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A C-terminus Mitochondrial-localization Region and BH3 Domain of Puma are Required for Apoptotic Function

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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by

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Abstract

Apoptosis is known to contribute to the loss of neurological function in brain injury and several neurodegenerative diseases. The Bcl-2 family of proteins consist of pro-apoptotic and anti-apoptotic members that interact physically and functionally to regulate apoptosis in a cell type and stimulus specific manner. We have previously demonstrated that the Bcl-2 family member Puma plays a key role in triggering neuronal apoptosis under diverse stress conditions. However, the mechanism by which Puma mediates apoptosis remains unclear. Here we found that Puma contains two domains required for its apoptotic function: the BH3 domain and a region in the c-terminus that regulates its localization to the mitochondria. We found that the BH3 domain is required to bind both the anti-apoptotic protein, Bcl-xl, and the pro-apoptotic protein, Bax. However, we found that the BH3 domain of the protein is not required for its mitochondrial-localization. Instead, we identified a region within the c-terminus of Puma that is essential for both mitochondrial-localization and apoptotic function of the protein. Although we found that the c-terminus of Puma is not required to bind Bcl-xl or Bax in vitro, it is required for co-localization and likely interaction with these proteins in the cellular context. In summary, we determined that Puma requires both its c-terminal mitochondrial-localization sequence and its BH3 domain to co-localize with and bind to Bcl-2 family members, and to induce neuronal cell death.

Keywords

Apoptosis, Bax, Bcl-2, Bcl-xl, BH3-only, BH3 domain, c-terminus, Mitochondrial localization, Protein interaction, Puma

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List of Abbreviations

APAF-1 - Apoptosis protease-activating factor 1

ART - Apoptosis regulating target

Bax – Bcl-2 associated X-protein

Bcl-2 – B-cell lymphoma type 2

Bcl-xl – B-cell lymphoma extra-large

BH – Bcl-2 Homology

BH3 - Bcl-2 homology domain three

CLAC - Cytochrome-c Liberation Associated Conformation

CLIC - Cytosolic Locked In Conformation

co-IP - co-immunoprecipitation

D33A – aspartate 33 to alanine

ER – endoplasmic reticulum

Hα1 – helix alpha 1

MESSA - MEta Server for protein Sequence Analysis

MOM – Mitochondrial outer membrane

N2A – Neuro2A

PCR – polymerase chain reaction

PI - Propidium Iodide

Puma – p53 up-regulated mediator of apoptosis

UV - ultra-violet

Preface

Apoptosis plays an crucial role in normal development and maintenance of tissue homeostasis. It is required to eliminate unnecessary tissue during development, and later on in an organism's lifespan it is necessary to destroy damaged or mutated cells (Kerr *et al.* 1972; Oppenheim 1991; Yoshida *et al.* 1998). One of the reasons that apoptosis is utilized for these functions is that unlike other modes of cell death, such as necrosis, apoptosis eliminates cells without eliciting an inflammatory response or causing damage to the surrounding tissue (Kerr *et al.* 1972). Disruption of the normal apoptotic process in animal models has been shown to cause significant and often lethal defects (Ashkenazi & Dixit 1998; Li *et al.* 2000; Motoyama *et al.* 1995; Strasser *et al.* 1990; Yoshida *et al*. 1998).

Perhaps due to its vital role in normal tissue, dysregulation of the apoptotic process also plays a major role in many diseases. Many cancer cells contain mutations in the apoptotic machinery that allow them to continue to grow when they would otherwise die (Adams & Cory 2007; Cory *et al.* 1999; Strasser *et al.* 1990; Yu & Zhang 2009; Vaux *et al.* 1988). Activation of apoptosis by acute trauma, as in ischemic injury or spinal cord injury, can result in significant neuronal loss (Engel *et al.* 2011; Kotipatruni *et al.* 2011; Nitatori *et al.* 1995; Steckley *et al.* 2007). Apoptotic cell death also plays a significant role in many neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, and Huntington's disease (Higgins *et al.* 2010; Portera-Cailliau *et al.* 1995; Vila & Przedborski 2003). Because it plays such a crucial role in both healthy and diseased tissue, a more complete understanding of the key proteins involved in the regulation of apoptosis will be important for the development of new treatments applicable to a variety of injury and disease conditions.

Chapter 1 – Introduction

1.1 – Apoptosis

Apoptosis, or programmed cell death, is perhaps the most prevalent and well-regulated means of cell death. The cell must precisely coordinate the actions of many proteins in order to destroy itself without causing damage to surrounding cells. This precise coordination presents a distinctly identifiable cellular morphology. In the initial stages of apoptosis, the cellular membrane shrinks and develops a rounded appearance (Kerr *et al.* 1972). At the same time, the chromatin condenses in the now shrinking nucleus, in a process termed pyknosis (Kerr *et al.* 1972). The cellular membrane then takes on the blebbed appearance characteristic of apoptotic cells (Kerr *et al.* 1972). In the final stage of apoptosis, the cell packages its remaining contents into small vesicles, known as apoptotic bodies, that can be easily engulfed by phagocytic cells for degradation (Kerr *et al.* 1972).

This carefully contained process can be triggered by both extracellular (extrinsic pathway) and intracellular (intrinsic pathway) cues. In the extrinsic apoptotic pathway, extracellular ligands bind to death receptors on the cellular surface in order to activate apoptosis (Ashkenazi & Dixit 1998). In the intrinsic apoptotic pathway, cellular stress causes expression or activation of apoptosis-inducing factors (Ashkenazi & Dixit 1998; Cory & Adams 2002). These factors activate pro-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family and result in the permeabilization of the mitochondrial

membrane (Cory & Adams 2002). The primary focus of this thesis is the intrinsic apoptotic pathway.

1.11 - Intrinsic apoptotic pathway

The intrinsic apoptotic pathway is activated by many different cellular stressors, including but not limited to: genotoxic, endoplasmic reticulum (ER), and oxidative stress (Engel *et al*. 2011; Galehdar *et al.* 2010; Reimertz *et al* 2003; Steckley *et al.* 2007). The B-cell lymphoma 2 (Bcl-2) family of proteins plays a key role in the regulation of the intrinsic apoptotic pathway, and downstream caspases are primarily responsible for the execution of the apoptotic process (Cory & Adams 2002; Salvesen & Dixit 1997). Activation of the pro-apoptotic Bcl-2 family members results in mitochondrial outer membrane permeabilization and releases pro-death factors, such as cytochrome-c, into the cytoplasm (Figure 1; Cory & Adams 2002; Li *et al.* 1997; Yoshida *et al.* 1998). Once released into the cytoplasm, cytochrome-c forms a complex with apoptosis protease-activating factor 1 (APAF-1) and caspase-9 known as the apoptosome (Figure 1; Li *et al.* 1997; Liu *et al.* 1996). The apoptosome cleaves and thereby activates caspase-9, which then cleaves and activates the downstream effector caspases, caspase-3 and caspase-7 (Figure 1; Li *et al.* 1997). This caspase cascade is responsible for the characteristic apoptotic events, including: chromatin condensation and fragmentation, membrane blebbing, and packaging of cellular remains into apoptotic bodies (Cory & Adams 2002; Liu *et al.* 1997).

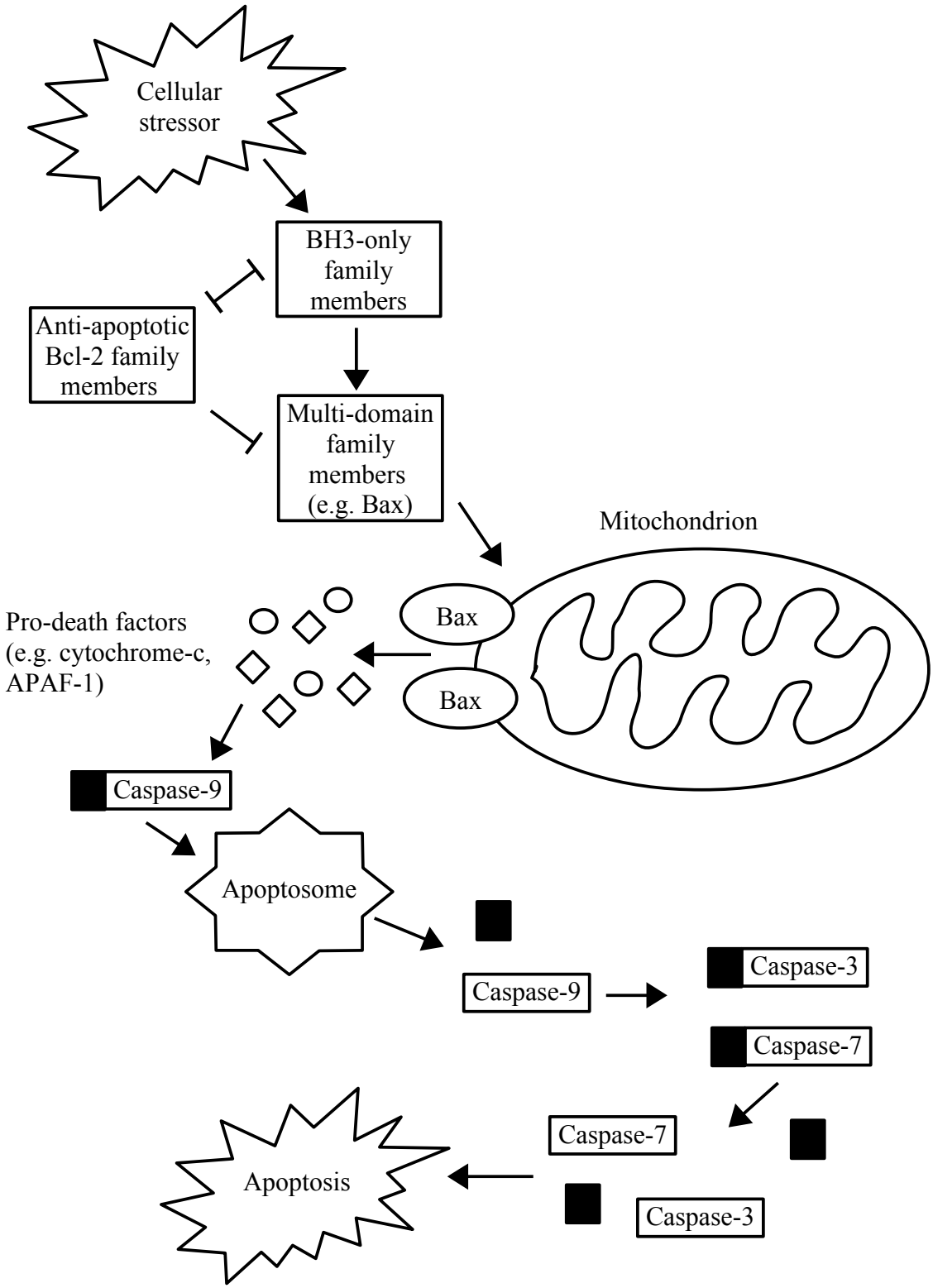

Cellular stressor
BH3-only family members
Anti-apoptotic Bcl-2 family members
Multi-domain family members (e.g. Bax)
Mitochondrion
Bax
Bax
Pro-death factors (e.g. cytochrome-c, APAF-1)
Caspase-9
Apoptosome
Caspase-9
Caspase-3
Caspase-7
Caspase-7
Caspase-3
Apoptosis

Figure 1 - The Intrinsic Apoptotic Pathway. The intrinsic apoptotic pathway is activated by a variety of cell stressors, including oxidative and genotoxic stress. These stressors trigger the up-regulation or activation of pro-apoptotic BH3-only Bcl-2 family members, which act both by inhibiting the anti-apoptotic family members and by activating the multi-domain pro-apoptotic family members. This allows the multi-domain Bcl-2 family members to translocate the the mitochondrial outer membrane, where they oligomerize and form channels that allow the release of pro-death factors into the cytoplasm. In the cytoplasm, these factors interact with caspase-9 in a structure called the apoptosome. The apoptosome cleaves and activates caspase-9, which goes on to activate downstream effector caspases, caspase-3 and caspase-7. These downstream effector caspases trigger the cellular changes characteristic of apoptotic cell death, such as chromatin condensation and membrane blebbing.

1.2 - Bcl-2 Family Members

The B-cell lymphoma 2 (Bcl-2) family of proteins are key regulators of the intrinsic apoptotic pathway (Cory & Adams 2002; Engel *et al.* 2011). These proteins share one or more Bcl-2 homology (BH) domains with their namesake, Bcl-2. Although they have different amino acid sequences and contain different BH domains, their three-dimensional fold structures are strikingly similar. Most Bcl-2 family members have two central hydrophobic alpha-helices surrounded by six or seven amphipathic alpha-helices (Petros *et al.* 2004). This similarity in structure may help to explain their ability to form homo and hetero-dimers with other family members (Petros *et al.* 2004).

The Bcl-2 family members are divided based on their functions into two main categories: the pro-apoptotic family members and the anti-apoptotic family members (Cory & Adams 2002). The pro-apoptotic family members are further sub-divided into Bcl-2 homology domain three (BH3) only proteins and multi-BH domain proteins (Cory & Adams 2002). Altering the expression or activation of one of the family members can kill cells or render them resistant to apoptotic cell death (Cory *et al.* 1999; Motoyama *et al.* 1995; Strasser *et al.* 1990; Steckley *et al.* 2007).

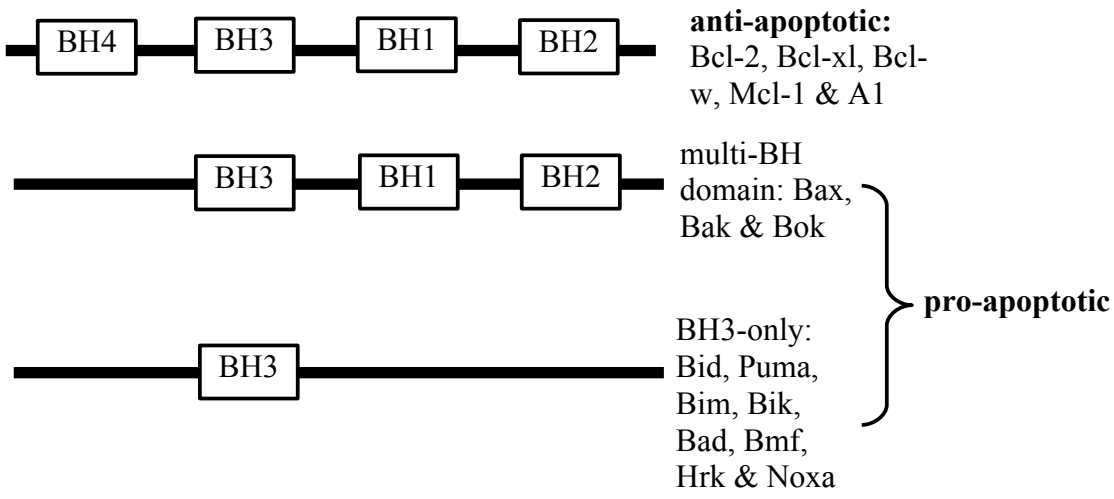

BH4
BH3
BH1
BH2
anti-apoptotic:
Bcl-2, Bcl-xl, Bcl-w, Mcl-1 & A1
BH3
BH1
BH2
multi-BH domain: Bax, Bak & Bok
BH3
BH3-only: Bid, Puma, Bim, Bik, Bad, Bmf, Hrk & Noxa
pro-apoptotic

Figure 2 - Bcl-2 Family Members. The Bcl-2 family of proteins are important regulators of the intrinsic apoptotic pathway. They are divided functionally into anti-apoptotic and pro-apoptotic proteins and the latter group is further subdivided into multi-domain and BH3 domain-only proteins. The BH3-only proteins promote apoptosis by activating the multi-domain pro-apoptotic proteins, while the anti-apoptotic proteins inhibit both the BH3-only and multi-domain proteins to suppress apoptosis.

