. As shown by the altered pattern of PARP cleavage seen in DKO MEFs, caspase-7 (and potentially caspase-3) is a target of Smac/DIABLO regulation.

To determine the effects of caspase-6 and caspase-7 knockdown on cell viability, we examined levels of cell survival following staurosporine treatment. Consistent with results observed for PARP cleavage, knockdown of caspase-7 enhanced levels of cell survival in Casp3 KO MEFs to levels similar to that seen in DKO MEFs (Figure 49D). These data further support the notion that loss of Smac/DIABLO promotes suppression of caspase-7 activity, which results in the enhanced cell survival seen in DKO MEFs during PCD. In wild-type and Diablo null MEFs, caspase-7 knockdown also enhanced PCD resistance, suggesting that levels of this executioner caspase play a role in determining levels of PCD in response to specific cellular stresses. Knockdown of caspase-7 in DKO MEFs was able to induce a small but significant increase in cell viability following staurosporine treatment. Consistent with PARP cleavage data, the effect of caspase-6 knockdown on cell viability was minimal (Figure 49D). Interestingly knockdown of caspase-6 in DKO MEFs resulted in some reduction in cell viability not seen in the scrambled siRNA control, suggesting that caspase-6 may exert some pro-survival functions when the total level executioner caspase activity is substantially inhibited.
The levels of siRNA-mediated knockdowns for caspase-6 and caspase-7 were assessed by immunoblotting and were found to consistently result in >80% knockdown for these proteases (Figure 49E).

3.3.3.6 Smac/DIABLO Acts Through XIAP, cIAP-1 and cIAP-2 to Alter Caspase-7 Function

The above results indicate that caspase-7 is responsible for initiating the PARP cleavage seen in the absence of caspase-3, and that loss of Smac/DIABLO inhibits this activity. Based upon previous biochemical and in vitro studies which identified IAP family members as potent suppressors of caspase activity, we examined the role of specific IAPs to inhibit PARP cleavage using siRNA-mediated knockdown.

Our analysis of IAP-mediated effects on executioner caspase activity has focused on XIAP, cIAP-1 and cIAP-2 as these IAPs exhibit the greatest sequence homology in their linker regions located at the amino-termini of their BIR2 domains (Eckelman et al., 2006). The effects of a structurally different subset of IAPs were also investigated (survivin and Naip1), however specific knockdown of survivin resulted in a significant reduction of cell viability even in the absence of PCD stimulation (likely due to effects on cellular architecture) while Naip1 knockdown (another IAP family member containing three BIR domains) did not result in any significant enhancement of executioner caspase activity (Figure 50F). To examine functional outcomes resulting from siRNA-mediated knockdown of the above IAPs, PARP cleavage was examined following staurosporine treatment (Figure 50A-C). Comparison of the pattern and extent of PARP cleavage seen in Casp3/Diablo DKO MEFs following XIAP, cIAP-1 or cIAP-2 knockdown (Figure
Figure 50. Ablation of Diablo Relieves Inhibition of Several IAP Family Members

Suppression of IAP activity was investigated by using siRNAs to specifically target IAP family members. Effect of siRNA-mediated knockdown of XIAP (A), cIAP-1 (B) or cIAP-2 (C) on staurosporine-induced PARP cleavage for each of the described genotypes. Inhibition of XIAP, cIAP-1 or cIAP-2 restores the reduction of PARP cleavage activity seen in Casp3/Diablo DKO MEFs; demonstrating that the observed reduction in executioner activity is mediated at least in part through these IAPs. (D)

Effect of staurosporine-induced PCD on cell viability in MEFs lineages following siRNA knockdown of each of the above IAP family members. Suppression of XIAP and cIAP-1 resulted in a significant reduction in cell viability following staurosporine addition for all genotypes examined. Knockdown of cIAP-2 also resulted in significant reductions in cell viability following staurosporine treatment, but to a lesser extent. (E) IAP knockdown efficiencies were measured by quantitative PCR. It was determined that the specific mRNA transcripts were reduced by siRNA treatment to approximately 25% of that in MEFs treated with scrambled control siRNA. Due to the equivalent knockdown efficiency observed between these IAP family members, results shown in (D) would confirm that XIAP and cIAP-1 have similar influence on cell viability through their inhibitory effects on executioner caspases, which is greater than that observed for cIAP-2. (F) Examination of DEVD-dependent caspase activity following staurosporine treatment in wild-type MEFs in the presence and absence of various siRNA treatments.

 Knockdown of XIAP resulted in a significant enhancement in DEVD-dependent executioner caspase activity, demonstrating its potential to suppress executioner caspase activity measured using the fluorogenic substrate. Data shown are representative of two
independent experiments using two independently derived MEF lineages for each genotype. # indicates statistical significance at levels of $p < 0.01$ compared to scrambled siRNA controls.
A) XIAP knockdown

Time (hours)

0 6 12 18

Casp3+/-, Diablo+/
Casp3+/-, Diablo-/
Casp3-/-, Diablo+/
Casp3-/-, Diablo-/

FL PARP (116 kDa)
C PARP (89 kDa)
β-actin (42 kDa)

B) cIAP-1 knockdown

Time (hours)

0 6 12 18

Casp3+/-, Diablo+/
Casp3+/-, Diablo-/
Casp3-/-, Diablo+/
Casp3-/-, Diablo-/

FL PARP (116 kDa)
C PARP (89 kDa)
β-actin (42 kDa)

C) cIAP-2 knockdown

Time (hours)

0 6 12 18

Casp3+/-, Diablo+/
Casp3+/-, Diablo-/
Casp3-/-, Diablo+/
Casp3-/-, Diablo-/

FL PARP (116 kDa)
C PARP (89 kDa)
β-actin (42 kDa)

D) Staurosporine-induced cell death (IAPs knockdown)

% survival

0 20 40 60 80 100

Casp3+/-, Diablo+/
Casp3+/-, Diablo-/
Casp3-/-, Diablo+/
Casp3-/-, Diablo-/

E) siRNA-mediated IAP knockdowns

Knockdown efficiency (relative to scrambled control siRNA)

XIAP KD cIAP-1 KD cIAP-2 KD

siRNA treatment (50 nM, 72 hours)

F) DEVDase activity (IAPs knockdown - staurosporine)

Caspase activity (RU/n)

0 500 1000 1500 2000 2500

Time (hours)

0 6 12 18

sc. siRNA XIAP siRNA cIAP-1 siRNA cIAP-2 siRNA
50A-C, panels 4) demonstrates that enhanced PARP cleavage is observed compared to control siRNA treatment (Figure 50A, panel 4). Inhibition of XIAP or cIAP-1 activity restores PARP cleavage to levels comparable to that seen in Casp3 KO MEFs (Figure 50A-C, panels 3), while knockdown of cIAP-2 is significantly less efficacious in restoring PARP cleavage in Casp3/Diablo DKO MEFs. These results demonstrate that siRNA-mediated inhibition of XIAP, cIAP-1 or cIAP-2 results in a significant restoration of caspase-6/7 activity. Consistent with this, analysis of cell survival following staurosporine treatment in IAP-inhibited MEF lineages (Figure 50D) demonstrates that for Casp3 KO, Diablo KO and Casp3/Diablo DKO MEFs, knockdown of XIAP or cIAP-1 results in a substantial and comparable reduction in cell survival compared to cIAP-2 inhibition or scrambled siRNA controls. These effects do not appear to be a result of differences in the relative degree of IAP inhibition between XIAP, cIAP-1 or cIAP-2 as each is inhibited to a comparable degree (~25% of scrambled siRNA control, Figure 50E). In wild-type cells, levels of endogenous XIAP, cIAP-1 and cIAP-2 are insufficient to alter levels of cell survival in the presence of PCD stimuli which promote extensive caspase activation; as evidenced by the effects seen in Casp3+/−, Diablo+/− MEFs following staurosporine treatment (Figure 50D). However under conditions of suboptimal PCD activation such as reduced Smac/DIABLO release or lowered activated caspase-3, XIAP and cIAP-1 levels can significantly alter functional outcomes such as cell survival (Casp3 KO, Diablo KO MEFs, Figure 50D).

As shown in Figure 50D, inhibition of XIAP, cIAP-1 and to a lesser extent cIAP-2, inhibits cell survival. In order to determine the nature of these effects with respect to executioner caspase activity, caspase activity was monitored following staurosporine
treatment using DEVD-Rho110 in wild-type MEFs lineages. As illustrated in Figure 50F, suppression of XIAP activity resulted in a substantial increase in executioner caspase activity, while cIAP-1 suppression lead to more nominal increases ($p = 0.06$ at 6 hours following staurosporine treatment). In contrast, suppression of cIAP-2 did not significantly alter executioner caspase activity.

3.3.3.7 Combinatorial Inhibition of Necroptosis Demonstrates an Enhanced Resistance of *Casp3/Diablo* DKO MEFs to PCD Stimuli Mediated by the Extrinsic Pathway

A novel caspase-independent pathway termed necroptosis had recently been described for ligand-mediated death receptor signaling involving RIPK1 (Degterev et al., 2005; Hitomi et al., 2008). Controlled principally through the activity of RIPK1 (Degterev et al., 2008), necroptosis is believed to induce the downstream actions of RIP3, resulting in a form of cell death which manifest itself with morphological properties similar to necrosis (i.e., oxidative burst, mitochondrial membrane hyperpolarization, lysosomal and plasma membrane permeabilization) (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). We have examined the contribution of necroptosis to the cell death triggered by Fas activation and TNF-$\alpha$ treatment by using the RIPK1-specific inhibitor necrostatin-1 (Degterev et al., 2008; Degterev et al., 2005). Concurrent inhibition of necroptosis using necrostatin-1 was examined in conjunction with our genetically modified MEFs lineages following treatment with Jo2 (antibody activating Fas receptor) or TNF-$\alpha$ (used in conjunction with cycloheximide) (Figure 51). Interestingly necrostatin-1 treatment revealed that cell death triggered by these two extrinsic pathway PCD stimuli was substantially reduced in *Casp3/Diablo* DKO MEFs compared to single
Figure 51. Inhibition of Necroptosis Reveals an Enhanced Resistance of 
*Casp3/Diablo* DKO MEFs in Response to Extrinsic Pathway PCD

Since death ligand-receptor signaling by Fas activation or TNF-α stimulation have been demonstrated to trigger a novel pathway of necroptosis, we have examined whether this programmed necrotic pathway may contribute to cell death observed in MEFs following Jo2 or TNF-α treatments. It was observed that inhibition of necroptosis by necrostatin-1 (50 µM) provided enhanced resistance to MEFs of all experimental genotypes in response to Fas activation or TNF-α stimulation, thus demonstrating a contribution by necroptosis to the observed cell death in Figure 1D-E. Furthermore it was observed that the enhancement in cell survival following Fas activation or TNF-α stimulation mediated by necrostatin-1 was significantly different between *Casp3/Diablo* DKO and control (wild-type and single KO) MEFs. Therefore, combinatorial deletion of *Casp3* and *Diablo* does provide enhanced resistance to extrinsic pathway stimuli, but this effect was effectively masked by cell death mediated via the necroptotic pathway and was not detected until this pathway was inhibited via the pharmacologic actions of necrostatin-1 on RIPK1. * indicates statistical significance of $p < 0.05$ between single knockout and wild-type control MEFs and # indicates statistical significance of $p < 0.05$ between DKO and single knockout MEFs.
A  Fas activation  
PCD vs. necroptosis

B  TNF-α stimulation  
PCD vs. necroptosis

% survival

Casp3+/-, Diablo+/-  Casp3+/-, Diablo-/-  Casp3-/-, Diablo+/-  Casp3-/-, Diablo-/-

Jo2 + CHX  Jo2 + CHX + Nec-1

Casp3+/-, Diablo+/-  Casp3+/-, Diablo-/-  Casp3-/-, Diablo+/-  Casp3-/-, Diablo-/-

TNF-α + CHX  TNF-α + CHX + Nec-1
KO and wild-type MEFs lineages. These results demonstrate that in contrast to intrinsic pathway PCD stimuli, death receptor-mediated signaling initiates both necroptotic and caspase-dependent signaling cascades. The further enhancement of cell survival in seen \textit{Casp3/Diablo} versus \textit{Casp3} null MEF lines in the presence of necrostatin-1 further demonstrates that Smac/DIABLO plays a physiologic role in tuning the cellular response to extrinsic pathway mediated PCD cues.
3.3.4 DISCUSSION

In the present study we provide the first direct in vivo evidence to demonstrate that endogenous Smac/DIABLO uniquely regulates the extent of both induced and developmental PCD in mammals. Because of the much greater catalytic activity of caspase-3 compared to caspase-6/7 (Stennicke et al., 2000), we utilized a null mutation of Casp3 to reduce total levels of executioner caspase activity to an extent that the modulatory effects of Smac/DIABLO could be readily examined. In conjunction with this sensitizing mutation, we observe that loss of Diablo results in lethality during the perinatal period. These findings are in contrast to suggestions that the nominal phenotype seen in Diablo null mice reflects functional redundancy between Diablo and factors such as Omi/HtrA2 (Martins et al., 2004) and that Smac/DIABLO does not play a significant physiologic role in PCD regulation (Martins et al., 2004; Okada et al., 2002). It is of interest to note that in addition to Smac/DIABLO and Omi/HtrA2, apoptosis-related protein in the TGF-β signaling pathway (ARTS) and XIAP-associated factor 1 (XAF-1) have been demonstrated to antagonize XIAP and other IAP family members in an IBM-independent manner (Larisch et al., 2000; Liston et al., 2001), and were reasoned to compensate for the absence of Smac/DIABLO in Diablo KO mice. However our current findings suggest that Smac/DIABLO nonetheless possesses pro-apoptotic functions which could not be fully compensated by other IAP antagonists encoded within the murine genome.

The results observed for Casp3/Diablo double null mutants exhibit both similarities and differences from that previously described for combinatorial null mutants of Casp3 and Casp7 (Lakhani et al., 2006). Both mutants appear to exhibit perinatal
lethality, however we do not observe any malformation of cardiac or supporting circulatory structures in Casp3/Diablo null mutants; structures suggested to be the cause of death in Casp3/Casp7 null animals. These morphologic differences may reflect differences in the temporal and spatial pattern of Smac/DIABLO versus caspase-7 expression in vivo. At present the physiologic mechanism underlying the late embryonic lethality observed in Casp3/Diablo double knockout is unknown. Histological examination of Casp3/Diablo DKO embryos collected from E15.5-18.5 demonstrates normal histologic appearance compared to control littermates. Given the period of embryonic lethality, these effects may be related to abnormalities in blood chemistry (pH, electrolytes, oxygen exchange, etc.), haematopoiesis or insulin production. Further analyses of these possibilities will form the basis for future investigation of Casp3/Diablo DKO animals in the laboratory.

With respect to derived MEF lines, Casp3/Diablo null mutants exhibit enhance resistance to PCD initiated by UVC irradiation and staurosporine treatment, similar to results seen Casp3/Casp7 null MEFs; as well a number of additional intrinsic pathway PCD stimuli. However in contrast to Casp3/Casp7 mutants, Casp3/Diablo null MEFs remained susceptible to both TNF-α and Fas activation-induced cell death. In addition Casp3/Diablo null MEFs continue to exhibit AIF nuclear translocation, albeit with delayed kinetics compared to controls in contrast to results reported with Casp3/Casp7 null MEFs (Lakhani et al., 2006). While such differences could reflect incomplete suppression of caspase-7 in Casp3/Diablo null mutants, several lines of evidence suggest that caspase-7 activity is depressed below any threshold required for PCD in this mutant. For one, there is a complete inhibition of PARP cleavage (a well-characterized target of
all three executioner caspases) in Casp3/Diablo double null MEFs over the entire 18 hour examination period in response to PCD induction by a variety of agents. Additional efforts to suppress caspase-7 activity using gene-specific siRNA clearly do not further reduce PARP cleavage in Casp3/Diablo null MEFs. The results observed match those seen in Casp3 null MEFs which have been treated with caspase-7 specific siRNA. We do observe a small increase in cell survival in Casp3/Diablo null MEFs following siRNA-mediated inhibition of caspase-7 following staurosporine treatment. Given the absence of observed changes in either PARP or DEVD-directed cleavage in Casp3/Diablo null MEFs following caspase-7 siRNA treatment, this small increase in cell survival may reflect an enhancement of IAP binding to non-caspase targets following suppression of “residual” (IAP-bound) capase-7. Thus in Casp3/Diablo null MEFs, caspase-7 activity is normally reduced (as a result of IAP binding) to levels below that required to propagated PCD. Further reduction of residual caspase-7 via siRNA-mediated inhibition would subsequently free some element of IAP activity, allowing it to bind other (lower affinity) cellular targets.

Several of the differences in cellular response seen in Casp3/Casp7 versus Casp3/Diablo DKO MEFs following PCD induction are intriguing. Despite evidence for functional inhibition of caspase-3 and -7 in our Casp3/Diablo DKO MEFs, we observe that these cells were not protected from either TNF-α or Fas ligand-induced PCD in contrast to results reported for Casp3/Casp7. In this regard it is interesting to note that Casp3/Casp7 null thymocytes were reported to be susceptible to Jo2-mediated Fas activation whereas MEFs from these animals were not (Lakhani et al., 2006). Such
finding highlights the potential for distinct PCD pathways and/or varied levels of PCD modulators in different tissue types.

Interestingly, we observe that blockade of programmed necrosis using the RIPK1-specific inhibitor necrostatin-1 altered the pattern of programmed cell death such that inhibition of caspase-3 now exerts a protective effect with respect to cell survival. This protective effect is further enhanced by the additional inhibition of Diablo. Casp3/Casp7 null MEFs were reported to exhibit enhanced resistance to several extrinsic pathway PCD stimuli, while similar effects were not observed in Casp3/Diablo null MEFs without the pharmacologic inhibition of necroptosis via necrostatin-1. In Casp3/Diablo null MEFs, necroptosis is clearly capable maintaining levels of PCD at or near that seen in wild-type MEFs following extrinsic pathway PCD stimulation. Even if one considered Casp3/Casp7 null MEFs to be completely devoid of all executioner caspase activity (with some residual caspase activity present in Casp3/Diablo null MEFs), it is unclear why such MEFs appear not to undergo necroptosis-mediated PCD. In this regard it is interesting to note that RIP3 expression levels and activity are highly correlated with the ability of different cell types to undergo necroptosis (He et al., 2009). Similarly, FADD has also recently been implicated as a negative regulator of necroptosis (Osborn et al., 2010).

Thus in contrast to results reported for Casp3/Casp7 null MEFs, we observe that TNF-α and Fas activation trigger both caspase-dependent as well as caspase-independent forms of PCD, consistent with findings on Fas-dependent signaling reported from several different cell lines (Holler et al., 2000; Matsumura et al., 2000; Vercammen et al., 1998a; Vercammen et al., 1998b). It appears that in our hands, treatment of MEFs with TNF-α or
Fas activation initiates at least three distinct PCD signaling events (extrinsic pathway apoptosis, intrinsic pathway apoptosis and necroptosis), in contrast to other intrinsic pathway PCD agents. It will therefore be of interest to see what aspects of apoptosis and/or necroptosis Smac/DIABLO may regulate beyond its strict modification of executioner caspase activity.