1.21 - Pro-apoptotic Bcl-2 Family Members

As suggested by their name, the pro-apoptotic members of the Bcl-2 family of proteins act to promote apoptosis. They are subdivided into BH3-only proteins and multi-domain proteins. The BH3-only proteins have only their BH3 domain in common with Bcl-2 and are the first response to cellular stress (Engel *et al.* 2011). Expression and activation of the BH3-only proteins results in the activation of the multi-domain Bcl-2 family members (Engel *et al.* 2011). The multi-domain Bcl-2 proteins share between one and three of the Bcl-2 homology domains and act by forming channels in the mitochondrial outer membrane and allowing the release of of pro-death factors, such as cytochrome-c (Cory & Adams 2002; Lalier *et al.* 2007; Li *et al.* 1997).

1.211 - BH3-only Bcl-2 family members

The BH3-only members of the pro-apoptotic Bcl-2 proteins consist of: Puma, Noxa, Bid, Bim, Bad, Bmf, and Hrk (Cory & Adams 2002; Engel *et al.* 2011). Although they are structurally similar, the BH3-only proteins are regulated by diverse mechanisms. Some, such as Puma, are regulated mainly through transcription; others, such as Bid, are regulated mainly post-transcriptionally via proteolytic cleavage (Cory & Adams 2002; Engel *et al.* 2011). Their affinity for binding to the anti-apoptotic proteins is also quite diverse; some BH3-only proteins, such as Noxa and Bid, are able to bind to only some of the anti-apoptotic proteins, whereas others, such as Puma, are able to bind to all of the anti-apoptotic proteins (Kuwana *et al.* 2005; Uren *et al.* 2007). The BH3-only proteins are differentially activated by various stimuli and in various cell types; one BH3-only

protein that is highly efficacious and of almost universal importance is Puma (Cory & Adams 2002; Engel *et al.* 2011; Hikisz *et al.* 2012).

In accordance with their name, the BH3-only proteins contain only the third of the Bcl-2 homology domains. It is a short, alpha-helical, amphipathic domain approximately 9-16 amino acids long (Adams & Cory 1998; Chittenden *et al.* 1995; Cory & Adams 2002; Engel *et al* 2011). The BH3 domain is the site of protein interactions (the binding site for either anti-apoptotic or multi-domain proapoptotic Bcl-2 family members) and is required for the apoptotic activity of BH3-only proteins (Chittenden *et al.* 1995; Engel *et al.* 2011; Kazi *et al.* 2011; Kelekar & Thompson 1998; Liu *et al*. 2009).

Two main mechanisms of action have been proposed for these proteins: the direct activation model and the indirect activation model. In the direct activation model, one of the BH3-only proteins directly binds to and activates one of the multi-BH domain pro-apoptotic Bcl-2 family members (Chipuk & Green, 2008; Cory & Adams, 2002; Engel *et al.* 2011). In the indirect activation model, a BH3-only protein binds to one of the anti-apoptotic family members and displaces a multi-BH domain pro-apoptotic Bcl-2 family member (Chipuk & Green 2008; Cory & Adams, 2002; Engel *et al.* 2011). Once the multi-BH domain pro-apoptotic Bcl-2 family member is freed from the anti-apoptotic protein, a few methods of activation have been suggested. Some believe that once released, the proteins can bind to membrane-bound, otherwise activated multi-BH domain pro-apoptotic Bcl-2 family members and form oligomers (Kazi *et al.* 2011; Valentijn 2008). Others have proposed that as-yet-unidentified intermediary proteins

bind directly to the multi-BH domain pro-apoptotic Bcl-2 family members once they are released from the anti-apoptotic proteins (Cory & Adams 2002). It has also been suggested that the binding and release of multi-BH domain pro-apoptotic Bcl-2 family members from anti-apoptotic proteins may in itself be sufficient to activate them (Gautier *et al.* 2011; Moreau *et al.* 2003).

There is evidence for both models, and it seems most likely that each of the BH3-only proteins functions slightly differently, allowing the cell to precisely regulate apoptosis. Some BH3 proteins, such as Bid, are widely accepted as direct activators of multi-BH domain pro-apoptotic Bcl-2 family members (Desagher *et al.* 1999; Eskes *et al.* 2000; Lovell *et al.* 2008). Others, such as Bad, appear to function only through the indirect model (Lalier *et al.* 2007). The conflicting evidence for other BH3-only proteins, such as Puma (Gallenne *et al* 2009; Gautier *et al.* 2011; Ming *et al.* 2006; Willis *et al.* 2007), may indicate that they function via a combination of both the indirect and direct activation models (Du *et al.* 2011; Engel *et al.* 2011; Kuwana *et al.* 2005; Steckley *et al.* 2007; Zhang *et al.* 2009).

1.212 - Multi-BH domain Bcl-2 family members

There are three multi-BH domain members of the pro-apoptotic Bcl-2 proteins: Bax, Bak, and Bok (Adams & Cory 1998; Cory & Adams, 2002). They contain the first three BH domains: BH1, BH2, and BH3 (Cory & Adams, 2002; Petros *et al.* 2004). Structurally, they consist of two core hydrophobic alpha-helices surrounded by seven

amphipathic alpha-helices (Petros *et al.* 2004). This structure allows them to exist in one conformation in the cytosol and to form oligomeric channels in the mitochondrial outer membrane when activated (Chipuk & Green 2008; Lalier *et al.* 2007).

Bax, Bak, and Bok are typically activated by the BH3-only proteins, either through a direct or an indirect interaction (Chipuk & Green 2008). Once activated, they oligomerize and form channels in the outer mitochondrial membrane (Cory & Adams 2002; Lalier *et al.,* 2007). These channels permeabilize the outer mitochondrial membrane and allow the release of pro-apoptotic factors, such as cytochrome-c, into the cytoplasm. When released into the cytoplasm, these pro-death factors are free to cleave and activate caspase-9 (Cory & Adams 2002; Li *et al.* 1997). Once activated, caspase-9 activates downstream caspases, resulting in apoptotic cell death (Li *et al.* 1997; Liu *et al.* 1996).

1.22 - Anti-apoptotic Bcl-2 Family Members

The anti-apoptotic Bcl-2 family members consist of: Bcl-xl, Bcl-2, Bcl-w, A1, and Mcl-1 (Adams & Cory 1998; Cory & Adams 2002). They contain all four of the BH domains as well as a c-terminal membrane-localization sequence (Lindsay *et al.* 2011; Petros *et al.* 2004; Reed *et al.* 1996). Their three-dimensional structure consists of two hydrophobic alpha-helices surrounded by five amphipathic alpha-helices (Petros *et al.* 2004). The first three BH domains form a hydrophobic binding pocket that is responsible for the interactions between anti-apoptotic proteins and other Bcl-2 family members

(Petros *et al.* 2004; Sattler *et al.* 1997). Some of the anti-apoptotic family members have also been shown to form soluble homodimers in the cytoplasm by sequestering their c-terminal membrane-localization sequence in this hydrophobic pocket until activated (Jeong *et al.* 2004; Petros *et al.* 2004).

Anti-apoptotic Bcl-2 family proteins function primarily by binding to and restricting both the BH3-only and multi-domain pro-apoptotic Bcl-2 family members. When anti-apoptotic proteins bind to the BH3-only proteins, it prevents them from binding to and/or activating the multi-domain pro-apoptotic Bcl-2 family members (Nakano & Vousden 2001; Steckley *et al.* 2007; Yu *et al.* 2001). When anti-apoptotic Bcl-2 proteins bind to the multi-domain pro-apoptotic Bcl-2 family members, it prevents the activation and/or oligimerization of the multi-BH domain pro-apoptotic proteins (Gallenne *et al.* 2009; Gautier *et al.* 2011; Jeong *et al.* 2004; Sattler *et al.* 1997).

1.3 – Bcl-2 family members in neuronal apoptosis

The wide variety of apoptotic regulators seems to suggest that each protein may be of differing importance in various cell types and for various cell-death stimuli. There is growing evidence to indicate that different cell types may regulate apoptosis in distinct ways (Chen *et al.* 2005; Chipuk *et al.* 2010; Cory & Adams 2002; Engel *et al.* 2011; Hikisz *et al.* 2012; Kim *et al.* 2006; Kuwana *et al.* 2005; Letai *et al.* 2002). For this reason it is increasingly important to determine the roles of the Bcl-2 family proteins in each cell type and under each apoptotic stimulus. Apoptosis has been shown to play an

important role in a number of neurodegenerative disorders including Alzheimer's and Huntington's disease, as well as in acute conditions, such as stroke and traumatic brain injury (Engel *et al.* 2011; Higgins *et al.* 2010; Portera-Cailliau 1995; Vila & Przedborski 2003). The Bcl-2 family of proteins appears to play an important role in the regulation of neuronal apoptosis under these conditions (Boise *et al.* 1993; Frankowski *et al.* 1995; Galehdar *et al.* 2010; Kotipatruni *et al.* 2011; Rubin *et al.* 1994; Steckley *et al.* 2007).

Anti-apoptotic members of the Bcl-2 family have been shown to inhibit neuronal cell death induced by a number of different stimuli, including: neurotrophic factor withdrawal, reactive oxygen species, and ischemia (Allsopp *et al.* 1993; Garcia *et al.* 1992; Martinou *et al.* 1994; Zhong *et al.* 1993). Of the anti-apoptotic Bcl-2 family members, Bcl-xl appears to play a particularly important role in preventing neuronal apoptosis (Boise *et al.* 1993; Frankowski *et al.* 1995; Rubin *et al.* 1994).

Of course, the role of the pro-apoptotic members of the Bcl-2 family in neuronal apoptosis cannot be neglected. The multi-domain pro-apoptotic Bcl-2 family member, Bax, has been shown to mediate neuronal apoptosis in a rat model of spinal cord injury (Kotipatruni *et al.* 2011), and neuronal apoptosis induced by oxidative stress (Steckley *et al.* 2007). The pro-apoptotic BH3-only protein, Puma, has been shown to be of particular importance in neuronal death induced by a number of factors, including: trophic factor deprivation, endoplasmic reticulum stress, ischemia, and oxidative stress (Ambacher *et al.* 2012; Galehdar *et al.* 2010; Niizuma *et al.* 2009; Reimertz *et al.* 2003; Steckley *et al.* 2007). It appears that the members of the Bcl-2 protein family are key

regulators of neuronal apoptosis in a number of conditions, both chronic (as in the case of neurodegenerative disorders) and acute (as with stroke or spinal cord injury).

The Bcl-2 family of proteins has also been shown to play a crucial role in the regulation of neuronal apoptosis during development. Anti-apoptotic Bcl-2 family members are highly expressed in developing neurons (Abe-Dohmae 1993; Frankowski *et al.* 1995). Bcl-xl knockout mice show increased neuronal apoptosis (Motoyama *et al.*1995) that can be mitigated by also knocking out the pro-apoptotic protein, Bax (Shindler *et al.* 1997). The pro-apoptotic members of the Bcl-2 family also play an important role in normal neuronal development. Bax/Bak double knockout mice have a number of neurological deficits, including apparent deafness and seizures (Lindsten *et al.* 2000). Histologically, the brains of these mice display accumulations of small, undifferentiated neuronal cells, most notably in the periventricular area, the hippocampus, the cerebellum, and the olfactory bulb; all regions where neural stem cells are common (Lindsten *et al.* 2000). Bax knockout mice show decreased sympathetic and neuronal apoptosis during development and are resistant to neuronal apoptosis induced by trophic factor deprivation (Deckwerth *et al.* 1996). The Bcl-2 family of proteins is also critical in maintaining tissue homeostasis by preventing the accumulation of damaged or dysfunctional cells (Adams & Cory 2007; Cory *et al.* 1999; Cory & Adams 2002). The pro-apoptotic members of the Bcl-2 family, most notably Puma, are activated by oncogenic stress in order to safely eliminate cancerous and potentially cancerous cells (Dudgeon *et al.* 2010; Ming *et al.* 2006; Yu *et al.* 2001; Yu *et al.* 2003). The accumulated evidence indicates that the Bcl-2 family of proteins play a crucial role in the

regulation of neuronal apoptosis, both during development and for maintenance of homeostasis.

1.4 - p53 Up-regulated Mediator of Apoptosis (Puma)

p53 up-regulated mediator of apoptosis (Puma) is perhaps the most prevalent and effective of the BH3-only Bcl-2 family members. Puma has been shown to play an essential role in apoptosis induced by many different factors, including: oxidative stress, genotoxic stress, endoplasmic reticulum (ER) stress, trophic-factor withdrawal, and oncogenesis (Ambacher *et al.* 2012; Engel, T *et al.* 2011; Galehdar *et al.* 2010; Niizuma *et al* 2009; Reimertz *et al.* 2003; Steckley *et al.* 2007). Although other BH3-only proteins appear to contribute to neuronal apoptosis in some of these conditions, none of them are as universally essential as Puma. Even under conditions where other BH3-only proteins are expressed, Puma still seems to be the essential factor for apoptosis to occur (Ambacher *et al.* 2012; Galehdar *et al.* 2010; Niizuma *et al* 2009; Reimertz *et al.* 2003; Steckley *et al.* 2007).

Puma was identified by several independent groups at almost the same time (Han *et al.* 2001; Nakano & Vousden 2001; Yu *et al.* 2001). As suggested by its name, Puma is transcriptionally up-regulated by the apoptotic protein p53 (Cregan *et al* 2004; Nakano & Vousden 2001; Yu *et al.* 2001), although it has also been shown to be induced independently of p53 expression (Wu *et al.* 2007; Yu & Zhang 2009). Other transcriptional regulators of Puma have been identified, including p73 (Ming *et al.* 2008;

Ray *et al.* 2011), FoxO3a (Dudgeon *et al.* 2010; You *et al.* 2006), CHOP (Galehdar 2010; Wu *et al.* 2007), E2F1 (Hershko & Ginsberg 2004; Ray *et al.* 2011), and c-Myc (Fernandez *et al.* 2003). This variety of regulators allows Puma expression to be controlled specifically in different cell types and under different forms of cell stress.

Puma is present at very low levels in healthy cells and is transcribed when the cell experiences a wide variety of stressors (Nakano & Vousden 2001; Puthalakath & Strasser 2002; Yu *et al.* 2001). The transcriptional up-regulation of Puma has been widely accepted as its main form of regulation, but recent evidence suggests that it may also be post-transcriptionally regulated by phosphorylation (Fricker *et al.* 2010; Lam *et al.* 2009; Puthalakath & Strasser 2002; Sandow *et al.* 2012). Phosphorylation of Puma at serine-ten has been shown to target the protein for degradation and reduce Puma-induced apoptosis (Fricker *et al.*2010; Sandow *et al.* 2012). Although other phosphorylation sites have been identified, their function in regulating Puma has not yet been determined (Hikisz & Kilianska 2012; Fricker *et al.* 2010; Lam *et al.* 2009; Puthalakath & Strasser 2002; Sandow *et al.* 2012).

There are two main splice-variants of Puma produced by cells: Puma-α and Puma-β (Nakano & Vousden 2001; Yu *et al.* 2001). Initially, these variants were thought to produce proteins that were similar in size and structure, both of which had full pro-apoptotic activity (Nakano & Vousden 2001; Yu *et al.* 2001). However, recent evidence has emerged to suggest that Puma-β may be smaller than Puma-α and lack full apoptotic activity (Tampio *et al.* 2009). Other splice-variants of Puma also exist, some of which

transcribe proteins without the BH3 domain or the full length c-terminus (Nakano & Vousden 2001; Yu *et al.* 2001). These proteins lack the apoptotic activity of full-length Puma and their cellular function is unknown, though perhaps also negligible because they are produced at such low levels (Nakano & Vousden 2001; Yu *et al.* 2001).