In the current study I observe that even in the presence of both caspase-3 and Smac/DIABLO inhibition, PCD signaling via extrinsic pathway stimuli appears to occur at a level comparable to that seen in wild-type MEFs. However in the presence of necrostatin-1 (a pharmacologic inhibitor of necroptosis), significant differences in cell survival are observed between Casp3/Diablo null and wild-type MEFs. These findings reveal the significant contribution necroptosis has in the regulation of extrinsic pathway PCD. Thus in contrast to cell death triggered by intrinsic PCD stimuli, cell death stimulated by extrinsic pathway PCD stimuli are more intimately connected with the process of necroptosis; despite displaying overtly similar apoptotic characteristics under normal (wild-type) circumstances. The ancillary position of necroptosis versus apoptosis in controlling extrinsic PCD has been suggested to be related to kinetic phenomena, given that apoptotic caspase activation rapidly inactivates key necroptotic mediators such as RIPK1. Specifically caspase-8 has been shown to cleave RIPK1 thus inactivating it (Lin et al., 1999). Hence necroptosis may be indirectly regulated by modulators of caspase-8 activity such as cFLIP. In addition, because caspase-3 is also capable of inducing RIPK1 cleavage at least in vitro, there is the potential for tremendous complexity regarding the interrelationship between these two modes of cellular death (Lin et al., 1999). Alternatively if substantial RIPK1 activity were present prior to (or in the absence of)
significant caspase activation as occurs in Casp3/Diablo null mice, one might expect
necroptosis to become the dominant form of PCD observed following the addition of
extrinsic PCD stimuli. An additional layer of complexity in this system due to the
potential regulation of RIPK1 ubiquitination by cIAPs, and by extension Smac/DIABLO
(Bertrand et al., 2008; Dynek et al., 2010; Vanlangenakker et al., 2010; Varfolomeev et
al., 2008), which may represent a crucial regulatory checkpoint between apoptotic and
necroptotic signaling systems (Wang et al., 2008).

With respect to the pattern of executioner caspase activity following the initiation
of PCD, several features emerge. Caspase-3, the caspase with the highest catalytic
activity, appears to be activated coincident with caspase-7. However activation of
caspase-7 appears to depend significantly upon the level of caspase-3 activity, as
inhibition of this caspase significantly reduces rates of procaspase-7 cleavage. Similarly,
amination of caspase-6 appears to depend entirely upon prior activation of caspase-3/7, as
Casp3 null MEFs treated with caspase-7-specific siRNA show no evidence of caspase-6
activity. Analysis of PCD in MEFs using a variety of agents indicates that caspase-6 is
activated at a far slower rate and to a lesser extent than either caspase-3 or -7. Each of
these executioner caspases shows significant structurally similarity and have been shown
to cleave a common set of cellular substrates (such as PARP) (Slee et al., 2001). However
each also possesses a unique set of cellular targets (Slee et al., 2001; Walsh et al., 2008).
In this context it should be noted that when isolated from caspase-3/7 activity (as in the
case of Casp3/Diablo null MEFs exposed to staurosporine), the most divergent caspase,
caspase-6, appears to exhibit a significant pro-survival effect. This is interesting given
recent studies on the role of caspase-6 in regulating dendritic pruning and huntingtin
cleavage (Graham et al., 2006; Nikolaev et al., 2009). It will therefore be important to our future understanding of PCD to carefully evaluate the relative kinetic and catalytic equivalency of each of the executioner caspases with respect to specific cellular outcomes (i.e., is a unit of activated caspase-6 functionally equivalent to caspase-3 with respect to PCD? Do different thresholds exist among the executioner caspases with respect to the propagation of PCD versus other cellular activities?). With respect to this latter question, the IAP-mediated effects observed in these studies are insufficient in magnitude to alter the nature or extent of PCD once initiated. What then is the purpose of such a system \textit{in vivo}? An answer is suggested by our titration of executioner caspase activity. I postulate that under normal circumstances, IAP inhibition provides an efficient means of insuring that low levels of cellular caspase activity do not errantly initiate a full-blown apoptotic response. Such low level caspase activity could arise as through either incidental activation, or as a consequence of non-PCD functions which are increasingly being recognized for executioner caspases (Fujita et al., 2008; Janzen et al., 2008; Nikolaev et al., 2009; Okuyama et al., 2004). Tight regulation of low levels of caspase activity using a set of independent regulators such as IAPs would be one means to safely distinguishing apoptotic versus non-apoptotic caspase functions. Of note, previously the closely related IAP family members XIAP, cIAP-1 and cIAP-2 have been shown to be expressed in the CNS (Duckett et al., 1996; Rothe et al., 1995). With respect to motor neurons, XIAP, cIAP-1 and cIAP-2 have been specifically detected in spinal motor populations (Ishigaki et al., 2002; Perrelet et al., 2004). The neuronal expression of these IAP members may be consistent with the suggested non-apoptotic roles that caspases have in the CNS (Aranha et al., 2009; Li et al., 2010; Ohsawa et al., 2010). Hence in such
cases, IAPs may act to restrict caspase activities to specific locations within the neuron to perform these non-apoptotic functions.

Small molecule Smac mimetics originally designed to target the XIAP BIR domain have been found instead to interact strongly with not only XIAP, but also cIAP-1/2 (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). Pharmacologic addition of such Smac mimetics and/or ectopic overexpression of Smac/DIABLO have been shown to potentiate PCD by modifying TNF and related TRAIL receptor signaling (Deng et al., 2002; Petersen et al., 2010); likely through an enhancement of cIAP-1/2-mediated ubiquitin degradation (Petersen et al., 2010; Varfolomeev et al., 2007; Vince et al., 2007). In our studies, altering levels of endogenous Smac/DIABLO did not alter cell survival for either Diablo or Casp3/Diablo null MEFs in TNF-α or Fas ligand-treated cells. Smac/DIABLO-mediated effects were only observed following the inhibition of RIPK1-mediated signaling in these systems. These differences in death receptor-mediated response suggest that the pharmacologic addition of Smac mimetics is able to overwhelm or override the normal necroptotic response in TNF-α or Fas ligand-treated cells.

Despite numerous recent studies describing the actions of Smac mimetics, the physiologic role of Smac/DIABLO had remained elusive. My current findings reveal that the principal physiologic role of Smac/DIABLO is to control the activity of caspase-3 and -7 through its selective inhibition of IAP family members such as XIAP, cIAP-1 and cIAP-2.
SECTION 4: DISCUSSION AND CONCLUSIONS
Through numerous studies over the past two decades a general picture of the molecular mechanisms governing PCD has emerged. The current challenge is to understand how regulator of this system is modified at different times during the life cycle and in different cell types under real *in vivo* conditions.

My thesis begun by examining the role of Bcl-2-mediated interactions regulating developmental and injury-induced cell death in motor and sensory neurons. This work demonstrated Bcl-2 is required to promote the survival of gamma spinal motor neurons during late embryonic and early postnatal development. My finding regarding the selective sensitivity of gamma motor neurons to of *Bcl2* is interesting given that GDNF (derived from muscle spindles innervated by gamma motor neurons) has also been shown to promote the survival of a similar population of spinal motor neurons (Buss et al., 2006; White et al., 1998; Whitehead et al., 2005). My second study provided evidence that (in contrast to previous suggestions) the inhibition of calcineurin inhibition explains the neuroprotective properties observed for the immunophilin ligands cyclosporin A and FK-506. I demonstrated for the first time that this motor neuron sparing effect could be reproduced using unrelated chemical agents which blocked calcineurin activity such as the insecticide cypermethrin. I further demonstrated that inhibition of calcineurin activity appears to influence PCD by inhibiting Bad dephosphorylation. These results suggest potential pharmacologic means of inhibiting injury-induced motor neuron death. The first two studies of this thesis focused on mitochondria-mediated events regulating PCD. The final study focuses on control of downstream PCD pathways and how they influence executioner caspase activity. Through the creation of an *in vivo* system exhibiting reduced executioner caspase activity, relative effects on executioner caspase activity
could be more closely monitored. These efforts led to the first demonstration of a physiologic role for Smac/DIABLO. Through the creation of mouse embryonic fibroblast lineages derived from Casp3/Diablo DKO, Casp3 KO, Diablo KO and control embryos I demonstrated that Smac/DIABLO functions in vivo to promote executioner caspase activity through the suppression of inhibitory effects contributed by inhibitor of apoptosis proteins. In addition the relative role of specific IAPs (XIAP, cIAP-1 and cIAP-2) in regulating executioner caspase activity for several distinct forms of PCD was determined. Collectively these studies identified and characterized a series of molecular interactions which are relevant to motor neuron PCD and survival in vivo for both developmental and injury-induced PCD. Understanding the physiologic context of these interactions required that these studies be performed in vivo, in conjunction with cellular and biochemical studies to attain a mechanistic appreciation of these biological phenomenon.

In vivo analyses are complex in nature, and their findings are often constrained due to the limits in controlling experimental variability. A significant limitation for in vivo genetic studies is potential physiologic adaptation following gene ablation. In traditional genetic knockout studies, gene ablation occurs at the level of embryonic stem cells. In this sense the ability of the system to adapt without the gene of interest is being examined, which may not necessarily reveal the true physiologic role for this gene. Conditional knockout approaches (e.g., Cre recombinase) address some of these issues by mediating the specific genetic change in a cell- and time-dependent manner. Thus such conditional knockout approaches may mitigate, but do not remove functional compensatory issues; in large part due to limitations in the specificity of promoters used to drive expression of Cre recombinase. An alternative approach is to establish the use of
a multi-conditional knockout system in which Cre recombinase expression is driven by a cell-specific promoter, whose expression places a tetracycline-dependent regulator in frame for transcription in specific cell-types. In addition, viruses (adeno-assoicated or lentivirus) with a tetracycline-responsive promoter (i.e., tetO) to drive short hairpin (sh)RNA expression to suppress expression of a gene of interest. Using this approach, cells expressing Cre recombinase are conferred with the ability to express the tetracycline-dependent regulator, but still requires tetracycline (or doxycycline) to be present to drive shRNA expression. This approach can provide more flexible control over the initiation of suppression via regulated doxycycline administration and multiple genes of interest could be examined simultaneously by using a combination of viruses. However this system is still largely unproven in an \textit{in vivo} CNS context. The viral vectors used can alter CNS function and stochastic mosaic expression using these (or even transgenic approaches) remains a significant problem.

Another complication involved in the traditional null mutation approach is that a given protein often process multiple functional domains to regulate distinct cellular activities. As such, genomic disruption of the entire gene may not allow an adequate opportunity to assess the function of a specific domain of interest. This can lead to misinterpretations regarding specific cellular roles of a given protein. However this can be appraised by the targeted gene knockin approach. An example of this would be the cytochrome c (K72A) mutant mouse in which a point mutation was utilized to distinguish the ability of cytochrome c to promote apoptosome formation in PCD regulation versus oxidative phosphorylation (Hao et al., 2005; Kanungo et al., 2008). In a similar vein, Smac/DIABLO has recently been proposed to possess cellular functions outside of its
amino-terminal IBM sequence (Roberts et al., 2001). A useful follow-up to my studies might therefore be to engineer an IBM mutant of Smac/DIABLO (lacking IAP antagonistic functions) and compare it to a complete knockout of Smac/DIABLO. Another approach (where applicable) is to disrupt cellular function via pharmacologic inhibitors. This approach is typically hindered by the availability of specific inhibitors of known function.

From our current understanding of PCD regulation, it is interesting to note that the convergence checkpoints in mammalian PCD pathways reveal similar architecture as depicted in Figure 52. In both instances “effector” molecules (i.e., Bax-like proteins and caspase-3/-6/-7) act to elicit a function (induction of MOMP or cleavage of cellular substrates), which can be suppressed by “inhibitor” molecules (i.e., Bcl-2-like proteins and IAPs). “Inhibitors” are present in both situations to ensure that under normal physiologic conditions “effector” activities are maintained at a minimal level to allow for non-PCD functions (Bax – mitochondrial dynamics; caspases – differentiation, remodeling, etc.). For most decision-making checkpoints in signaling pathways, “effectors” and “inhibitors” (kinase vs. phosphatase, ubiquitin ligase vs. deubiquitinase, etc.) are often sufficient to construct a switch between progressing through the checkpoint and prohibiting the signal from being transmitted further down the pathway. Incorrect PCD decision-making can ultimately result in demise of the cell or organism. Hence, two additional components have been integrated into both checkpoints: “activators” and “de-repressors”. It has been proposed in recent years that BH3-only proteins in the Bcl-2 protein family can be functionally assigned to two classes,
The most fundamental PCD signaling network identified to date belongs to that established originally *C. elegans*. This simple signaling network consists of four components. During evolution, PCD signaling evolved novel mechanisms to specifically regulate the activity of executioner caspases (CED-3, drICE and caspase-3, -6 and -7). In *Drosophila* a similar four-component network evolved involving slightly different regulatory interactions. In order to provide the cell with finer regulation over PCD, the signaling pathway split into two distinct networks in mammalian cells. One network functions at the mitochondria to regulate the release of factors which subsequently are influenced by the second regulatory network. This second network acts downstream of the mitochondria to control executioner caspase activity levels.
“activators” or “de-repressors”, depending on whether they can directly influence the activities of Bax-like proteins or promote this activity indirectly by suppressing the inhibitory functions of Bcl-2-like proteins (Kim et al., 2006; Kim et al., 2009; Kuwana et al., 2005; Letai et al., 2002). Similarly, two proteins released from the mitochondria during MOMP, cytochrome c and Smac/DIABLO, possess similar “activator” and “de-repressor” functions in that cytochrome c activates downstream executioner caspases via induction of apoptosome formation while Smac/DIABLO operates to promote executioner caspases by removing their endogenous inhibitors, IAP family members. The fundamentals of this four-component regulatory system were already present in the PCD signaling network of C. elegans (Figure 52). As organisms became more specialized with respect to cellular functions, PCD signaling also appears to have become more diverse in its regulatory control, diversifying but still maintaining the fundamental signaling system of the initial network.

In recent years, programmed cell death has become distinguished into both apoptotic and necroptotic components. The networks regulating cell death signaling processes such as autophagy and necroptosis have recently been described as distinct from apoptosis to facilitate cellular destruction. It has also been proposed that autophagy and necroptosis may not strictly be cell death pathways, but rather a form of signaling which results in death upon overstimulation (Vandenabeele et al., 2010). For instance in numerous cases autophagy has been demonstrated to serve cytoprotective functions through the degradation of chemically damaged organelles or protein aggregates. Excessive stimulation may overload the autophagic system, thus initiating a stress response which ultimately results in cell death. Similarly, it has been proposed that
necroptosis may actually be a pathway to enhance survival through increased energy production in the cell. Overactive stimulation of energy metabolism may result in cellular toxicity through reactive oxygen species (ROS) formation and the generation of advanced glycation end products (AGEs). It has been demonstrated that the signaling networks between PCD and these pathways are at some levels intertwined. Future studies will therefore no doubt probe the concerted interaction of these three pathways in greater detail when examining cell death responses. Given the growing connections between these three cellular process, it is of interest to note that though PCD has traditionally been considered to be the principal regulatory pathway, recent evidence in *Drosophila* demonstrates that autophagy is a key regulator in the removal of specific tissues during development (Denton et al., 2009). It is interesting that my own analyses of MEFs treated with TNF-α or Fas stimulation (Study 3) also demonstrated necroptotic component of cell death. Thus with respect to therapeutic measures to enhance cell survival following injury, it may be necessary to modulate the networks of several “programmed cell death pathways” in order to effectively rescue injured neurons. Thus it will be interesting to elucidate the relative contribution between these three cell death pathways in various pathologic conditions and identify critical regulatory checkpoints in each of these pathways for which pharmacologic agents could be designed against.

Elucidation of these new PCD regulatory networks underlying processes such as necroptosis will be of interest. Current opinion is that RIPK1 represents a central point of necroptotic regulation following death ligand-receptor (Fas, TNFR, etc.) binding. However RIPK1 is involved in several other signaling pathways and therefore the network systems encompassing necroptosis may be extended in the next several years.
For example, RIPK1 has been shown to be required for generation of ceramide (Thon et al., 2005), which is involved in MOMP via Bax/Bak-dependent and -independent mechanisms (Ganesan et al., 2010; Novgorodov et al., 2005; von Haefen et al., 2002). Autophagosome formation has also been shown to occur downstream of RIPK1 activation, suggesting a functional overlap between these cell death programs (Degterev et al., 2005). Thus while recent evidence suggesting that RIPK1-RIP3 interaction is crucial to necroptosis regulation, as more downstream targets of RIPK1 emerge, we may have to redefine our view of the relationship between necroptosis, PCD and autophagy. It is with much hope and enthusiasm that regulatory mechanisms underlying these pathways will be elucidated in coming years, much as it was before for PCD. Only through such research will we understand the complex cellular processes which govern the molecular decision of life and death, and grant us knowledge of how best to treat CNS injuries.
SECTION 5: REFERENCES


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SECTION 6: APPENDIX
APPENDIX 1: LIST OF PUBLICATIONS AND ABSTRACTS

Publications


Oral Presentations


Graduate Research In Progress, Graduate Department of Pharmaceutical Sciences, University of Toronto (May 12th, 2004). Analysis of Bcl2 family protein-protein interactions in PCD regulation. Hui, K., and Henderson, J.T.
**Poster Presentations**

Models of Human Diseases First International Conference, Toronto (June 29\textsuperscript{th}, 2010). Calcineurin inhibition enhances motor neuron survival following injury through modulation of mitochondrial signaling. Hui, K., Liadis, N., Robertson, J., Kanungo, A., Henderson, J.T. (Best Scientific Poster Award 2\textsuperscript{nd} Prize)

Association of Faculties of Pharmacy of Canada (AFPC) Annual Conference 2009, Halifax, (June 3\textsuperscript{rd}-6\textsuperscript{th}, 2009). Molecular mechanism of neuroprotection mediated by immunophilin ligands. Hui, K., Liadis, N., Robertson, J., Kanungo, A., Henderson, J.T. (National Poster Award winner)


Canadian Association for Neuroscience Meeting, Toronto (May 23\textsuperscript{rd}-25\textsuperscript{th}, 2007). Differential requirements between skeleto- and fusimotor neurons for Bcl2 in mediating their survival during CNS development. Hui, K., Kucera, J., Henderson, J.T.

Graduate Research In Progress, Graduate Department of Pharmaceutical Sciences, University of Toronto (May 18th, 2005). Pial artery occlusion as an *in vivo* model of neural programmed cell death. Hui, K. and Henderson, J.T.