Like the other BH3-only proteins, Puma contains a BH3 domain that is required for its interaction with other Bcl-2 family members (Day *et al.* 2008; Nakano & Vousden 2001; Yu *et al.* 2001). It is amphipathic and alpha-helical in structure, spanning amino acids 137 to 150 of the protein (Day *et al.* 2008). The BH3 domain of Puma also has a similar function to the BH3 domains of the other BH3-only proteins; it is required to bind to the anti-apoptotic Bcl-2 proteins, Bcl-xl, Bcl-2, Bcl-w, A1, and Mcl-1 (Day *et al*. 2008; Gautier *et al.* 2011; Nakano & Vousden 2001; Yu *et al.* 2001). The BH3 domain of Puma is the site of Puma's putative direct interaction with the multi-BH domain pro-apoptotic Bcl-2 family members. The BH3 domains of Bid and Bim, two of the other BH3-only proteins, have been shown to interact directly with the multi-BH domain pro-apoptotic protein, Bax (Kuwana *et al.* 2005; Lovell *et al.* 2008). Many groups have found evidence that the BH3 domain of Puma can directly bind to and activate Bax in the same manner (Cartron *et al.* 2004; Du *et* al. 2011; Gallenne *et al.* 2009; Steckley *et al.* 2007; Yee & Vousden 2008; Zhang *et al.* 2009). However, unlike the other members of the Bcl-2 family, Puma does not contain a classic c-terminal membrane localization sequence (Nakano & Vousden 2001; Yee & Vousden 2008; Yu *et al.* 2001; Yu *et al.* 2003). Puma has been shown to localize to the mitochondria, but the amino acids

involved and their exact function have not been well-defined (Hikisz & Kilianska 2012; Nakano & Vousden 2001; Yee & Vousden 2008; Yu *et al.* 2001).

The reasons for the importance of Puma in cellular apoptosis are currently unclear. It has been suggested that the reason for Puma's efficacy is its ability to bind all of the anti-apoptotic family members, or that Puma may bind with greater strength than the other Bcl-2 family members (Chen *et al.* 2005; Kuwana *et al.* 2005; Yu & Zhang 2009). One important difference between Puma and the other BH3-only proteins is that Puma can bind directly to all of the anti-apoptotic Bcl-2 family members with high affinity (Chen *et al.* 2005; Kuwana 2005). This is likely part of the reason that Puma is such an efficacious and important protein in neuronal apoptosis. However, even these ideas fail to explain what it is about Puma that enables it to be such an effective and essential mediator of apoptosis.

1.5 – Bcl-2-associated x protein (Bax)

Bcl-2-associated x protein (Bax) is a multi-domain pro-apoptotic Bcl-2 family member. Similar in structure to the other Bcl-2 family members, Bax consists of two hydrophobic alpha-helices surrounded by seven amphipathic alpha-helices (Lalier *et al.* 2007; Petros *et al.* 2004; Suzuki *et al.* 2000). Like the other multi-domain pro-apoptotic members of the Bcl-2 family, Bax contains three BH domains: BH1, BH2, and BH3 (Petros *et al.* 2004; Suzuki *et al.* 2000). Several regions of Bax have been shown to be important for the function of the protein: the BH3 domain; the first alpha-helix (Hα1); the

central helices, Hα5 and Hα6; the n-terminus; and the c-terminus (Carton *et al.* 2004; Lalier *et al.* 2007; Reed *et al.*1996; Suzuki *et al.* 2000; Wolter *et al.* 1997).

The number of important domains in Bax is unsurprising, considering the overall significance of the protein in apoptosis. Our lab and others have shown that Bax plays a particularly important role in Puma-mediated neuronal apoptosis (Engel *et al*, 2011; Shindler *et al.* 1997; Steckley *et al.* 2007). Under normal conditions, Bax is present in the cellular cytoplasm in an inactive conformation. Upon activation, Bax translocates to the mitochondrial outer membrane (MOM) and forms homo- or hetero-oligomers with other Bax proteins or with Bak. These oligomers act as channels in the MOM and allow the release of cytochrome-c and other death factors into the cytoplasm.

Like most of the BH3 domains found in Bcl-2 family members, the BH3 domain of Bax has been implicated in its interactions with other Bcl-2 family proteins. A peptide modeled on the BH3 domain of Bax has been shown to interact with the hydrophobic binding pocket on the anti-apoptotic proteins, Bcl-xl and Bcl-2; the same peptide also interferes with Bax binding to Bcl-xl and Bcl-2 (Moreau *et al.* 2003). Mutations of the BH3 domain of Bax appear to interfere with its ability to bind Bcl-xl, Bcl-2, and Mcl-1 (Cartron *et al.* 2004; Wang *et al.* 1998). The Bax BH3 region also seems to be necessary for its interaction with and activation by the pro-apoptotic BH3-only protein, Bad (Cartron *et al.* 2004). However, the BH3 region of Bax does not appear to be responsible for all of its interactions with Bcl-2 family members.

Indeed, the BH3-only proteins Puma and Bid appear to interact with Bax via its Hα1 domain rather than its BH3 domain (Cartron *et al.* 2004). The first alpha helix at the n-terminus of Bax, Hα1, is one of the amphipathic helices sequestered in the unactivated, cytoplasmic conformation of the protein. It has been suggested that the exposure of the Hα1 domain may be a critical step in the activation and conformational change of Bax (Lalier *et al.* 2007). The aspartate at position 33 of Bax is required for Bax to interact with the BH3 regions of Bid and Puma; mutation of this acidic residue into a neutral residue, alanine, abrogates these interactions (Cartron *et al.* 2004). However, the Hα1 domain of Bax is not required for its interactions with Bcl-xl and Bcl-2, and mutation of the Hα1 domain does not affect the ability of Bax to bind the anti-apoptotic proteins Bcl-xl and Bcl-2 (Cartron *et al.* 2004).

Although they do not appear to play a role in protein interactions, the central helices of Bax, Hα5 and Hα6, are important for the pro-apoptotic function of the protein. These central hydrophobic alpha-helices are tightly-sequestered in the inactive cytoplasmic conformation of Bax, but are exposed upon its activation (Lalier *et al.* 2007; Suzuki *et al.* 2000). Their resemblance to the pore-forming domains of diptheria toxin and colicins inspired research into their potential for a similar function in Bax (Nouraini *et al.* 2000). Structurally, Hα5 and Hα6 of Bax appear to be trans-membrane domains; peptides based on these helices have been shown to form pores in a synthetic lipid membrane (Annis *et al.* 2005; Garcia-Saez *et al.* 2006). Although Hα5 and Hα6 are required to form the channel in the mitochondrial outer membrane that allows activated Bax to release pro-apoptotic factors into the cytoplasm, they do not appear to regulate its

localization to the mitochondria (Carton *et al.* 2005; Lalier *et al.* 2007). Instead, the localization of Bax appears to be regulated by both its c-terminus and its n-terminus.

At the c-terminus of Bax, its ninth alpha-helix, Hα9, occupies the hydrophobic binding pocket formed by the first three BH domains of Bax in its inactive conformation (Suzuki *et al.* 2000). Activation of Bax exposes this helix, which resembles the c-terminal membrane-localization domains in Bcl-xl and Bcl-2 (Lalier *et al.* 2007). The c-terminus of Bax has been shown to play an important role in the localization and function of the protein (Gardia *et al.* 2004; Nechustan *et al.* 1999). However, some studies have demonstrated that Bax retains its ability to translocate to the mitochondria despite c-terminus deletion (Antonsson *et al.* 2000; Carton *et al.* 2003). Perhaps this is because the n-terminus of Bax also appears to play a role in the mitochondrial-localization of the protein (Carton *et al.* 2003). The n-terminus of Bax has been shown to contain a mitochondrial-localization sequence, and deletion of this sequence has been shown to abrogate its localization (Carton *et al.* 2003). Interestingly, the first 20 amino acids of Bax appear to inhibit its mitochondrial-localization, and have been termed the Apoptosis Regulating Target (ART) domain (Goping *et al.* 1998). Deletion of this domain results in a protein constitutively localized to the mitochondria (Goping *et al.* 1998). However, the complete n-terminus alone is unable to insert into the mitochondrial outer membrane (Carton *et al.* 2003). It appears that the n-terminus and the c-terminus of Bax co-operate to regulate the localization of the protein (Lalier *et al.* 2007).

Mitochondrial localization is of particular importance in the apoptotic function of Bax. Without its ability to localize to the mitochondrial outer membrane, Bax cannot induce apoptosis. The precise reasons for the importance of membrane-localization in the apoptotic function of Bax are still a subject of investigation, but some recent studies have suggested a role for the membrane in protein interactions (Bogner *et al.* 2010; Lalier, L. *et al.* 2007, Lovell, J.F. *et al*. 2008; Roucou *et al.* 2002; Yethon *et al.* 2003). The BH3-only protein, tBid, requires the presence of a membrane in order to bind to and activate Bax (Lovell, J.F. *et al*. 2008). It has been suggested that contact with the membrane may open the conformation of Bax and encourage protein interactions (Yethon *et al.* 2003), or that mitochondrial proteins may play a role in Bax oligomerization (Roucou *et al.* 2002). Of course, in order for Bax to form the channels in the mitochondrial outer membrane that are required for release of pro-apoptotic factors into the cytoplasm, it must be in the mitochondrial outer membrane. The mitochondrial-localization of Bax plays an essential role in the apoptotic function of the protein.

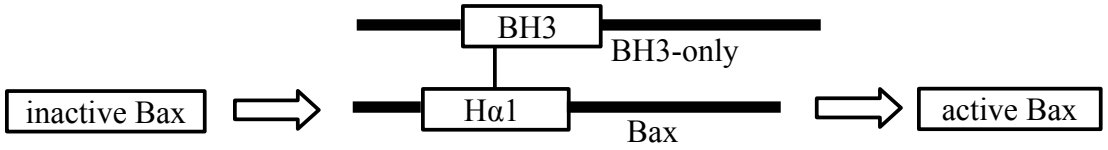
Bax is constitutively expressed in cells in its inactive form and is regulated post-transcriptionally by interactions with the pro-apoptotic BH3-only proteins as well as the anti-apoptotic members of the Bcl-2 family (Lalier *et al.* 2007). Bax has two major conformations: a cytosolic, inactive conformation and a membrane-bound, active conformation (Lalier *et al.* 2007; Wolter *et al.* 1997). These two conformations have been dubbed CLIC (for Cytosolic Locked In Conformation) and CLAC (for Cytochrome-c Liberation Associated Conformation) for easier reference (Lalier *et al.* 2007).

In its cytosolic conformation, Bax is tightly-packed and the regions responsible for protein interaction and membrane-localization are sequestered within the structure of the protein. The three BH domains of Bax form a hydrophobic binding pocket, similar to the one seen in the anti-apoptotic proteins Bcl-2 and Bcl-xl, that facilitates the binding of other Bcl-2 family members via the BH3-domain (Jeong *et al.* 2004; Suzuki *et al.* 2000). When in its cytosolic conformation, the ninth alpha-helix of Bax (Hα9), at the c-terminus of the protein, occupies the hydrophobic binding pocket formed by the three BH3 domains (Suzuki *et al.* 2000). This interaction effectively sequesters two important regions of Bax: the BH3 domain and the c-terminus. Occupation of the hydrophobic binding pocket, and specifically of the BH3 domain, deters protein interactions; sequestration of the c-terminus prevents Bax from anchoring in the mitochondrial outer membrane (Suzuki *et al.* 2000; Lalier *et al.* 2007). The third region of importance for the function of Bax, the Hα1 helix at the n-terminus of the protein, forms bonds with the hydrophobic helices at the centre of the protein and acts to stabilize the protein structure in its cytoplasmic conformation (Suzuki *et al.* 2000; Lalier *et al.* 2007). CLIC Bax is a closely-packed protein unlikely to interact with other proteins or membranes. In order for Bax to promote apoptosis, it must be changed into its active conformation so that it is able to both insert into membranes and bind to other proteins.

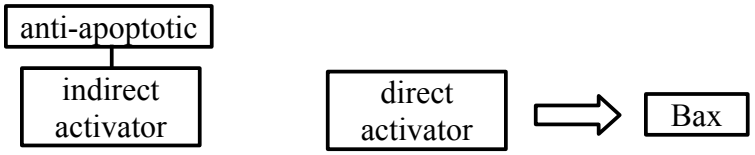
There are two proposed models of Bax activation by BH3-only pro-apoptotic proteins: the indirect and the direct model of Bax activation. In the direct activation model, BH3-only proteins directly interact with Bax in order to alter its conformation and activate the protein (Figure 3; Cartron *et al.* 2004; Du *et al.* 2011). Interactions have

been shown to occur between the BH3 domain of some BH3-only proteins and the Hα1 domain of Bax (Carton *et al.* 2004). It is believed that a BH3-only protein binding to the Hα1 domain of Bax may open up the structure of the protein and encourage the switch to its active conformation (Carton *et al.* 2004). There is an extensive body of evidence to show that Bid, one of the BH3-only proteins, can directly interact with and activate Bax in order to promote apoptosis (Billen *et al.* 2009; Eskes *et al.* 2000; Lovell *et al.* 2008). Bid appears to require a membrane in order to bind to and activate Bax (Lovell *et al.* 2008; Veresov & Davidovskii 2009). Upon binding to the mitochondrial outer membrane, Bid is able to bind and activate Bax, which then inserts into the mitochondrial outer membrane and forms oligomeric channels (Lovell *et al.* 2008; Veresov & Davidovskii 2009). Many studies have also shown that Puma can directly activate Bax in a manner similar to Bid (Cartron *et al.* 2004; Du *et al.* 2011; Melino *et al.* 2004; Ren *et al.* 2010). In the indirect model, BH3-only proteins activate Bax through a variety of proposed mechanisms, such as the inhibition of anti-apoptotic Bcl-2 family proteins (Kazi *et al.* 2010; Willis *et al.*2007). The indirect activation of Bax by BH3-only proteins has been explained by three slightly different sub-models: the sensitizer model, the derepressor model, and the neutralization model (Figure 3; Chipuk *et al.* 2010; Kazi *et al.* 2011; Kuwana *et al.* 2005; Ming *et al.* 2006; Uren *et al.* 2007; Willis *et al.* 2007). In each of these models, the indirect activators interact directly with anti-apoptotic proteins in order to activate Bax. Evidence has been given for both the indirect and the direct activation models; it has also been suggested that the activation of Bax may be regulated by a combination of the two models, or that different BH3-only proteins may follow

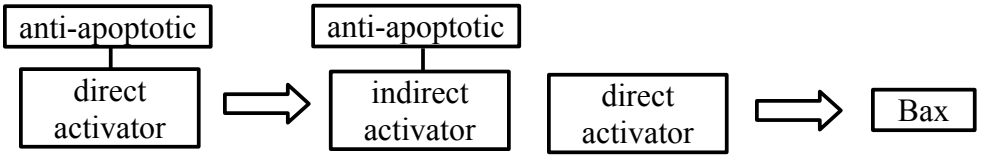
different models (Juin *et al.* 2005; Kim *et al.* 2006; Kuwana *et al.* 2005; Letai *et al.* 2002). The ability of the various BH3-only proteins to induce apoptosis via the different models of Bax activation may help to explain the differences in their efficacy.