APPENDIX 2: CELL-BASED HIGH-THROUGHPUT SMALL MOLECULE SCREEN FOR INHIBITORS OF BAX ACTIVATION
Cell-based high-throughput small molecule screen for inhibitors of Bax activation

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Condensed title: Small molecule inhibitors of Bax activation

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Abstract
Programmed cell death (PCD) or apoptosis plays a critical role in the formation and homeostasis of a wide array of cellular systems. Aberrant regulation of PCD signaling has also been connected to an assortment of human pathologies ranging from autoimmune disease and cancer to acute and chronic neurodegeneration. Pharmacologic modulation of PCD signaling is therefore of great interest for a variety of clinical and biomedical research applications. Analysis of PCD signaling over the past two decades has demonstrated that many of the most far-reaching and influential alterations in PCD response involve molecular modification at the level of the mitochondria, where the actions of anti-apoptotic Bcl-2-family proteins are opposed by Bax and/or Bak. Given that Bax undergoes translocation to the mitochondrial outer membrane following PCD induction; monitoring of this event can be used as a real-time detector of PCD. In the present study we have utilized a high-throughput screening approach to examine the subcellular redistribution of an EGFP-Bax fusion protein following PCD induction. Examination of the activities of 5006 compounds in four chemical libraries added to cells 8 hours following the induction of PCD resulted in the identification of two compounds which significantly prevent p53-dependent Bax translocation. Both compounds act through the modulation of cellular cAMP levels. Subsequent analysis identified one further hit which also acted to influence cAMP levels. Consistent with their effects on Bax translocation, each of the identified compounds inhibit downstream executioner caspase activity and enhance cell viability following the induction of several forms of PCD. The results demonstrate the viability of using Bax translocation to monitor mitochondrial PCD, identify a key role for cAMP in regulating P53-dependent PCD, and describe the activity of a related group of small molecules which inhibit post-mitochondrial actions of PCD.
Introduction

In its many varied forms, programmed cell death (PCD) has been shown to play a critical role in regulating the development, cell homeostasis, and injury-mediated responses of organisms throughout metazoan evolution (Hakem et al., 1998; Kuida et al., 1998; Kuida et al., 1996; Woo et al., 1998; Yan and Shi, 2005; Yoshida et al., 1998). Consistent with this, aberrant PCD is associated with a variety of cellular abnormalities ranging from neurodegeneration to cancer (Taylor et al., 2008). Indeed the most frequent gene mutations in human cancers which are found in p53, PTEN, Bcl-2, Bcl-xl, Bax, Fas/CD95 and Akt, each directly regulate a component of programmed cell death (Lowe and Lin, 2000; Luo et al., 2009). The molecular interactions governing PCD have thus generated considerable interest as potential targets of pharmacologic modulation. Studies performed over the past two decades have elucidated details of the two major pathways governing PCD (Brenner and Mak, 2009). The first, termed the intrinsic pathway, regulates cellular responses to genotoxic stress, growth factor withdrawal, calcium influx and disruption of the cytoskeletal network; while the extrinsic pathway is initiated in response to extracellular binding of death receptor (DR) ligands such as Fas ligand, TNF-α and TRAIL (TNF-related apoptosis-inducing ligand). Programmed necrosis, sometimes termed necroptosis, is also activated by this ligand-mediated system (Degterev et al., 2008; Degterev et al., 2005).

Though the intrinsic and extrinsic pathways are regulated by distinct sets of cellular stimuli, both converge at the level of the mitochondria where interactions between pro- and anti-apoptotic Bcl-2 family proteins regulate mitochondrial outer membrane permeability (MOMP) (Cory and Adams, 2002). Induction of MOMP promotes changes in the mitochondrial matrix, accelerating mitochondrial fission and reducing levels of oxidative phosphorylation (Tait and Green, 2010). Mechanistically, the multi-domain pro-apoptotic Bcl-2 proteins Bax and Bak have been shown to play a key role in MOMP, facilitating release of cytochrome c, Smac/DIABLO, AIF, and endonuclease G from the mitochondrial intermembranous space. Holo-cytochrome c and Smac/DIABLO subsequently act to promote downstream caspase activity, while AIF and endonuclease G initiate caspase-independent modes of cell death (Du et al., 2000; Kim et al., 2009; Li et al., 2001; Li et al., 1997; Liu et al., 1996; Lovell et al., 2008; Susin et al.,
1999; Verhagen et al., 2000). Mechanistically Bax and Bak initially undergo activation through interactions with BH3-only proteins, which function as intrinsic sensors of cell stress (Puthalakath and Strasser, 2002). Once conformationally activated, Bax and Bak are competent to undergo oligomerization at the mitochondrial outer membrane to release mitochondrial proteins. This activity is opposed by anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-xL, Bcl-w and Mcl-1 which are believed to prevent Bax/Bak pore formation through either inhibitory interactions with a subgroup of BH3-only proteins and/or through direct suppression of Bax/Bak oligomerization (Chen et al., 2005; Gavathiotis et al., 2008; Kim et al., 2006; Kim et al., 2009; Lovell et al., 2008; Willis et al., 2007). The critical role of Bax and Bak in this process is demonstrated by findings observed in Bax/Bak double knockouts, in which cells are substantially protected from PCD induced through the mitochondrial (intrinsic) pathway (Wei et al., 2001). Further analysis of the relative roles of Bax and Bak in PCD has demonstrated that a majority of cell types principally depend upon Bax to execute MOMP (Deshmukh and Johnson, 1998; Miller et al., 1997; Putcha et al., 2000; Putcha et al., 2002). Thus for these cell types, Bax function as the primary gatekeeper of mitochondrial PCD regulation. In its inactive state Bax exists as a cytoplasmic monomeric protein, its hydrophobic C-terminal tail resting in a hydrophobic groove delineated by the BH1, 2 and 3 domains (Suzuki et al., 2000). Interaction with the appropriate BH3-only proteins (truncated Bid, Bim and Puma) induces a conformational change in both the N- and C-termini of Bax, freeing the C-terminus and allowing it to interact with the mitochondrial outer membrane (Kim et al., 2009). This translocation event is critical for the pro-apoptotic functions of Bax (Kim et al., 2009) and promotes three critical aspects of cell death: the induction of mitochondrial fission with depletion of cellular ATP, the activation of downstream executioner caspases, and stimulation of the release of proteins promoting caspase-independent forms of cell death.

Genetic manipulation of Bcl-2 family protein-protein interactions in vivo had demonstrated that interference with Bax oligomerization results in substantial enhancements in neuronal survival following acute or chronic neural injury (Deckwerth et al., 1996; Dubois-Dauphin et al., 1994; Farlie et al., 1995; Kostic et al., 1997; Parsadanian et al., 1998; Sun and Oppenheim, 2003; Yin et al., 2002). Similarly, previous
studies have shown that peptides derived from two proteins known to interact with Bax (Ku70 and Bax inhibitor-1/BI-1) also suppress Bax activation and cell death. However the *in vivo* administration of such agents presents considerable challenges clinically due to their peptidic nature and low bioavailability/stability. Given this, small molecule antagonists appear the most viable option to induce Bax inhibition.

In recent years efforts have focussed on the identification of small molecule agonists of apoptosis in order to enhance levels of cell death for various cancers over-expressing Bcl-2 family proteins (Cragg et al., 2009; Labi et al., 2008). As a result, remarkably specific small molecules (ABT-737 and its derivative ABT-262) have been identified which inhibit Bcl-2, Bcl-xL and Bcl-w interactions at nanomolar concentration and show considerable promise in early clinical trials (Chonghaile and Letai, 2008). In contrast, the search for pharmacologic agents to inhibit Bax activation and suppress PCD progression for conditions such as stroke and spinal cord injury thus far have not been met with comparable success.

Previous studies have demonstrated the feasibility of using EGFP-Bax and similar PCD fusion proteins as a real-time detector of apoptotic progression (De Giorgi et al., 2002; Deng and Wu, 2000; Lartigue et al., 2008). Because the translocation of Bax from the cell cytoplasm to the mitochondria serves as an early and readily detectable event in the progression of apoptosis, we utilized EGFP-Bax to monitor levels of Bax translocation in conjunction with a high-throughput chemical screen to identify potential small molecule inhibitors of Bax. Following our initial screening of over 5000 compounds, we have identified three which demonstrated significant reductions in Bax translocation following p53-mediated PCD stimulation. We have further validated the ability of these compounds to suppress Bax activation, inhibit downstream caspase activation and enhance cell viability. Interestingly two of the initial hits modulate levels of the same cellular constituent. Subsequently investigation of this process identified a third modulator which also inhibited Bax translocation. Taken together, our results demonstrate the viability of using Bax translocation as a high-throughput measure of mitochondrial PCD, and identify several new small molecule inhibitors suggesting a novel means of inhibiting intrinsic pathway PCD.
Materials and Methods

Construction of EGFP-Bax expression vector
Mouse Bax cDNA was amplified by PCR from IMAGE clone (3968903) and inserted into pEGFP-C1 mammalian expression plasmid (Clontech Laboratories, Inc., 6084) via BglII and EcoRI restriction sites. Insert sequence was subsequently verified by DNA sequencing.

Cell culture and transfection
Chinese Hamster Ovary (CHO) cells were maintained at 37°C, 6% CO₂ in Dulbecco's Modified Eagle Medium (DMEM, 25 mM HEPES) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corp., 12483020), 2 mM glutamine and 1% antibiotics (penicillin and streptomycin) (Invitrogen Corp., 10378016). For generation of stable cell lines, EGFP-Bax was linearized at the MluI site and transfected into CHO cells by the standard calcium phosphate transfection (Kingston et al., 2001). G418 (Sigma-Aldrich Co., G8168) was utilized at 2.5 mg/ml for selection of stably-transfected clones in culture media 48 hours following transfection. Culture media containing G418 was changed every three days for a period of two weeks before sub-cloning individual colonies to 24-well plates. Independent isolates were subsequently analyzed for EGFP-Bax expression by fluorescence imaging. Optimal EGFP-Bax expressing lines were expanded through 3-5 serial passages on 10 cm plates, retested and frozen for long-term cryostorage until use.