BH3
BH3-only
inactive Bax
Hα1
Bax
active Bax

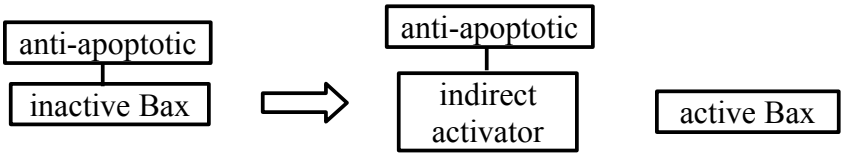
(a)


anti-apoptotic
indirect activator
direct activator
Bax

(b)


anti-apoptotic
direct activator
anti-apoptotic
indirect activator
direct activator
Bax

(c)


anti-apoptotic
inactive Bax
anti-apoptotic
indirect activator
active Bax

(d)

Figure 3 - Bax Activation Models. (a) Direct activation model: Direct activator BH3-only proteins, such as Bid and perhaps Puma, bind directly to the Hα1 domain of Bax via their BH3 domain; this switches Bax into its active conformation (Cartron *et al.* 2004; Du *et al.* 2011; Eskes *et al.* 2000; Lalier *et al.* 2007). (b) Sensitizer model: Indirect activator BH3-only proteins sequester anti-apoptotic proteins so that they cannot inhibit direct activator BH3-only proteins; this leaves direct activator BH3-only proteins free to activate Bax (Chipuk *et al.* 2010; Willis *et al.* 2007). (c) De-repressor model: Direct activator proteins are bound to anti-apoptotic proteins in cytoplasm. Indirect activator BH3-only proteins compete with direct activator BH3-only proteins for binding to anti-apoptotic proteins; this releases direct activator BH3-only proteins so they are free to activate Bax (Chipuk *et al.* 2010; Chipuk *et al.* 2008; Kuwana *et al.* 2005). (d) Neutralization model: Bax is bound to anti-apoptotic proteins in the cytoplasm. Indirect activator proteins compete with Bax for binding to these anti-apoptotic proteins; this frees and, in the process, activates Bax (Chipuk *et al.* 2010; Gautier *et al.* 2011; Kazi *et al.* 2011; Ming *et al.* 2006; Moreau *et al.* 2003; Uren *et al.* 2007).

1.6 - B-cell lymphoma extra-long (Bcl-xl)

Bcl-xl is a 233 amino acid protein, consisting of seven alpha-helices (Boise *et al.* 1993; Muchmore *et al* 1997). Like the other anti-apoptotic proteins, Bcl-xl contains multiple BH domains. Specifically, Bcl-xl contains a BH1, a BH2, a BH3, and a BH4 domain. In the normal configuration of the protein, the first three BH domains are in close proximity and appear to form a hydrophobic binding pocket (Muchmore *et al* 1997). All three of these BH domains seem to be essential for the anti-apoptotic function of Bcl-xl, as mutation or deletion of any one of them abrogates its anti-apoptotic activity (Muchmore *et al* 1997). The BH3 domain is the most common site for the interaction of Bcl-2 family members; the proximity of the other BH domains in the hydrophobic binding pocket likely enhances the ability of other Bcl-2 family members to bind to this region. The BH3 domain of Bcl-xl has been shown to interact with both multi-domain and BH3-only pro-apoptotic proteins (Kazi *et al.* 2011; Kelekar & Thompson 1998; Muchmore *et al* 1997).

Under normal conditions, Bcl-xl is found in both the cytosol and the mitochondrial outer membrane (Hsu *et al.*1997; Wolter *et al.* 1997). Free cytosolic Bcl-xl appears to exist mainly in homodimers. These dimers are formed by one Bcl-xl protein inserting its hydrophobic c-terminus into the pocket formed by the BH domains of another Bcl-xl protein (Jeong *et al* 2004). This sequesters the c-terminal membrane-localization domain and allows the protein to remain cytosolic, while also blocking the BH3 domain. However, the bonds of the Bcl-xl homodimers are easily broken when the anti-apoptotic activity of Bcl-xl is required. Expression of a BH3-only protein, such as

Bad, has been shown to break apart the Bcl-xl homodimers and allow Bcl-xl to translocate to the mitochondrial outer membrane (Jeong *et al* 2004). When Bcl-xl inserts its c-terminal membrane domain into the mitochondrial outer membrane, the hydrophobic binding pocket is free to bind to other Bcl-2 family members (Jeong *et al* 2004). In fact, the c-terminus of Bcl-xl appears to be necessary to bind to and sequester the pro-apoptotic multi-BH domain protein Bax (Jeong *et al* 2004).

Bcl-xl has been shown to be of particular importance in neuronal apoptosis (Boise *et al.* 1993; Frankowski *et al.* 1995; Motoyama *et al.* 1995; Rubin *et al.* 1994) and is considered by some to be the most effective anti-apoptotic protein (Ming e*t al.* 2006). Part of the reason for its efficacy and crucial role in apoptosis may be its ability to specifically target the mitochondrial outer membrane, a crucial site for the regulation of apoptotic events. Unlike the other anti-apoptotic Bcl-2 family members, Bcl-xl contains a membrane localization sequence specific to the mitochondrial outer membrane (Kaufmann *et al.* 2003; Lindsay *et al.* 2011). Bcl-xl contains a classic c-terminal membrane targeting sequence flanked by groups of basic amino acids (Kaufmann *et al.* 2003; Lindsay *et al.* 2011). This sequence allows Bcl-xl to localize specifically to the mitochondrial membrane, while Bcl-2, which contains a similar membrane localization sequence without the flanking groups of basic amino acids, does not specifically target mitochondria (Kaufmann *et al.* 2003; Lindsay *et al.* 2011). Because of its overall importance and its specific importance in neuronal cells, we will focus on Bcl-xl as our main anti-apoptotic protein of interest.

1.7 – Rationale

Neuronal apoptosis plays a crucial role in normal development and in a number of diseases. In normal development, the Bcl-2 family of proteins are important regulators of neuronal apoptosis, and alteration of these proteins can result in significant abnormalities (Lindsten *et al.* 2000; Motoyama *et al.* 1995; Shindler *et al.* 1997). Bcl-2 family members have also been shown to play a key role in the regulation of neuronal apoptosis in both chronic conditions, such as Alzheimer's disease (Higgins *et al.* 2010) and Parkinson's disease (Vila & Przedborski, 2003), and in acute trauma, such as ischemia (Niizuma *et al.* 2009; Steckley *et al.* 2007) and spinal cord injury (Kotipatruni *et al.* 2011).

We have previously shown that the BH3-only Bcl-2 family member, Puma, plays an important role in the mediation of neuronal apoptosis due to a number of cell stressors (Ambacher *et al.* 2012; Galehdar *et al.* 2010; Steckley *et al.* 2007). The BH3 domain has been shown to be the site of Puma's interactions with both multi-domain (e.g. Bax) and anti-apoptotic (e.g. Bcl-xl) members of the Bcl-2 family (Cartron *et al.* 2004; Day *et al*. 2008; Du *et* al. 2011; Gautier *et al.* 2011; Nakano & Vousden 2001; Steckley *et al.* 2007; Yu *et al.* 2001 Gallenne *et al.* 2009; Yee & Vousden 2008; Zhang *et al.* 2009). Both Bax and Bcl-xl have been shown to localize at the mitochondria when the cell is undergoing apoptosis (Carton *et al.* 2003; Gardia *et al.* 2004; Goping *et al.* 1998; Kaufmann *et al.* 2003; Lalier *et al.* 2007; Lindsay *et al.* 2011; Nechustan *et al.* 1999). We and others have found that Puma also localizes to the mitochondria during apoptosis, but the precise

mechanism of this membrane targeting and whether it is important for apoptotic function of the protein remain unclear. However, our preliminary studies indicate that the c-terminus of Puma contains a motif that may be involved in targeting the protein to the mitochondrial membrane. In this work, we will attempt to identify the region of Puma responsible for its mitochondrial-localization. We will also examine the importance of this region and the BH3 domain of Puma in its interactions with other Bcl-2 family members and its apoptotic activity.

1.8 - Hypothesis and Objectives

We hypothesize that both the BH3 domain and a distinct mitochondrial localization region of p53-upregulated mediator of apoptosis (Puma) are required for Puma-mediated neuronal apoptosis.

Objectives:

1. To determine whether BH3 domain mediated BCL-2 family interactions are required for mitochondrial localization of Puma
2. To determine whether the c-terminus is required for mitochondrial targeting of Puma
3. To determine whether the c-terminus is required for Puma-induced neuronal apoptosis
4. To determine whether the c-terminus is required for Puma interaction with Bcl-xl and/or Bax
5. To determine whether the c-terminus is required for co-localization of Puma and Bcl-xl

Chapter 2 – Materials and Methods

2.1 – Cell Culture

Neuro-2A (N2A) cells are an adherent neuronal cell line, derived from a neuroblastoma in a strain A albino mouse (Augusti-Tocco & Sato 1969; Klebe & Ruddle 1969). Differentiation of the cell line into a more neuronal cell-type via serum-withdrawal has been well-documented (Amano *et al.* 1972; Evangelopoulos *et al.* 2005). Upon differentiation, cells develop extensive neurites and produce levels of caspase-3 comparable to primary neurons (Amano *et al.* 1972; Evangelopoulos *et al.* 2005). Early studies demonstrated that N2A cells express choline acetyltransferase and tyrosine hydroxylase, enzymes required for neurotransmitter synthesis (Amano *et al.* 1972; Augusti-Tocco & Sato 1969). They have also been shown to contain glutamate (NMDA) receptors (LePage *et al.* 2005; Van der Valk JB & Vijverberg 1990) and voltage-gated sodium channels (LePage *et al.* 2005). In cell-death studies, these cells have been shown to behave in a similar manner to primary neurons (Klebe *et al.* 1969; Gotoh *et al.* 2012). Because these cells are easy to transfect and exhibit neuronal behaviour, we chose differentiated N2As as our preferred cell-type for these experiments.

Neuro-2A (N2A) cells (ATCC #CCL131) were cultured in complete media (Minimum Essential Medium (MEM) (Invitrogen #11095), 10% Fetal Bovine Serum (FBS) (Invitrogen #10437-028), 10 µg/ml gentamycin (Invitrogen #15710), and 1% penicillin/streptomycin (Invitrogen #15070063)) at 37ºC in 5% CO_2. To plate, cells were washed once with 0.25% Trypsin-EDTA (Invitrogen #25200) and then incubated with

0.25% Trypsin-EDTA (Invitrogen #25200) at 37ºC in 5% CO_2 (humidified incubator) for five minutes. Cells were then suspended in complete media at an appropriate density for plating. For live-cell microscopy experiments, cells were plated at a density of 15000 cells per glass-bottomed 35mm dish. For fixed-cell microscopy experiments, cells were plated at 5000 cells per well on glass cover slips (Fisher Scientific #12-545-83) in 4-well dishes. For co-immunoprecipitation (co-IP) experiments, cells were plated at a density of 45000 cells per 60mm dish. The day after plating, cells for all experiments were differentiated by serum-withdrawal (Amano *et al.* 1972; Evangelopoulos *et al.* 2005). In summary, cells were washed once in 1x PBS then incubated in serum-free media (Minimum Essential Medium (MEM) (Invitrogen #11095)) at 37ºC in 5% CO_2 (humidified incubator) for 24 hours to induce differentiation. Cells were then maintained in complete media for the duration of treatment.

2.2 – Plasmid constructs

HA-Puma and HA-Puma (ΔBH3) cloned in pCEP4 vector were generously donated by Dr. Vogelstein (Yu *et al.* 2001). The plasmids and the desired vector, GFP C1 (Clontech #6084-1), were digested with Kpn I (New England Biolabs #R0142L) and BamH 1 (New England Biolabs #R0136L) restriction enzymes (Table 1). The digested DNA was then ligated into the digested GFP C1 vector with T4 DNA Ligase (New England Biolabs #M0202S) (Table 2) at 4ºC overnight.

The other GFP-Puma plasmids, GFP-Puma (1-160) and GFP-Puma (151-93) were generated by PCR amplification (Table 3) and cloned into GFP C2 vector between Eco RI and Sal I. The DNA template for Puma was diluted to 10ng/µl and and a PCR was run using Qiagen Taq (Qiagen #201203) and High Fidelity Taq (Qiagen #202602) (Table 3). Ten µl of the PCR product was then run on a 1.5% agarose gel and purified with the Qiagen Gel Purification kit as per manufacturer's instructions (Qiagen #28704). The purified product and 5µg of the GFP C2 vector were then digested with Eco RI (New England Biolabs #R0101L) and Sal I (New England Biolabs #R0138L) restriction enzymes (Table 1). The digested vector and DNA were then ligated with T4 DNA Ligase (New England Biolabs #M0202S) (Table 2) overnight at 4ºC. All plasmids are based on the Puma-α isoform and were confirmed by sequencing.

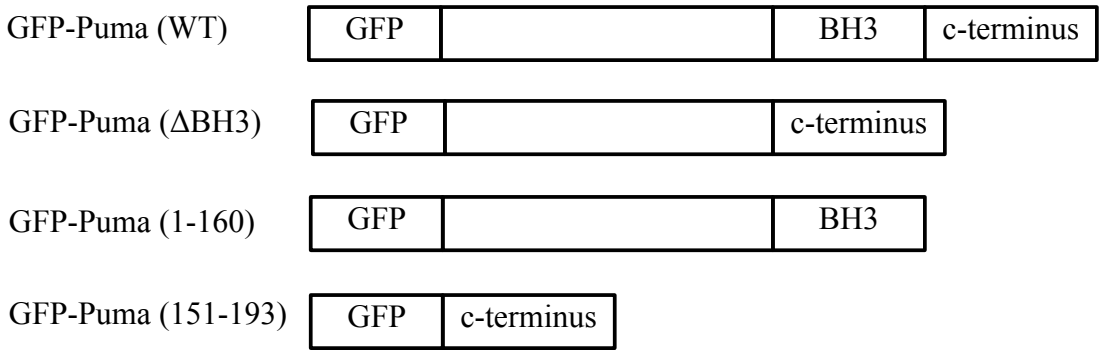

GFP-Puma (WT)
GFP
BH3
c-terminus
GFP-Puma (ΔBH3)
GFP
c-terminus
GFP-Puma (1-160)
GFP
BH3
GFP-Puma (151-193)
GFP
c-terminus

Figure 4 - GFP-Puma plasmids. A summary of the sequence of the various GFP-Puma plasmids. GFP-Puma (WT) consists of a GFP tag and the full Puma sequence; GFP-Puma (ΔBH3) consists of a GFP tag and the Puma sequence with the BH3-domain deleted; GFP-Puma (1-160) consists of a GFP tag and the Puma sequence without the c-terminus; and GFP-Puma (151-193) consists of a GFP tag and the Puma c-terminus only.

2.3 - Transfection

Differentiated N2As were transfected using calcium/phosphate. For live-cell confocal microscopy, cells in 35mm glass-bottomed dishes were transfected using 1ug of plasmid DNA (GFP, GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), or GFP-Puma (151-193)) per dish. Plasmid DNA was diluted in 45µl ddH20 and then mixed with 5µl of 2.5M CaCl2. Fifty microlitres of 2xHBS (0.28M NaCl, 0.05M HEPES, 1.5mM Na2HPO4, at pH 7.1) was added drop-wise and the resulting mixture was carefully inverted two to three times. To each 35mm dish, 100µl of this solution was added drop-wise.

For fixed-cell confocal microscopy and live-cell microscopy, cells in 4-well dishes were transfected using 0.25ug of plasmid DNA (GFP, GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193) and/or 3x Flag-Bcl-xl) per well. One microgram of plasmid DNA was diluted in 60ul of ddH20 and then mixed with 6.7ul of 2.5M CaCl2. To this solution, 66.7ul of 2xHBS (0.28M NaCl, 0.05M HEPES, and 1.5mM Na2HPO4, at pH 7.1) was added drop-wise and the resulting mixture was carefully inverted two to three times. Thirty-three microlitres of this solution was added drop-wise to each well in a 4-well plate.

For co-immunoprecipitation experiments, cells in 60mm dishes were transfected using 3ug of plasmid DNA (GFP, GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), 3x Flag-Bcl-xl, and/or 3xFlag) per dish. Three micrograms of plasmid DNA was diluted in 300µl distilled water and then mixed with 33.5ul of 2.5M

CaCl2. To this solution, 334ul of 2xHBS (0.28M NaCl, 0.05M HEPES, 1.5mM Na2HPO4, at pH 7.1) was added drop-wise and the resulting mixture was carefully inverted two to three times. The entirety of this solution was added drop-wise to each 60mm dish.

2.5 - Peptide treatment

For experiments with GFP or GFP-Puma (WT) transfected cells, cells were treated with 1μM of either Bax-Hα1 (CanPeptide #CP08640) or Bax-Hα1(D33A) (CanPeptide #CP08641) Tat-fused peptide per well 12 hours after transfection. Tat is a short sequence of basic amino acids derived from human immunodeficiency virus that allows the transport of proteins across cell membranes (Fawell *et al.* 1994; Ruben *et al.* 1989; Vives *et al.* 1997). See Table 5 for complete peptide sequences. Peptide treatment was repeated every six hours thereafter, for a total of 28 hours post-transfection, at a concentration of 0.2μM of either Bax-Hα1 or Bax-Hα1(D33A) tat-peptide per well.

2.6 – Cell death assesment by Hoechst 33342 and Propidium Iodide staining

Hoechst 33342 (Sigma #B2261) was added to cells at a final concentration of 1μg/ml and samples were incubated for 20 minutes at 37ºC in 5% CO_2 (humidified incubator). For staining with Propidium Iodide, Propidium Iodide (Sigma # P1470) was added to cells at a final concentration of 1μg/ml and samples were incubated for 20 minutes at 37ºC in 5% CO_2 (humidified incubator).

To examine apoptosis and cell death after transfection with GFP-Puma plasmids, cells were stained with Hoechst 33342 and/or Propidium Iodide at 28 hours post-transfection or 36 hours after the initial drug treatment. Nuclear morphology was visualized with a 40x objective lens under ultra-violet (UV) light (for Hoechst 3342) and/or with a rhodamine (TRITC) filter (for Propidium Iodide) using a Zeiss Axiovert 200 fluorescent microscope. At least five representative images per well were captured using Zeiss Axiovision 4.8 software and at least 500 nuclei were counted for each treatment. For analysis of apoptosis by Hoechst staining, nuclei were categorized as either healthy or apoptotic (blebbed, shrunken, and/or fragmented) as previously described (Steckley *et al.* 2007). For each treatment, the percentage of apoptotic cells was calculated as the number of GFP-positive apoptotic cells out of the total number of cells. For analysis of cell death by Propidium Iodide and Hoechst staining, Hoechst-stained nuclei were categorized as either live (Propidium Iodide-negative) or dead (Propidium Iodide-positive). Percent cell death was calculated as the number of Propidium Iodide-positive (dead cells) out of the total number of cells for each treatment.

2.7 - Mitotracker ® Red CMXRos staining

Neuro2A cells were transfected with GFP, GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), or GFP-Puma (151-193) plasmids (see Transfection). Twenty-four hours post-transfection, cells were stained with Mitotracker ® Red CMXRos (Invitrogen #M-7512). at 1ug/ml in complete MEM culture media to identify mitochondria and counterstained with Hoechst 33342 to visualize cell nuclei. Hoechst 33342 and

Mitotracker ® Red CMXRos were added to the cells simultaneously and samples were incubated for 20 minutes at 37ºC in 5% CO_2 (humidified incubator). Samples were visualized with a 63x objective lens on an LSM 510 Meta Zeiss confocal microscope and images were captured using Zeiss software. To detect Hoechst 33342 staining, an excitation wavelength of 405nm at 3% intensity was used with a bandpass filter for 420-480nm. For GFP, the fluorophore was excited at 3% intensity at 488nm with a bandpass filter for 505-530nm. Mitotracker ® Red CMXRos fluorescence was excited at a wavelength of 633nm at 10% intensity and viewed through a 650nm longpass filter.

2.8 - Immunofluorescence

Cells were incubated at 37ºC in 5% CO_2 (humidified incubator) for 20 minutes with Hoechst 33342 (Sigma #B2261) diluted to 1ug/ml in complete media. Cells were then fixed with Lana's fixative (4% paraformaldehyde (Sigma-#P6148), 160mM dibasic and monobasic sodium phosphate, and 0.2% picric acid (Sigma-#80456)) for 20 minutes at room temperature, then washed twice with 1xPBS. Cells were incubated at room temperature for 20 minutes with permeabilizing solution (0.2% Triton-X in 1xPBS), and then in blocking solution (0.2% Triton-X and 2% BSA in 1x PBS) for 45 minutes at room temperature. Cells were then incubated overnight at 4ºC with primary antibody at a 1:500 dilution in blocking solution (1x PBS, 0.2% Triton-X, 2% BSA). Primary antibodies used for immunofluorescent staining were Anti-Tomm-20 (Abcam #ab56783), or Anti-Flag M2 (Sigma #F-3165). The following day, cells were washed twice in 1x PBS and incubated at room temperature for one hour with fluorescent secondary antibody

at a 1:500 dilution. Secondary antibody used in this experiment was Alexa Fluor® 594 goat anti-mouse IgG (Invitrogen #A11005). Cells were washed twice in 1x PBS and then the glass coverslips were affixed to slides using Immu-mount (Thermo Scientific #9990402). Samples were visualized with a 63x objective lens on an LSM 510 Meta Zeiss confocal microscope and images were captured using Zeiss software. To detect Hoechst 33342 staining, an excitation wavelength of 405nm at 3% intensity was used with a bandpass filter for 420-480nm. For GFP, the fluorophore was excited at 3% intensity at 488nm with a bandpass filter for 505-530nm. Alexa Fluor® 594 fluorescence was excited at a wavelength of 543nm at 10% intensity and viewed through a 585nm longpass filter.

2.9 – Co-immunoprecipitation

Anti-Flag M2 agarose beads (Sigma #A2220) were aliquoted into a 15ml falcon tube and washed with 1xPBS, then centrifuged at 500xg for five minutes. The PBS was then carefully aspirated off and the rinse was repeated twice. Flag-bead/CHAPS slurry was prepared by re-suspending the rinsed Flag-beads in an equal volume of 1% CHAPS buffer (10mM HEPES, 150mM NaCl, 0.5mM EDTA, 1% CHAPS (Sigma #C9426), 0.5mM phenylmethyl sulfonyl fluoride (PMSF) (Bioshop #PMS123), 10mg/ml Aprotonin, 2mg/ml Leupeptin).

Twenty-four hours after transfection, cells were rinsed twice with ice-cold 1x PBS and then lysed with 300ul of 1% CHAPS on ice for 30 minutes. The resulting lysate was

centrifuged at 14000xg for 12 minutes at 4ºC to pellet cell debris. Protein concentration in the supernatant was determined using the Pierce © BCA protein determination assay (Thermo Fisher Scientific © #22325). Five hundred micrograms of protein from each sample was added to 50µl of the Flag-bead/CHAPS slurry and rotated at 4ºC for one hour. Twenty-five micrograms of protein from each sample was not exposed to beads and reserved for Western blotting to assess protein expression.

After incubation, beads were rinsed three times with 1x PBS, centrifuged at 14000xg for 20 seconds and aspirated with a syringe. Beads were resuspended in 50µl 1% CHAPS and 10µl of 6x sodium dodecyl sulfate (SDS) (Bioshop #SDS101). The resulting mixture was boiled at 100ºC for 10 minutes to denature the protein and release it from the beads. Twenty-five microlitres of each of the bead-incubated samples and the entirety of the 25µg lysate samples were analysed by Western blot (see Western Blot).

2.10 - Western Blot

Protein samples were run on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane by semi-dry electroblotting. The membrane was washed three times in 1x TBS-T (150mM NaCl, 10mM Tris, 0.05% Tween-20 (Fisher Biotech #BP1605)) and blocked for an hour in blocking buffer (10% milk (w/v) in 1x TBS-T) at room temperature on a rocker. The membrane was again washed three times in 1x TBS-T, and then incubated overnight at 4ºC with primary antibody at 1:10,000 dilution in

blocking buffer (5% milk (w/v) in 1x TBS-T). Primary antibody used in this experiment was anti-GFP (Invitrogen #A6455).

The following morning, membranes were washed three times in 1x TBS-T and incubated with secondary antibody at 1:10,000 dilution in blocking buffer (5% milk (w/v) in 1x TBS-T) for one hour at room temperature on a rocker. The secondary antibody used for this experiment was a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Biorad #170-6515). The membrane was once again washed three times in 1x TBS-T, and then Pierce ECL Western Blotting Substrate (Thermo Scientific #32106) was used to detect immunoblotted proteins by chemiluminescence. The membrane was exposed to Kodak Biomax maximum sensitivity film (Kodak #8294985) to capture an image of the immunoblotted proteins.

2.11 - Data Analysis

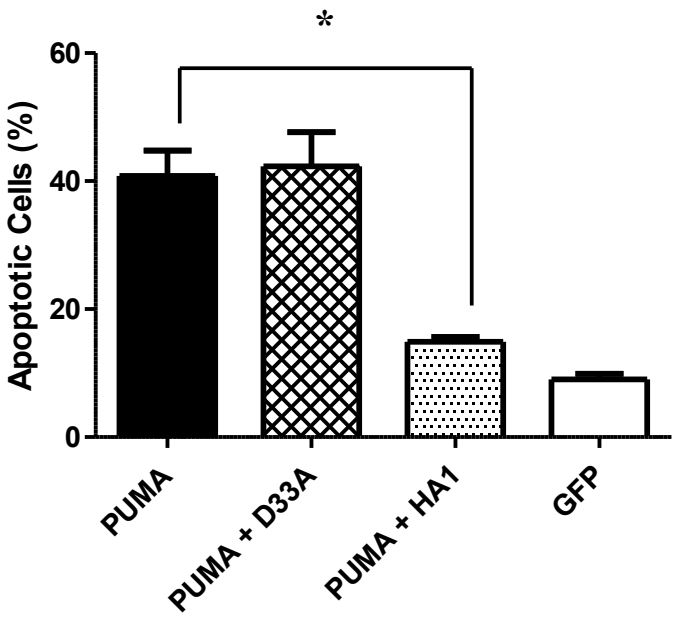
Data were reported as mean and standard deviation. The value n represents experiments conducted using independent passages of Neuro-2A cells. Differences between groups were calculated using one-way ANOVA followed by Dunnet's post hoc test. Data were considered statistically significant when $p < 0.01$. Statistical analyses were conducted with GraphPad Prism software version 5.0 (GraphPad software, San Diego, CA).

Chapter 3 - Results

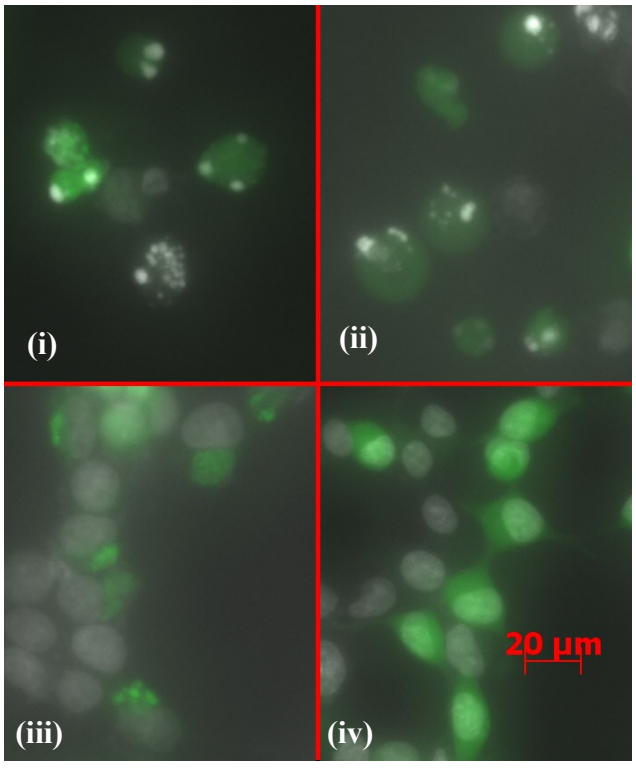
3.1 – Interaction with other BCL-2 family proteins is required for Puma's apoptotic activity but not its localization.

We first sought to determine whether Puma localization to the mitochondria was mediated through interactions with other Bcl-2 family proteins. The BH3 domain of Puma is known to be required for its interaction with other Bcl-2 family proteins (Chen *et al.* 2005; Day *et al*. 2008; Gautier *et al.* 2011; Kuwana 2005). It has previously been demonstrated that the first α helix (Hα1) of Bax can interact with the BH3 domain of Puma, but not other Bcl-2 family proteins (Cartron *et al.* 2004; Lalier *et al.* 2007). Furthermore, it was shown that a peptide mimicking the Bax-Hα1 region could inhibit the interaction of Puma with the Bcl-2 protein, Bax, *in vitro*, and that an aspartate to alanine mutation at amino acid residue 33 of this peptide abrogates its binding to the Puma-BH3 domain (Cartron *et al.* 2004; Lalier *et al.* 2007). To determine whether Puma's interactions with other Bcl-2 family proteins are required for its localization and apoptotic activity we synthesized Tat-fused Bax-Hα1 and Bax-Hα1(D33A) peptides to facilitate their uptake into neurons. Differentiated Neuro2A cells were transfected with either GFP-Puma or GFP and after 12 hours cells were treated with 1μM of either Tat-Bax-Hα1 or Tat-Bax-Hα1(D33A) peptide. The tat-peptide treatment was repeated every six hours at a concentration of 0.2μM to compensate for potential degradation. Twenty-eight hours following transfection, nuclei were Hoechst 33342 stained to identify apoptotic cells.

We found that N2A cells expressing GFP-Puma exhibited a marked increase in the fraction of apoptotic cells as compared to cells expressing GFP alone (Figure 5). Furthermore, we found that cells transfected with GFP-Puma and treated with the Tat-Hα1 peptide exhibited significantly less apoptosis than those treated with Tat-Bax-Hα1(D33A) peptide or no peptide (Figure 5). We observed that N2A cells expressing GFP-Puma exhibited a punctate, cytoplasmic GFP staining pattern. Interestingly, this staining pattern was not affected by treatment of cells with the Tat-Bax-Hα1 peptide (Figure 5). Taken together these results suggest that while interaction of Puma with Bcl-2 family proteins is required for its apoptotic activity these interactions are not required for its characteristic localization.