High-throughput screening for small molecule inhibitors of Bax traslocation
For high-throughput chemical screening, EGFP-Bax CHO cells were plated in 384-well plates at a density of 4,000 cells per well. Following overnight incubation, cells were treated with 50 µg/ml cisplatin (Sigma-Aldrich #P4394) in cell culture media for 8 hours prior to drug addition. Cisplatin-containing cell culture media was then removed and replaced with fresh cell culture media. Vehicle alone (DMSO) or that containing chemical library components were then added to wells at a final concentration of 5 µM (200 nl of 1 mM stock added to a total volume of 40 µl). Cells were incubated further for 24 hours before fixation in 4% paraformaldehyde in 100 mM phosphate-buffered saline.
(0.9% NaCl, pH 7.4) with Hoechst-33258 (Sigma-Aldrich Co., B2883) at a final concentration of 2 µg/ml for 10 minutes to provide a fluorescent nuclear detection signal. Cells were then washed with 0.1 M PBS, pH7.4, and tiled arrays of EGFP-Bax CHO cells from each well were captured using a Cellomics ArrayScan HCS Reader (Thermo Fisher Scientific, Inc.). For each 384-well plate, results from 16 controls wells were first normalized against one another and the results plotted against the composite data.

*Analysis of Bax translocation*

To determine levels of cellular Bax translocation in each cell, Cellomics ArrayScan HCS images were automatically analyzed using a modified Spot Detection algorithm. Briefly, cells with a registered EGFP profile lying adjacent to an observed DAPI profile (XF53 dichroic filter, Omega Optical; ex. 475 nm, em. 525; ex. 365, em. 525; respectively) were examined as function of their EGFP distribution and signal intensity. Cells undergoing PCD demonstrated a redistribution of EGFP from the cell cytoplasm to punctuate distributions adjacent to the nucleus. In the presence of such redistribution, a dramatic rise in EGFP pixel signal intensity is also observed. For each well, results from a minimum of 250 separate cell profiles were obtained from 20-30 image fields. Results for individual cells were recorded as either *translocated* or *non-translocated* as determined by parameters range obtained from analyses of >2,000 vehicle or cisplatin-treated cells derived from >600 individual well assays. Compound and control results are reported as the percentage of cell exhibiting translocation. For cisplatin-treated controls, observed levels of translocation were typically 60-70%. As an additional internal control, 16 vehicle and 16 cisplatin-treated wells were included for examination for each assay plate as negative and positive controls, respectively.

*Executioner caspase activity measurement*

Executioner caspase (DEVDase) activity was measured using SensoLyte Homogeneous Rh110 Caspase-3/7 Assay Kit (Anaspec, Inc., 71114) according to the manufacturer’s instructions. Briefly, 10,000 cells were plated into each well of a 96-well plate and subjected to the treatments indicated. At the indicated time points, 33 µl of a freshly prepared solution containing fluorogenic caspase substrate (DEVD-Rho110) was added
to wells containing 100 µl cell culture media, then incubated at room temperature for 18 hours. Following incubation, fluorescence was determined using a microplate fluorometer (ex. 496 nm, em. 520 nm; Molecular Devices, Inc., SpectraMax M2).

Trypan Blue exclusion
For trypan blue exclusion assay, 2.5×10^4 cells were plated into each well of a 24-well plate and subjected to the treatments indicated. At the time points indicated, cells were lifted using 0.25% trypsin in saline/EDTA and the solution re-combined with the initial culture media appropriate for that well (which may contain potential floating dead cells). Samples were then pelleted by centrifugation at 800 × g for 5 minutes. Pellets were re-suspended in 100 µl cell culture media and diluted 1:1 with 0.4% trypan blue (Invitrogen Corp., 15250061). Counts were performed using a hemocytometer and cell viability reported as the percentage of trypan blue excluding cells divided by total cell number.

Lactate dehydrogenase release assay
LDH was measured using CytoTox-ONE Homogenous Membrane Integrity Assay (Promega Corp., G7890) according to the manufacturer’s instructions. Briefly, 4.0×10^4 cells were plated into each well of 96-well plates and subjected to the treatments indicated. At the indicated time points, samples were equilibrated to room temperature and 100 µl freshly prepared resazurin-containing solution was added to individual wells containing 100 µl cell culture media. Samples were incubated at room temperature for 10 minutes and fluorescent measurements made using a fluorescence microplate reader (excitation wavelength = 560 nm, emission wavelength = 590 nm; Molecular Devices, Inc., SpectraMax M2).

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
For MTT reduction assays 10×10^4 cells were plated onto 96-well plates and subjected to the treatments indicated. At the specified time points 25 µl of MTT solution (5 mg/ml in PBS) was added to individual wells (containing 100 µl cell culture media) and incubated at 37°C for 2 hours. Following incubation 100 µl of MTT extraction buffer (20% SDS (w/v), 50% dimethyl formamide in deionized distilled water, pH 4.7) was added to
individual wells. Following overnight incubation at room temperature colorimetric measurements were recorded using a multi-well microplate reader (absorbance = 570 nm, reference wavelength = 690 nm; Molecular Devices, Inc., SpectraMax M2).
Results

*Generation and analysis of EGFP-Bax expressing cells*

In order to monitor Bax translocation following PCD stimulation we first constructed an EGFP-Bax expression plasmid. Transient transfection of our EGFP-Bax construct was first performed in CHO and HEK 293T cells to confirm wild-type function of the fusion protein and monitor the temporal localization of EGFP-Bax compared to endogenous Bax following PCD stimulation. For these initial studies, PCD was stimulated using staurosporine and UVC irradiation. As shown in Figure 1A-B, EGFP-Bax is homogeneously distributed in cell cytoplasm prior to stimulation. In contrast, EGFP-Bax re-localized to discreet cellular puncta in a time-dependent manner following PCD induction (Figures 1C-F). Notably, HEK 293T cells appeared more resistant to staurosporine-induced Bax activation (data not shown). Due to the nature of the stimulus, time-dependent induction of PCD using UVC demonstrated that irradiation periods longer than 10 minutes resulted to a significant diminution of the available fluorescent signal. We therefore reduced the duration of subsequent UVC irradiation experiments to 5 minutes, a period which displayed little reduction in EGFP-Bax signal intensity.

Given that anti-apoptotic Bcl-2 family proteins have been shown to suppress Bax activation either indirectly through inhibitory effects on BH3-only proteins, or directly by interacting with Bax (Kim et al., 2009; Lovell et al., 2008), we were interested to determine whether PCD induced Bax relocation could be suppressed through co-transfection of anti-apoptotic Bcl-2 family members. To test this, we co-transfected Bcl-w with EGFP-Bax in HEK 293T cells. Forty-eight hours following transfection, PCD was induced using staurosporine. As shown in Figure 2A-B, cells transfected only with EGFP-Bax, exhibited substantially higher rates of translocation at 8 hours following staurosporine induction compared to Bcl-w/EGFP-Bax transfected cell. Thus the devised EGFP-Bax construct translocates in a spatial and temporally appropriate manner following induction of PCD and is sensitive to interference by anti-apoptotic Bcl-2 family members.

Following these initial studies, lines of stably transfected EGFP-Bax CHO cells were developed. Clones were selected for expansion based upon the above criteria and their level of EGFP-Bax expression. Stable lines which exhibited a degree of Bax
translocation even in the absence of PCD induction (due to high levels of EGFP-Bax expression) were discarded as were low-expressing lineages.

Prior to using stably-transfected EGFP-Bax CHO cells for screening purposes we performed several experiments of ectopic Bax activity. First we compared the activation kinetics of non-transfected and stably-transfected CHO cells at different times following UVC irradiation. As shown in Figure 3A the percentage of cells undergoing Bax translocations were similar in each case between groups, demonstrating that Bax activation is not significantly altered in EGFP-Bax transfected cells. Second we confirmed localization of translocated EGFP-Bax to the mitochondria. As shown in Figure 3B-D, subcellular localization of translocated EGFP-Bax at 6 hours following UVC irradiation is coincident with Tom20, a well-characterized component of the translocase outer membrane (TOM) complex of the mitochondria. Thus EGFP-Bax undergoes correct appropriate relocalization following PCD induction.

*Induction of Bax translocation by cisplatin-mediated DNA damage*

In order to generate a targeted and reproducible model of PCD appropriate for high-throughput screening, we examined several chemical PCD inducers due their uniformity of application. Though experiments with staurosporine indicated that this general serine/threonine kinase inhibitor induced substantial levels of Bax translocation, it was not utilized for high-throughput screening due to its broad ranging effects on cell morphology; which impaired automated cell detection and the analysis of EGFP signal distribution. Cisplatin was therefore utilized to induce p53-dependent induction of Bax translocation. Cisplatin addition resulted in a dose- and time-dependent increase in Bax translocation such that 60±1% and 69±1% cells exhibited complete translocation following 24 hours exposure to 50 µg/ml and 100 µg/ml cisplatin (Figure 4A-B). Because our analyses demonstrated that majority of cisplatin-induced Bax translocation actually occurs between 8 and 24 hours following treatment (Figure 4B), the effect of cisplatin washout following 8 hours of incubation on cell viability was examined (Figure 4C). Levels of Bax translocation as a percent of population in cell incubated with cisplatin for 8 hours followed by an additional 24 hours in cisplatin-free media were in the range of 54±1%. As a result we developed an assessment protocol in which potential small
molecule PCD inhibitors were added to EGFP-Bax expressing CHO cells to a final concentration of 5 µM at 8 hours following incubation in cisplatin. Analyses were performed 24 hours following small molecule addition. The introduction of candidate inhibitors in this manner achieved three desirable screening features: (1) effects arising due to potential drug-cisplatin interactions were avoided; (2) screens identified only candidate molecules capable of inhibiting PCD subsequent to its initiation (8 hours) and; (3) analyses of Bax translocation occurred at a time point prior to significant overt cell loss thus insuring the values reported reflected the true response of the treated population in terms of observed percent.