*
60
40
20
0
Apoptotic Cells (%)
PUMA
PUMA + D33A
PUMA + HA1
GFP

(a)


(i)
(ii)
(iii)
(iv)
20 µm

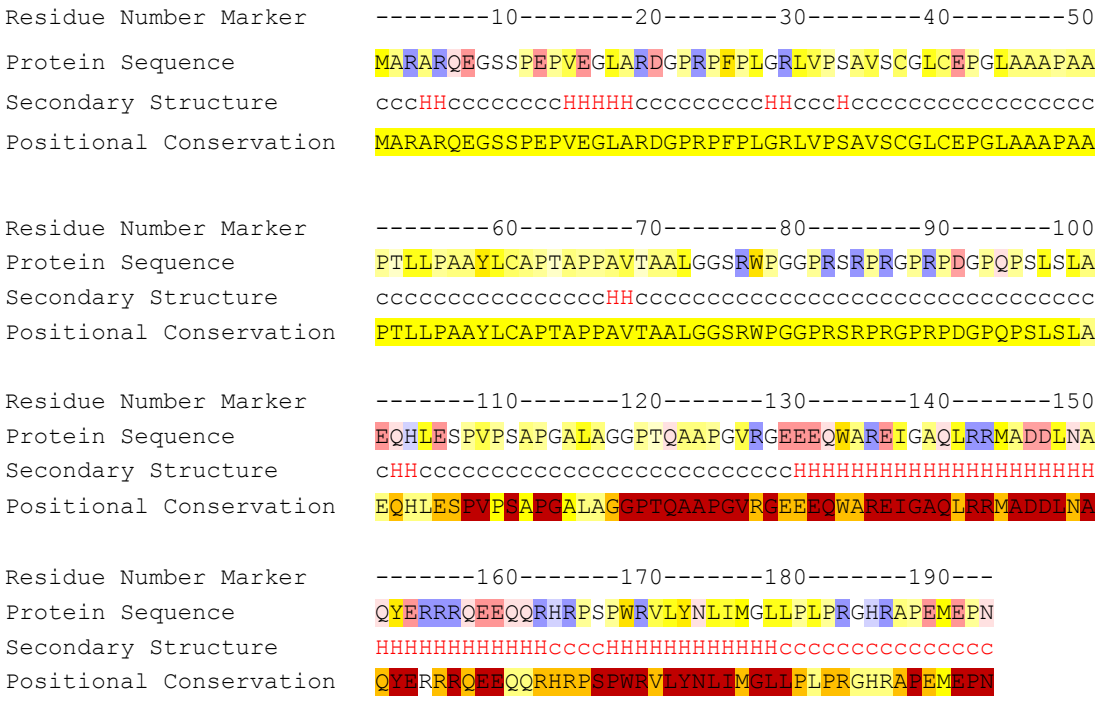
(b)

Figure 5 - Hα1 tat-peptide interferes with Puma-induced apoptosis but not its localization. Differentiated Neuro2A cells were transfected with GFP-Puma (WT) or GFP. Cells transfected with GFP-Puma were treated with Bax-Hα1 or Bax-Hα1(D33A) tat-peptide beginning with 1μM at 12 hours post-transfection followed by 0.2μM every six hours thereafter. At 28 hours post-transfection, cells were stained with Hoecsht 33342 and viewed by fluorescence microscopy to assess nuclear morphology. (a) Cells that displayed characteristics of apoptosis (nuclear condensation and blebbing) were counted as apoptotic. Apoptotic Cells (%) was calculated as GFP-positive apoptotic cells out of total GFP-positive cells (*$p<0.01$, $n=4$). Data were analyzed using one-way ANOVA and Dunnet's Multiple Comparison test. (b) (i) Cells transfected with GFP-Puma. (ii) Cells transfected with GFP-Puma and treated with Bax-Hα1(D33A) tat-peptide. (iii) Cells transfected with GFP-Puma and treated with Bax-Hα1 tat-peptide. (iv) Cells transfected with GFP. Representative images from four independent experiments are shown ($n=4$). Scale bar = 20μm.

3.2 - Identification of a potential membrane targeting sequence in the Puma protein.

Having determined that interactions between the BH3 domain of Puma and Bcl-2 family proteins are not required its characteristic localization, we analyzed the Puma protein sequence for potential membrane targeting motifs using MEta Server for protein Sequence Analysis (MESSA) software. MESSA is a meta-server designed to identify local sequence properties, three-dimensional structure, and overall function(s) of proteins using a consensus from multiple existing computer programs (Cong & Grishin 2012).

By examining the results from the MESSA analysis, we identified two regions of interest in the Puma protein: the well-established BH3 domain (amino acids 137-150), and a putative localization sequence at the c-terminus of the protein (approximately amino acids 165-185) (Figure 6). The BH3 domain is well-conserved, amphipathic, and alpha-helical in structure (Figure 6). Although MESSA did not identify a classic membrane-localization sequence in the Puma protein, we did identify sequence features that suggest an important region at the c-terminus of Puma (Figure 6). The putative localization region is well conserved and alpha-helical in structure (Figure 6). It is a hydrophobic region flanked by basic amino acids, a sequence which resembles the mitochondrial-localization sequence of Bcl-xl (Figure 6; Kaufmann *et al.* 2003).



Protein Sequence: The input protein sequence shown in this line is highlighted according to amino acid property: positively charged residues in blue (light blue for partially positive), negatively charged residues in red (pink for partially negative), hydrophobic residues in yellow and no highlight for small residues.

Secondary Structure (Consensus): The 3-state secondary structure of the query is predicted, alpha-helix (including 3-turn and 5-turn helix), beta-strand and coils (including any secondary structure other than helix and strand). In the result, "H" represents alpha-helix; "E" stands for beta-strand; and "c" refers to coils.

Positional Conservation: Multiple Sequence Alignment of confident BLAST hits, filtered by less than 90% identity and more than 40% coverage, are used to calculate the positional conservation indices of residues in the sequence. The conserved residues usually plays important in maintaining the function or structure of a protein. Here the residues are highlighted from white, through yellow to dark red as the conservation level increases.

Figure 6 - Regions of interest in the Puma protein. MEta Server for protein Sequence Analysis (MESSA) analysis of Puma protein (Cong & Grishin 2012). The important regions of interest are the BH3 domain, amino acids 137-150, and the putative c-terminal localization sequence. Here we can see that the BH3 domain falls in a highly conserved region, appears to be amphipathic, and alpha-helical in structure. There is also a region of interest in the c-terminus of the protein, appromixately amino acids 165-185. This region is highly conserved and appears to contain an alpha-helical structure. It is hydrophobic, which would aid in membrane localization and insertion, and is flanked by basic amino acids. This resembles the localization sequence on Bcl-xl put forward by Kaufmann *et al.* that is purported to localize the protein specifically to the mitochondrial membrane (2003). Although it is not identified as a classic membrane localization sequence, this region may be similarly responsible for the localization of Puma.

3.3 - Analysis of protein expression confirms that GFP plasmids express proteins at equal levels and expected molecular weights.

In order to examine the roles of the BH3 domain and the putative c-terminal localization sequence of Puma, a number of GFP-tagged mutant plasmids were created: GFP-Puma (WT), the GFP tag followed by full length Puma; GFP-Puma (ΔBH3), the GFP tag followed by a variant of Puma with the BH3 domain deleted; GFP-Puma (1-160), the GFP tag followed by a truncated variant of Puma containing only the first 160 amino acids; and GFP-Puma (151-193), the GFP tag followed by a truncated variant of Puma containing only the last 42 amino acids. In order to ensure proper and equal expression of these Puma variants, differentiated Neuro2A cells were transfected with equal amounts of the plasmids, and protein samples were collected for analysis by Western blot. GFP primary antibody was used to evaluate protein expression levels because there is no single Puma antibody capable of detecting all of the different Puma variants. Calnexin was used to ensure equal loading of the protein samples.

The GFP-Puma (WT) protein should be approximately 49 kDa (22 kDa from Puma plus 27 kDa from the GFP tag). GFP-Puma (ΔBH3) should be about 46 kDa and GFP-Puma (1-160) should be approximately 44 kDa. GFP-Puma (151-193) should be about 28kDa, only a little more than GFP alone. All plasmids express protein at similar levels and at the expected molecular weights (Figure 7).

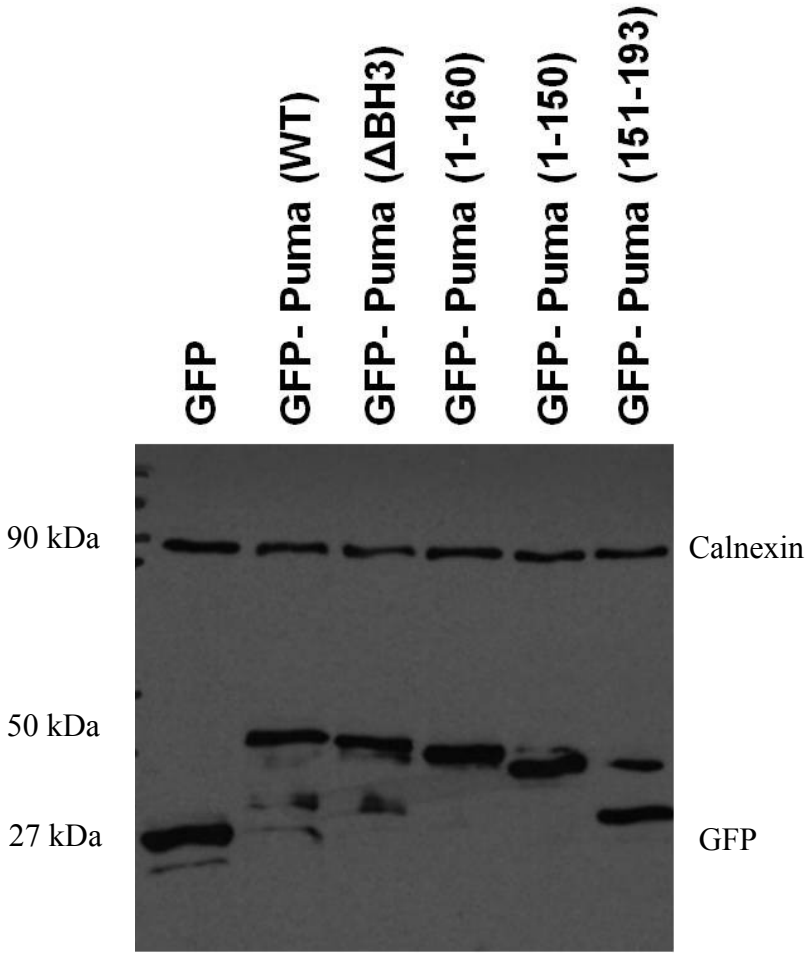

GFP
GFP- Puma (WT)
GFP- Puma (ΔBH3)
GFP- Puma (1-160)
GFP- Puma (1-150)
GFP- Puma (151-193)
90 kDa
50 kDa
27 kDa
Calnexin
GFP

Figure 7 - Expression of GFP-Puma plasmids. Differentiated Neuro2A cells were transfected with equal amounts (1μg) of the various GFP plasmids. Twenty-four hours after transfection, protein samples were collected and expression of Calnexin and the GFP tagged proteins was analyzed by western blot. Representative image of four independent experiments is shown (n=4).

3.4 – An element of the Puma c-terminus is required and sufficient for cellular localization of Puma.

Next, we wanted to examine potential roles of the BH3-domain and the c-terminus in regulating the cellular localization of Puma. In order to do so, differentiated Neuro2A cells were transfected with GFP or one of the various GFP-Puma mutants. Twenty-four hours after transfection, cells were stained with Hoechst 33342 as a nuclear marker and visualized by fluorescence microscopy. Cells were counted in two categories: those that displayed punctate, cytoplasmic GFP fluorescence and those that displayed diffuse GFP fluorescence.

As expected, the vast majority of cells expressing GFP alone exhibited a diffuse GFP fluorescence throughout the nucleus and cytoplasm (Figure 8). In contrast, N2A cells expressing GFP-Puma (WT) displayed mainly punctate, cytoplasmic GFP fluorescence (Figure 8). Cells expressing GFP-Puma (ΔBH3) and GFP-Puma (151-193), the plasmids with intact c-termini but lacking the BH3 domain of Puma, displayed punctate, cytoplasmic GFP fluorescence similar to the cells expressing wild-type GFP-Puma (Figure 8). However, cells expressing GFP-Puma (1-160), the plasmid with an intact BH3 domain but lacking the c-terminus of Puma, did not display punctate GFP fluorescence and instead showed diffuse GFP fluorescence similar to cells expressing GFP (Figure 8). This suggests that an element in the c-terminus is required and sufficient for the cellular localization of Puma. Furthermore, it suggests that the BH3 domain is not required for cellular localization.

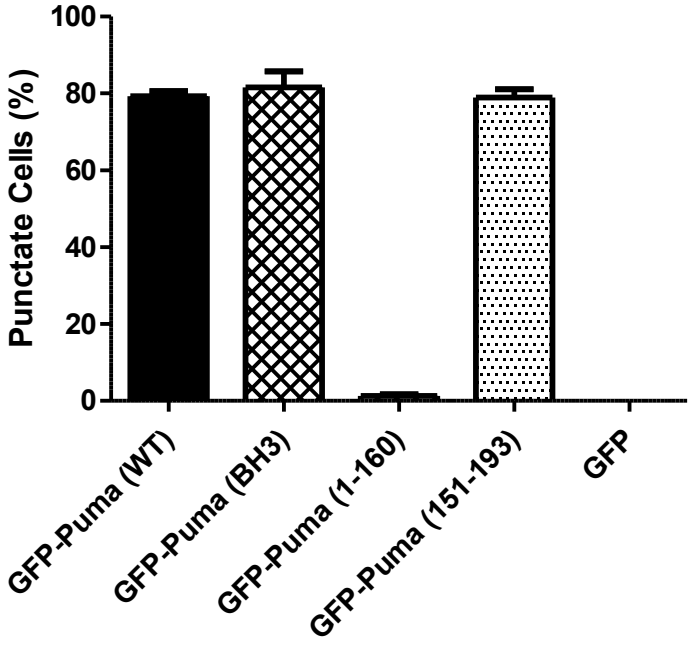

Punctate Cells (%)
100
80
60
40
20
0
GFP-Puma (WT)
GFP-Puma (BH3)
GFP-Puma (1-160)
GFP-Puma (151-193)
GFP

Figure 8 - Puma c-terminus is required and sufficient for cellular localization.
Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP. Twenty-four hours post-transfection, cells were stained with Hoechst 33342 as a nuclear marker and the pattern of GFP fluorescence was examined. Punctate Cells (%) was calculated as the number of cells with GFP-puncti out of total number of GFP-positive cells (n=3).

3.5 – Puma c-terminus is required and sufficient for mitochondrial-localization.

The next step was to determine the roles of the BH3 domain and c-terminus of Puma in its ability to localize to the mitochondria. In order to do so we examined the co-localization of the various GFP-Puma proteins with Mitotracker ® Red CMXRos using confocal microscopy. Mitotracker ® Red CMXRos is a fluorescent compound that stains mitochondria in live cells.

Differentiated Neuro2A cells were transfected with the various GFP-Puma plasmids. Twenty-four hours post-transfection, mitochondria were stained with Mitotracker ® Red CMXRos and co-localization of the GFP and Mitotracker ® Red CMXRos fluorescence was examined by confocal microscopy.

In cells transfected with GFP-Puma (WT), the majority of the characteristic GFP-Puma puncti appear to co-localize with mitochondria (Figure 9, panel (b)). The GFP-Puma variants with intact c-termini but without BH3 domains, GFP-Puma (ΔBH3) and GFP-Puma (151-193), also appear to mostly co-localize with mitochondria (Figure 9, panels (c) and (e)). However, the GFP-Puma protein with an intact BH3 domain but lacking the c-terminus, GFP-Puma (1-160), does not appear to co-localize with mitochondria (Figure 9, panel (d)).