**Determination of intra- and inter-plate variability**

Due to the single-pass, high-throughput nature of the chemical screen, estimates of intra- and inter-plate variability of EGFP-Bax translocation were required in order to determine the relative sensitivity of the assay. Replicates of stable EGFP-Bax CHO cells were plated into six 384-well plates and subjected to PCD stimulation using 50 µg/ml cisplatin for 8 hours. As illustrated in Figure 5A, following washout and additional incubation in cisplatin-free media for 24 hours, levels of Bax translocation were determined in >250 cells for each of the 2304 wells. An analysis of the cumulative data sets reveals a normal distribution with a standard deviation of 4.53 (Figure 5B). Of the 2304 control translocation levels obtained, only 5 were situated greater than three standard deviations away from the mean. By contrast 78.2% of control Bax translocations lay within one standard deviation of the normalized mean, thus demonstrating the relative variability of the assay as implemented.

**Screening of molecule inhibitors of Bax translocation.**

B score analysis (Brideau et al., 2003; Malo et al., 2006) of four separate chemical libraries (LOPAC, NIH Clinical Collection, Prestwick, Spectrum) consisting of 5006 distinct chemical entities initially identified 18 compounds which significantly reduced Bax translocation compared to cisplatin-treated controls (Figure 6). Following secondary replicate of each compound we investigated whether the results obtained truly reflected an inhibition of cell death, or were a result of other forms of cell toxicity. Three measures
of cell toxicity were employed: trypan blue exclusion, LDH release and MTT reduction assays. Cells were treated for 24 hours in each of the indicated compounds, then analyzed in each of the above assays. As shown in Figure 7A-C, four of these compounds proved to be intrinsically toxic and were removed from further analysis.

Next we examined the potential of the remaining 14 compounds to reduce levels of Bax activation in response to cisplatin-induced DNA damage over a concentration range of 100 nM to 5 µM. As shown in Figure 8, two compounds (isolates number 5 and 29) suppressed Bax translocation >10% compared to vehicle controls at concentrations of 0.5 µM or greater. Thus from pharmacologic and toxicologic profiling it was determined that compounds number 5 and 29 warranted further investigations. Intriguingly both compounds are known to enhance levels of cyclic adenosine monophosphate (cAMP). These findings prompted us to analyze a number of related compounds which act either directly or indirectly to modulate cAMP levels. These investigations resulted in the identification of one further compound (rolipram) which alters levels of Bax translocation by modulating cAMP levels (data not shown).

Small molecule inhibitors of Bax reduce levels executioner caspase activity and enhance cell survival

In order to assess the abilities of compounds 5, 29 and rolipram to suppress PCD, executioner caspase activity was measured in treated and untreated cultures following cisplatin treatment. As shown in Figure 9A, executioner caspase activity was significantly inhibited following treatment with compound 5, 29 or rolipram. The observed inhibition of caspase activity is consistent with a role for these agents in acting upstream of Bcl-2/Bax interaction to inhibit the progression of apoptosis. Consistent with this, we further examined the ability of these compounds to protect against cell death induced by DNA damage. Both LDH release and MTT reduction assays were used to assess plasma membrane integrity and mitochondrial activity, respectively. As shown in Figure 9B, compounds 5 and 29 significantly reduced levels of PCD-induced LDH release, while rolipram trended towards a reduction in cisplatin-induced LDH release but did not achieve statistical significance. In contrast, MTT assays revealed only minor enhancements of mitochondrial activity following addition of compound 5 and 29 to
cisplatin treated cells; and no such enhancement was seen following rolipram addition (Figure 9C). The results suggest that despite reducing levels of executioner caspase activity and enhancing plasma membrane integrity, addition of compounds 5 and 29 did not prevent other Bax- and caspase-independent forms of cell death which occur in the presence of cisplatin.

In the absence of compound 5 or 29 addition, cisplatin treated CHO cells resulted in cells displaying markers of classical apoptosis (Barry et al., 1990). However in the presence of the above compounds, CHO cells continued to undergo cell death displayed several properties of programmed necrosis. Recent studies have demonstrated that receptor-interacting protein 1 kinase (RIP1K) plays a central role in regulating this process, and pharmacologic inhibitors RIP1K have been identified (Degterev et al., 2008; Degterev et al., 2005). To determine the relative influence of this alternative signaling pathway on the cell death seen in cisplatin-treated CHO cells, cell survival was examined in the presence and absence of necrostatin-1. As shown in Figure 10A, addition of necrostatin-1 enhanced levels of cell survival compared to untreated controls but did not achieve statistical significance. Interestingly co-treatment of cisplatin-treated CHO cells with a combination of necrostatin-1 and compound 5 resulted in a further additive enhancement in cell viability (statistically significant from untreated controls), suggesting the presence of at least two distinct forms of cell death mechanisms are operational in cisplatin-treated cells. Combinatorial treatment did not result in a significant increase in mitochondrial activity as measured by MTT reduction assay (Figure 10B). Therefore the enhancement of cell viability seen using combinatorial treatment is unlikely to be mediated through a direct effect on mitochondrial activity. Such results highlight the complexities likely to be encountered in attempting to inhibit PCD in vivo and demonstrate the importance of utilizing a combinatorial approach in undertaking such efforts.
Discussion

In the present study we developed a high-throughput system to examine real-time Bax translocation following PCD stimulation. We have utilized CHO cell lines stably expressing EGFP-Bax to screen for small molecule inhibitors of Bax translocation, a central and requisite step in the execution of mitochondrial-mediated programmed apoptotic cell death. Examination of over 5000 compounds from four distinct chemical libraries resulted in the initial identification of eighteen compounds which reduced cisplatin-induced Bax translocation. Further testing and analysis of these initial hits ultimately revealed two pharmacologic agents which significantly reduced levels of pro-apoptotic Bax translocation, even when added 8 hours subsequent to PCD stimulation by cisplatin. Interestingly, uncoding of these agents revealed them to be two distinct modulators of cAMP signaling. Secondary screening of cAMP modulatory agents revealed that the phosphodiesterase inhibitor rolipram also provided modest inhibition of Bax translocation. To our knowledge this is the first high-throughput screen aimed at identifying small molecule inhibitors of Bax activation/translocation in order to inhibit progression of programmed cell death. Conversely, several high-throughput screens have previously been performed attempting to identify inhibitors of Bcl-2 family proteins such as Bcl-xL and Bcl-B for use as anti-cancer therapeutics (Chan et al., 2003; Qian et al., 2004; Yip et al., 2008). For these studies we have incorporated several screening features aimed at enhancing the identification of clinically useful therapeutic agents. First, we have constructed our primary screen around a cell-based system instead of utilizing a direct in vitro biochemical assay. Such systems select for compounds with appropriate membrane permeability and quickly filter out those possessing significant acute toxic effects. In addition, screening using dynamic core markers of apoptosis such as Bax allow one to test compounds functionally, providing an unbiased means of identifying any and all agents which influence the given process regardless of mechanism rather than narrowly focusing on a particular protein-protein interaction. Second, we have chosen to examine the ability of small molecules to inhibit Bax translocation and enhance cell rescue 8 hours subsequent to cisplatin-induced cell injury. Such a screening protocol therefore aims to identify only those compounds which possess inhibitory capabilities within a realistic and clinically useful therapeutic window. This is in contrast to screening
protocols in which the functional inducer and small molecule therapeutic are tested coincident with one another, a staging which clinically is unrealistic. Predictably such screening has lead to the identification of a number of lead compounds (particularly in the area of neural injury) which subsequently fail in follow-up clinical evaluation. (Diguet et al., 2004; Tymianski et al., 1993). Conversely by taking a post-injury approach for the current screen, there is a greater probability that the cell-based effects observed would translate into significant cellular rescue when applied in a more physiologic context. With respect to this it is important to note that despite demonstrating relatively modest effects on Bax inhibition in the high-throughput screen, the identified apoptotic inhibitors may exert significant neuroprotective effects in vivo. In this context it is interesting that cyclosporin A, which has previously been shown by ourselves and others to mediate significant neuroprotection in vivo in several neural injury paradigms, was observed to reduced Bax activation by only 11% in our high-throughput screen despite our demonstration that it acts upstream of Bax activation (Hui et al., 2009; Snyder et al., 1998; Uchino et al., 1998; Uchino et al., 2002; Wang et al., 1997).

Screening for small molecule inhibitors of Bax translocation in response to cisplatin-induced DNA damage identified two different modulators of cAMP signaling. Previously, several investigators have described protective effects resulting from elevated cAMP levels against p53-mediated PCD (Naderi et al., 2009; von Knethen and Brune, 2000; von Knethen et al., 1998). However the precise molecular mechanism underlying these observations remains unclear. Multiple pathways have been suggested, including modulation of p53 phosphorylation; altered expression of Bax and related pro-apoptotic proteins such as Puma; induction of BDNF expression through its cAMP responsive element binding protein (CREB), and direct alteration of Bax phosphorylation - which has been shown to inhibit its translocation into the mitochondria (Riccio et al., 1999; Safa et al., 2010; Xin et al., 2007). In the present study we show that the identified cAMP modulators significantly reduce levels of downstream executioner caspase activity.

Analysis of the pattern of PCD seen in cisplatin-treated CHO cells suggested the presence of alternative PCD pathways such as necroptosis. In addition, as we have only monitored the activation of Bax, cAMP-independent Bax activation (e.g., direct interaction with p53) and Bax-independent mechanisms (i.e., Bak-mediated MOMP)
could still have triggered cell death as illustrated by the absence of any protection against the loss of mitochondrial activity shown through MTT reduction assays. Hence, in order to realistically rescue neurons damaged during acute neural injuries, pharmacologic modulation of multiple pathways are likely required.