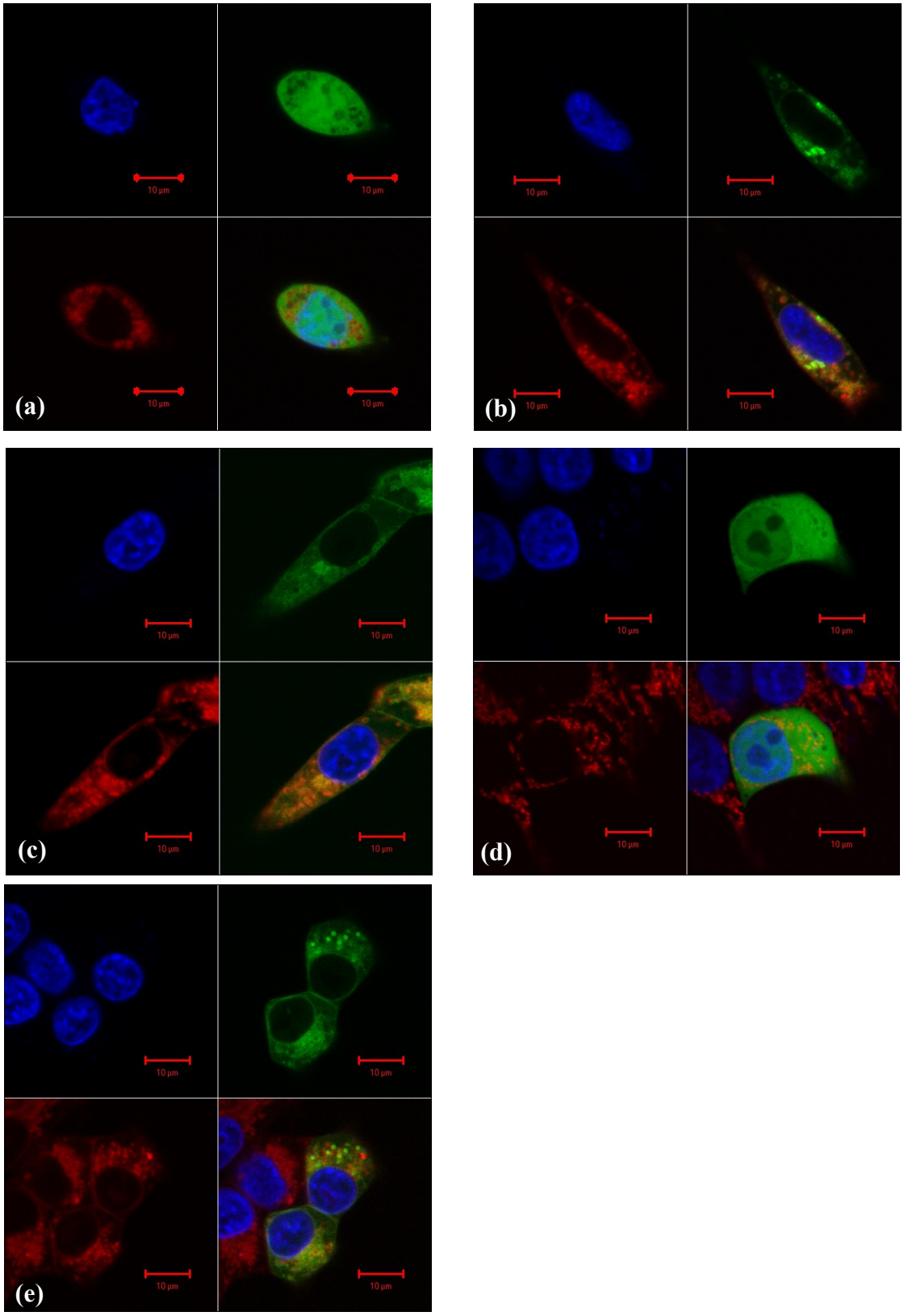

(a)
(b)
(c)
(d)
(e)
10 µm

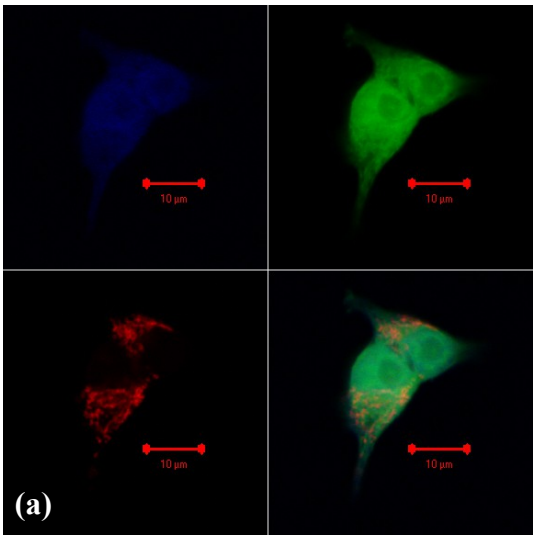
Figure 9 - Co-localization of different GFP-Puma proteins with Mitotracker ® Red CMXRos. Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP. 24 hours post-transfection, cells were stained with Hoechst 33342 and Mitotracker ® Red CMXRos. Co-localization of GFP-Puma proteins with mitochondria was assessed by confocal microscopy. Blue = Hoechst 33342; Green = GFP proteins; Red = Mitotracker ® Red CMXRos. (a) GFP appears diffuse and does not appear to co-localize with the mitochondria. (b) GFP-Puma (WT) appears punctate and appears to co-localize with the mitochondria. (c) GFP-Puma (ΔBH3) appears punctate and appears to co-localize with the mitochondria. (d) GFP-Puma (1-160) appears diffuse and does not appear to co-localize with the mitochondria. (e) GFP-Puma (151-193) appears punctate and appears to co-localize with the mitochondria. Representative images from three independent experiments are shown (n=3). Scale Bar = 10μm.

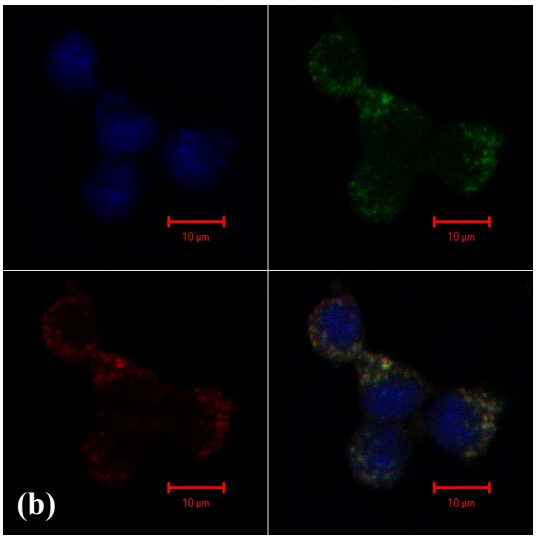
Although the majority of the GFP puncti in cells transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), and GFP-Puma (151-193) co-localize with mitochondria labeled by Mitotracker ® Red CMXRos (Invitrogen #M-7512), there are some GFP puncti remaining that are not accompanied by Mitotracker ® Red CMXRos stain. One possible explanation for this is that Mitotracker ® Red CMXRos stains live cell mitochondria and we are investigating cell death processes. It is possible that those GFP-Puma puncti are co-localized with mitochondria that have lost their mitochondrial membrane potential and therefore do not retain the Mitotracker ® Red CMXRos. In order to investigate this possibility, we examined the co-localization of the various GFP-Puma proteins with a mitochondrial protein, translocase of outer mitochondrial membrane (Tomm20). Tomm20 is a mitochondrial import receptor protein that is located on the mitochondrial membrane and can therefore be used to identify mitochondria.

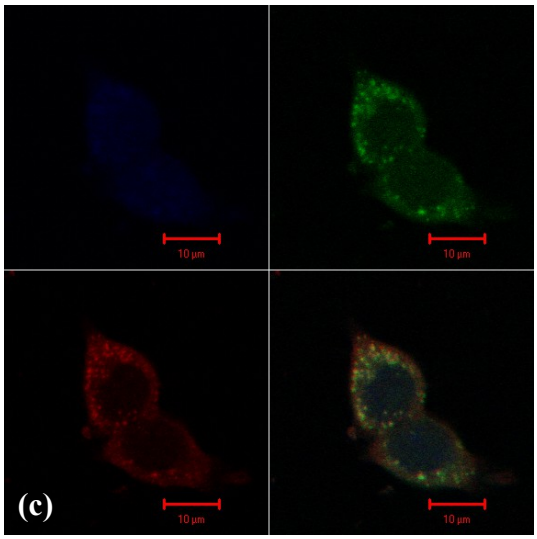
Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP. Twenty-four hours post-transfection, mitochondria were immunostained for Tomm20 and co-localization of GFP-Puma and Tomm20 was assessed by confocal microscopy.

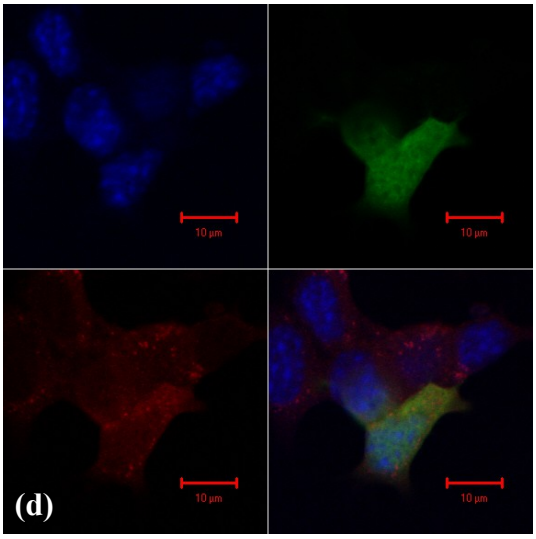
We found that GFP-Puma (WT) co-localized with the mitochondria stained for Tomm20 (Figure 10, panel (b)). Similarly, GFP-Puma (ΔBH3) and GFP-Puma (151-193), the Puma variants in which the C-terminal domain remains intact also exhibited marked co-localization with Tomm20 (Figure 10, panels (c) and (e)). However, GFP-

Puma (1-160), the Puma variant that retains the BH3-domain but lacks the c-terminus, does not co-localize with Tomm20-stained mitochondria (Figure 10, panel (d)). These results indicate that a sequence at the c-terminus, but not the BH3 domain of Puma, is both necessary and sufficient for the mitochondrial localization of Puma.


10 µm
(a)


10 µm
(b)


10 µm
(c)


10 µm
(d)

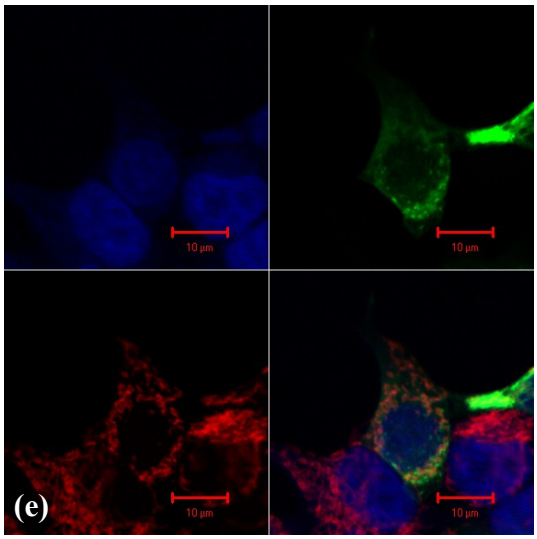
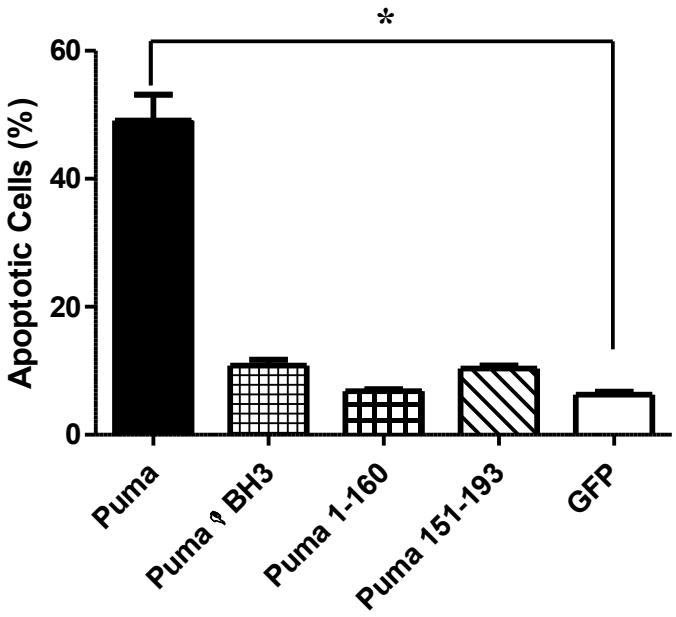

10 µm
(e)

Figure 10 - Co-localization of different GFP-Puma proteins with Tomm20.
Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP. Twenty-four hours post-transfection, cells were stained with Hoechst 33342, fixed, and immunostained for Tomm20. Co-localization of GFP-Puma proteins with mitochondria was assessed by confocal microscopy. Blue = Hoechst 33342; Green = GFP plasmids; Red = Tomm20. (a) GFP appears diffuse and does not appear to co-localize with the mitochondria. (b) GFP-Puma (WT) appears punctate and appears to co-localize with the mitochondria. (c) GFP-Puma (ΔBH3) appears punctate and appears to co-localize with the mitochondria. (d) GFP-Puma (1-160) appears diffuse and does not appear to co-localize with the mitochondria. (e) GFP-Puma (151-193) appears punctate and appears to co-localize with the mitochondria. Representative images from three independent experiments are shown (n=3). Scale Bar = 10μm.

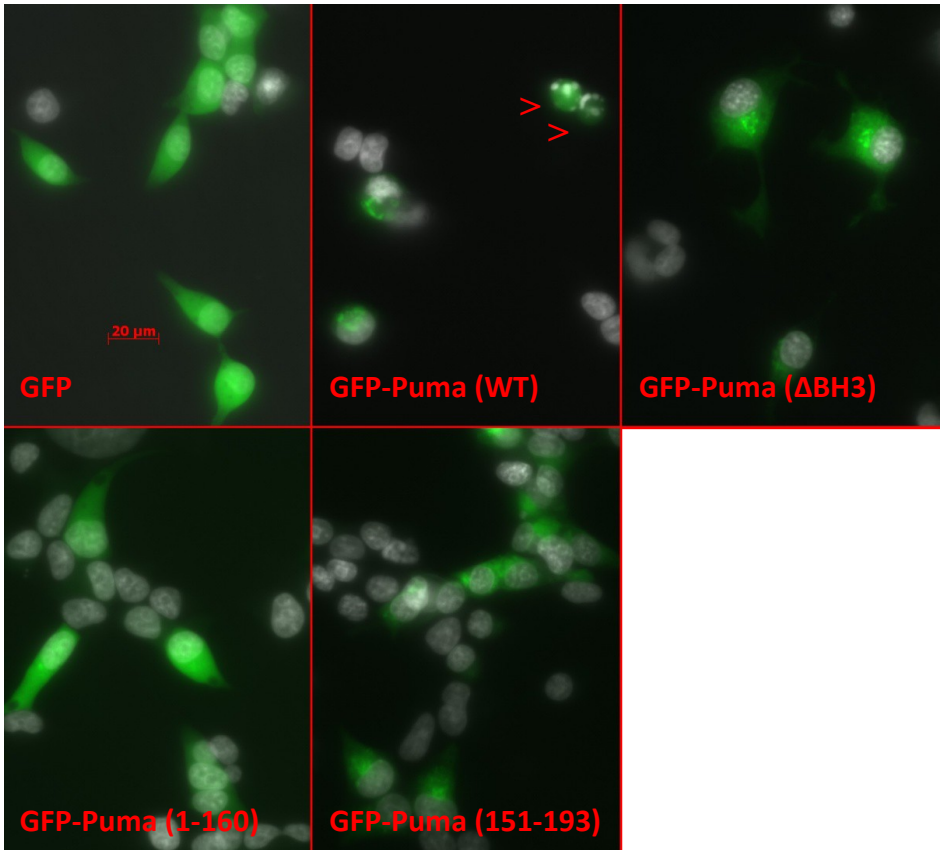
3.6 – Both the BH3 domain and a distinct c-terminal domain of Puma are required for Puma-induced apoptosis.