In the present study we describe the development of a robust high-throughput screening approach to identify small molecule inhibitors of Bax translocation. Such screening resulted in the identification of two small molecules regulating cAMP signaling which significantly inhibit Bax translocation and sub-micromolar concentrations. Such findings demonstrate a practical pharmacologic means of manipulation cAMP levels in order to alter levels of neural apoptosis. However, our findings also highlight the potential complexities in treating such injuries due to the potential presence of multiple forms of programmed cell death. Thus inhibitors of several PCD pathways may ultimately need to be employed in order to effective therapy \textit{in vivo}. 
References


Figure 1. Time-dependent translocation of EGFP-Bax to the mitochondria following initiation of PCD by staurosporine in CHO and HEK 293T cells. Following transfection with EGFP-Bax, CHO and HEK 293T cells were stimulated 48 hours following transfection with 2 µM staurosporine or UVC irradiation (data not shown) to initiate programmed cell death. Subcellular localization of EGFP-Bax was then examined in transfected cells at 0, 4 or 8 hours following treatment. As indicated in the photomicrographs, EGFP-Bax was initially homogeneously distributed throughout the cell cytoplasm prior to PCD stimulation in CHO (A) and HEK 293T (B) cells. As seen at 4 (C, D) and 8 hours (E, F) following staurosporine treatment, both CHO (C, E) and HEK 293T (D, F) cells exhibited re-localization of EGFP-Bax to a punctuate perinuclear pattern of distribution.
Figure 2. Co-transfection of Bcl-w inhibits EGFP-Bax re-localization following staurosporine treatment in HEK 293T cells. The ability of anti-apoptotic Bcl-2 family proteins to suppress translocation of EGFP-Bax was examined using co-transfection of Bcl-w in transfected EGFP-Bax cells. At 48 hours following transfection, cells were stimulated with 2 µM staurosporine to initiate programmed cell death. No differences in the initial cytoplasmic distribution of EGFP-Bax was observed prior to staurosporine treatment regardless of Bcl-w status (A, C). However at 8 hours following staurosporine treatment, substantially fewer cells co-transfected with Bcl-w exhibited a punctate redistribution of EGFP-Bax compared to EGFP-Bax cells transfected with control vector alone (B, D).
Figure 3. Ectopically expressed EGFP-Bax does not alter the temporal pattern redistribution of endogenous Bax. High levels of Bax over-expression have previously been shown to be capable of initiating MOMP. The temporal profiles of Bax activation were therefore examined in stably-transfected EGFP-Bax expressing CHO cells with that seen in non-transfected controls. As demonstrated using anti-Bax antisera, the percent Bax translocation seen as a function of time in non-transfected CHO cells was examined via direct immunofluorescence and compared to that seen in stably transfected EGFP-Bax expressing cells. (A) When examined at 0, 3, 6 and 9 hours following UVC treatment no significant difference in Bax translocation was observed between untransfected CHO cells and cells expressing EGFP-Bax. Data represent mean ± SEM of triplicate experiments in which n ≥ 100 cells per well were examined for all treatment groups. In order to determine the fidelity of EGFP-Bax translocation, the subcellular localization of EGFP-Bax (B) was compared in cells to that seen for mitochondrial marker Tom20 (C) as determined by immunofluorescence. (D) Overlay of EGFP and Tom20-specific signals demonstrates that ectopic EGFP-Bax is appropriately translocated to the mitochondria following PCD stimulation.
**Figure 4. Cisplatin induces EGFP-Bax translocation in a time- and dose-dependent manner.** In addition to staurosporine and UVC treatment, the ability of cisplatin to induce EGFP-Bax translocation was examined. *(A)* When examined at 24 hours following cisplatin treatment Bax translocation was observed to occur in a dose-dependent manner. *(B)* To temporally assess the redistribution of Bax in the presence of cisplatin, Bax translocation was examined at 0, 8 and 24 hours following cisplatin treatment. Nominal levels of Bax translocation are observed 8 hours following cisplatin treatment as compared to translocation levels seen in vehicle treated controls; indicating the principle phase of Bax translocation occurs between 8 and 24 hours following cisplatin addition under the conditions utilized. *(C)* Removal of cisplatin 8 hours following its addition to CHO cells does not significantly alter the subsequent course of EGFP-Bax translocation. Levels of EGFP-Bax translocation at various time points following cisplatin addition were examined in the presence and absence of cisplatin washout following 8 hours of treatment. Time indicated on the abscissa in *(C)* represents total time between initiation of cisplatin treatment and time of analysis. As indicated in the figure, no significant differences in the level of EGFP-Bax translocation are observed in the presence or absence of cisplatin at 8 hours. Thus exposure of cells to cisplatin for 8 hours is sufficient to initiate PCD regardless of subsequent culture conditions. Data represent mean ± SEM from six independent replicates in which $n \geq 250$ cells per well were examined for all treatment groups.
Figure 5. Intra- and inter-plate variability observed for automated EGFP-Bax translocation screening of 384-well high-throughput assay. Prior to screening small molecule inhibitors of Bax translocation, intra- and inter-plate variability was assessed by examining levels of cisplatin-induced EGFP-Bax translocation in six 384-well plates (2304 wells) following cisplatin treatment. For each well, \( n \geq 250 \) cells were examined. (A) Data points represent level of observed EGFP-Bax translocation as determined in single assay wells. Data collected from each plate are represented by different colours normalized to a mean set arbitrarily to 100%. Blue and red dotted lines represent mean ± 3 standard deviations and mean ± 1 standard deviation, respectively. (B) Frequency distribution of the data presented in (A), demonstrating normalized distribution of the collected data. Of the data collected, 99.8% falls within three standard deviations of the normalized mean. Dotted lines indicate standard deviations away from the normalized mean. Red line represents normal distribution curve.
Figure 6. Identification of inhibitors of Bax translocation. High-throughput assay screening was utilized to examine compounds from four chemical libraries to identify small molecule inhibitors of Bax translocation. In the initial screen a total of 18 hit compounds, each represented by a (+) sign were identified from B score analysis in comparison to controls. Control data are shown for reference. For each well, n ≥ 250 cells were examined. Red dotted lines represent the mean plus or minus three standard deviations. Secondary re-screening of the identified hits verified their action with respect to Bax translocation.
Figure 7. Determination of intrinsic toxicity of identified compounds. Because low molecular weight compounds can potentially inhibit Bax translocation through a variety of mechanisms, the intrinsic cell toxicity of identified hits was evaluated using three distinct assays of cell viability. Cells were incubated for 24 hours at a concentration of 5 μM prior to analysis. (A) Analysis of cells for trypan blue exclusion; (B) examination of levels of lactate dehydrogenase (LDH) release; (C) analysis of mitochondrial activity as measured through the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Compounds 3, 18, 21 and 22 (indicated by black arrows) were observed to possess intrinsic toxicity and therefore were not examined in subsequent analyses. Data represent mean ± SEM from four independent replicates for all treatment groups. For trypan blue exclusion experiments, n ≥ 100 cells per well were examined for all treatment groups.
Figure 8. Dose-response relationships for identified compound hits. To further assess the ability of identified small molecule compounds to inhibit Bax translocation following cisplatin treatment, identified hits were examined at concentrations ranging from 100-5000 nM. Compounds 5 and 29 were found to be significantly more effective than other identified hits as suppressing levels Bax translocation at sub-micromolar concentrations. Subsequent unmasking of compound hits revealed that both compounds 5 and 29 are modulators of cAMP signaling, suggesting a link between cAMP signaling and Bax activation. Data represent mean ± SEM of four independent replicates in which n ≥ 250 cells per well were examined for all treatment groups.
Figure 9. Inhibition of executioner caspase activity and enhancement of cell viability measures by compounds 5, 29 and rolipram. Given that two hits identified through high-throughput screening were known modulators of cAMP signaling, we also examined the potential of the phosphodiesterase inhibitor rolipram to suppress Bax activation and cell death following cisplatin treatment. Cells were exposed to cisplatin for 8 hours followed by washout and addition of test compounds. (A) Treatment with compounds 5, 29 and rolipram each significantly reduced levels of executioner caspase activity. (B) Alterations in plasma membrane integrity as measured by LDH release demonstrated that compounds 5 and 29 significantly reduce levels of LDH release, while rolipram exhibit a non-significant trend toward reduced LDH release. (C) Analysis of mitochondrial activity as measured by MTT reduction reveals a small but significant increase by both compounds 5 and 29. Data represent mean ± SEM of triplicate experiments for all treatment groups. * and # indicate statistical significance at \( p < 0.05 \) and \( p < 0.01 \) between treatments with compound and vehicle.
Figure 10. Combinatorial pharmacologic inhibition of PCD and necroptosis further enhances cell survival in the presence of cisplatin. To examine the influence of additional cell death mechanism in the presence of cisplatin, we examined the role of necroptosis in cells following DNA damage. Cells were initially exposed to cisplatin for 3 hours followed by washout and compound addition. (A) As examined by trypan blue exclusion at 24 hours following washout and compound addition, necrostatin-1 treatment resulted in a non-significant trend toward reduced levels cell death following cisplatin exposure. Interestingly, co-treatment of compound 5 plus necrostatin-1 resulted in a further increase in cell viability (statistically significant compared to vehicle treatment), suggesting that these compounds exerting their protective effects via independent mechanisms (i.e., PCD versus necroptosis). (B) The observed enhancement in cell viability examined at 24 hours following washout and compound addition was not a associated with an increase in mitochondrial activity. Data represent mean ± SEM of triplicate experiments for all treatment groups. For trypan blue exclusion experiments, n ≥ 100 cells per well were examined for all treatment groups. * indicates statistical significance at p < 0.05 between treatments with compound and vehicle.