To examine the roles of Puma's BH3 domain and c-terminus in its ability to induce apoptosis, differentiated Neuro2A cells were transfected with the various GFP-Puma plasmids. Twenty-eight hours after transfection, cells were stained with Hoechst 33342 and visualized by fluorescence microscopy. Hoechst 33342 is a fluorescent stain that binds to nuclear DNA and allows the examination of nuclear morphology. Characteristics of apoptosis are chromosome condensation and nuclear blebbing; the use of Hoeschst 33342 stain to assess nuclear morphology is a well-established method for evaluating apoptosis. The proportion of GFP-positive cells with blebbed and/or pyknotic nuclei out of the total number of GFP-positive cells was calculated in order to assess the levels of apoptosis induced by expression of the various Puma proteins.

Cells expressing the wild-type Puma protein had significantly higher levels of apoptosis (with a mean of 49% apoptotic cells) than cells expressing GFP alone (with a mean of 6% apoptotic cells) (Figure 11). On the other hand, expression of GFP-Puma (ΔBH3), GFP-Puma (1-160) or GFP-Puma (151-193) did not result in a significant increase in apoptosis as compared to expression of GFP alone (Figure 11). The variants of Puma lacking either the BH3 domain or the c-terminus were unable to induce apoptosis. Taken together, these data suggest that both the BH3 domain and the c-terminus of Puma are required for its apoptotic activity.


*
60
40
20
0
Apoptotic Cells (%)
Puma
Puma Δ BH3
Puma 1-160
Puma 151-193
GFP

(a)


20 μm
GFP
GFP-Puma (WT)
GFP-Puma (ΔBH3)
GFP-Puma (1-160)
GFP-Puma (151-193)

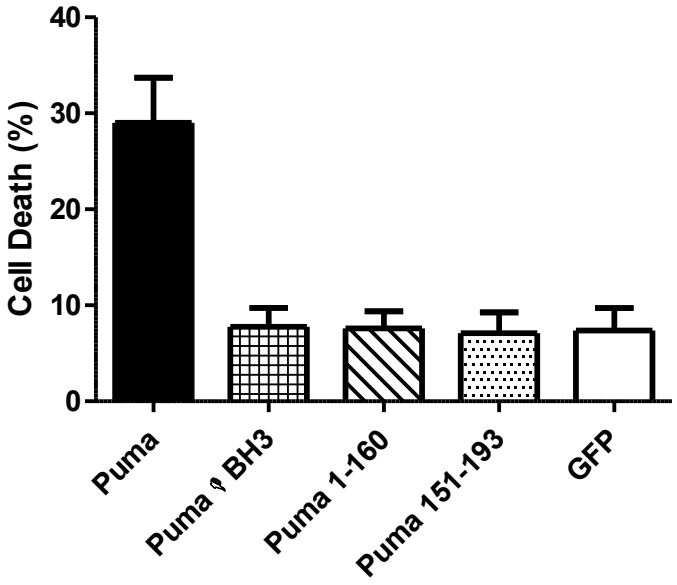
(b)

Figure 11 - Differing levels of apoptosis induced by various GFP-Puma proteins. Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP. Twenty-eight hours post-transfection, Hoechst 33342 was used to stain cellular nuclei. Nuclear morphology was examined and cells that displayed characteristics of apoptosis (nuclear condensation and blebbing) were counted as apoptotic. (a) Apoptotic Cells (%) was calculated as GFP-positive apoptotic cells out of total GFP-positive cells (*$p<0.01$, $n=4$). Data were analyzed using one-way ANOVA and Dunnet's Multiple Comparison test. (b) Representative images of the quantified results in panel (a) are shown. Apoptotic nuclei are marked with (>). Scale Bar = 20μm.

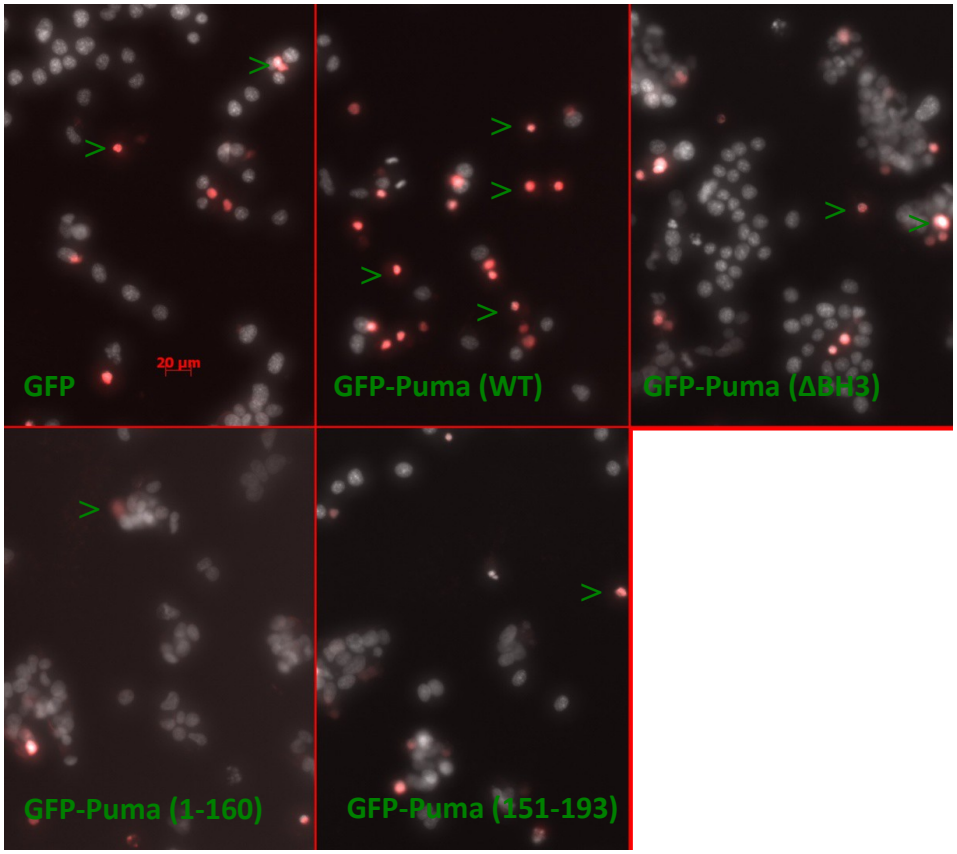
In order to ensure that the Puma variants were not inducing cell death by a process other than apoptosis and to ensure that assessment of nuclear morphology was sufficient to evaluate cell death, we used Propidium Iodide stain to compare the ability of different Puma variants to induce cell death. Propidium Iodide is an intercalating agent that binds DNA, but is excluded from viable cells by intact cellular membranes. However, Propidium Iodide can permeate dead cells where it binds to and fluorescently labels DNA, allowing visualization by fluorescence microscopy.

Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP. Twenty-eight hours after transfection, cells were stained with Hoechst 33342 as a nuclear marker and Propidium Iodide to identify dead cells. Cells were then visualized by fluorescent microscopy and the proportion of Propidium Iodide-positive cells to total number of cells was calculated in order to determine the level of cell death induced by each plasmid.

Expression of wild-type Puma resulted in an increase in cell death, with a mean of 29%, as compared to GFP alone, with a mean of 7% (Figure 12). However, expression of GFP-Puma (ΔBH3), GFP-Puma (1-160), and GFP-Puma (151-193) did not increase cell death as compared to expression of GFP alone (Figure 12). These results and the results from the Hoechst stain analysis combine to show that Puma requires both its BH3 domain and c-terminus to induce apoptosis.


Cell Death (%)
40
30
20
10
0
Puma
Puma Δ BH3
Puma 1-160
Puma 151-193
GFP

(a)


GFP
20 μm
GFP-Puma (WT)
GFP-Puma (ΔBH3)
GFP-Puma (1-160)
GFP-Puma (151-193)

(b)

Figure 12 - Differing levels of cell death induced by various GFP-Puma proteins. Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP. Twenty-eight hours post-transfection, cells were stained with Hoechst 33342 and Propidium Iodide. Cells positive for Propidium Iodide were counted as dead. (a) Cell death (%) was calculated as Propidium Iodide-positive cells out of the total number of cells (n=3). (b) Representative images of the quantified results in panel (a) are shown. Examples of Propidium Iodide-positive cells are marked with (>). Scale Bar = 20μm.

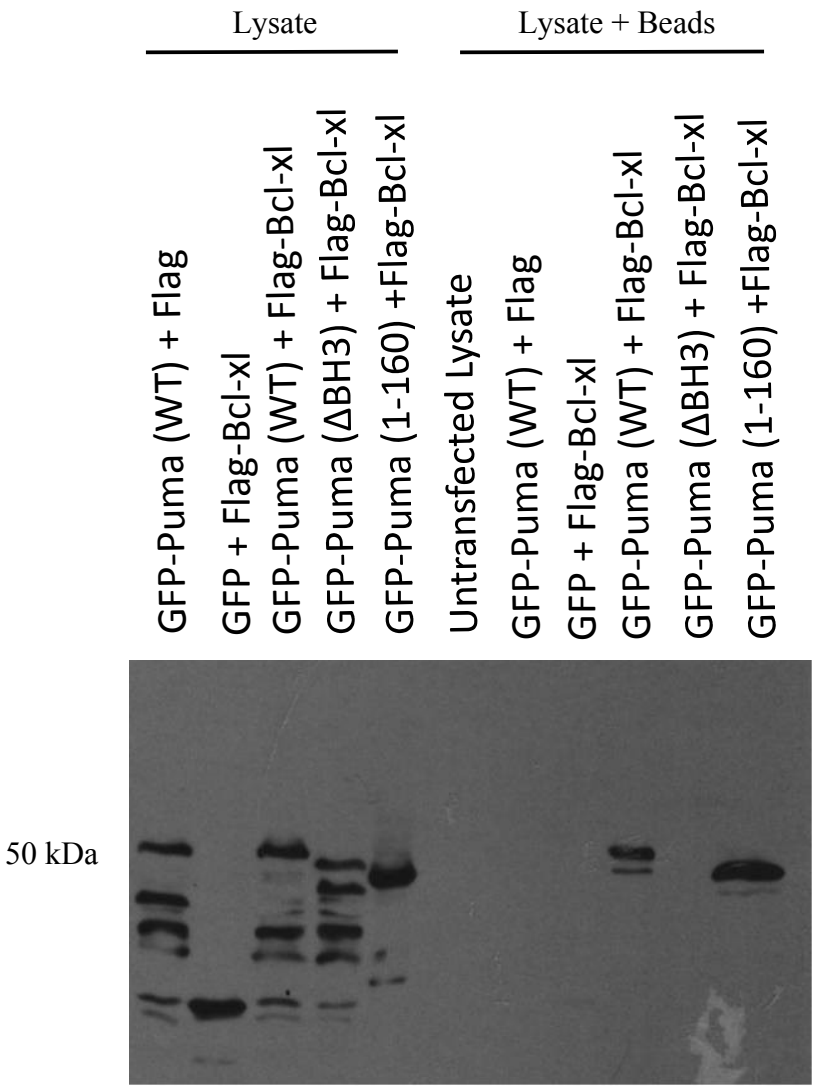
3.7 – Puma BH3 domain, but not Puma c-terminus, is required to bind to Bcl-xl *in vitro*.

One of the ways that BH3-only proteins, such as Puma, are able to promote apoptosis is through the indirect activation of Bax. In this model of Bax activation, BH3-only proteins bind to anti-apoptotic proteins so that the anti-apoptotic proteins cannot bind to and inhibit Bax. The anti-apoptotic protein Bcl-xl has been shown to play a particularly important role in neuronal apoptosis (Motoyama *et al.* 1995). Bcl-xl also appears to localize specifically to the mitochondria, which seems to be an important site for Bcl-2 family member interactions (Kaufmann *et al.* 2003; Lindsay *et al.* 2011). Therefore, we examined whether the c-terminal region of Puma, in addition to the BH3 domain, is required for binding to Bcl-xl.

Differentiated Neuro2A cells were co-transfected with one of the various GFP-Puma plasmids and either Flag-tagged Bcl-xl or empty Flag vector. Protein was collected from the transfected cells and was immunoprecipitated using Flag-beads. The ability of the various Puma proteins to bind the Flag-tagged Bcl-xl was assessed by immunoblot with GFP-antibody.

As shown in Figure 13, both GFP-Puma (WT) and GFP-Puma (1-160), which contain the BH3 domain, immunoprecipitated with Bcl-xl. However, GFP-Puma (ΔBH3), which retains the c-terminal region but lacks the BH3 domain, did not co-immunoprecipitate with Bcl-xl (Figure 13). We did not detect any non-specific binding between GFP and Flag-Bcl-xl, GFP-Puma (WT) and Flag, or cell lysate and beads

(Figure 13). These results suggest that the Puma c-terminal region is not required for binding to Bcl-xl *in vitro*.



IP: Flag
IB: GFP

Figure 13 - Puma BH3 domain, but not Puma c-terminus, is required to bind to Bcl-xl *in vitro*. Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), or GFP, as well as Flag-tagged Bcl-xl or Flag-tag. 24 hours post-transfection, protein was collected and immunoprecipitated with Flag-beads. An immunoblot was then performed on the samples using GFP-antibody to identify any co-precipitated GFP-Puma proteins. Representative blot from three independent experiments is shown (n=3).

3.8 – Puma c-terminus is required for co-localization with Bcl-xl in N2A cells *in situ*.

The co-immunoprecipitation experiments were performed in whole-cell lysate, which makes the localization of proteins irrelevant by creating a homogeneous mixture of the cellular components. Proteins may be able to bind in cell lysate, but might not do so in an intact cellular environment if they localize to different areas of the cell and therefore do not have the opportunity to interact. Perhaps the localization sequence at the c-terminus of Puma is not required to bind to Bcl-xl in cell lysate but is required to co-localize with and bind to Bcl-xl in the intact cellular environment. In order to investigate this possibility, we used confocal microscopy to examine the ability of the various Puma mutants to co-localize with Flag-Bcl-xl.

Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP, as well as Flag-tagged Bcl-xl. Twenty-four hours post-transfection, cells were stained with Hoechst 33342 as a nuclear marker and fixed. Flag-tagged Bcl-xl was immunofluorescently labeled and the co-localization of Flag-tagged Bcl-xl and the various GFP-Puma mutants was assessed using confocal microscopy.

As shown in Figure 14 (panel b) GFP-Puma (WT) protein exhibits marked co-localization with Flag-Bcl-xl. Similarly, GFP-Puma (ΔBH3) and GFP-Puma (151-193), which possess the c-terminal region but lack the BH3 domain, also co-localize with Flag-Bcl-xl (Figure 14, panels (c) and (e)). However, GFP-Puma (1-160), which retains the BH3-domain but lacks the c-terminus, does not appear to co-localize with Flag-Bcl-xl

(Figure 14, panel (d)). Taken together these results suggest that the c-terminus, but not the BH3 domain, is required for Puma to co-localize with Bcl-xl in N2A cells and likely for Puma-Bcl-xl interaction in the cellular context.

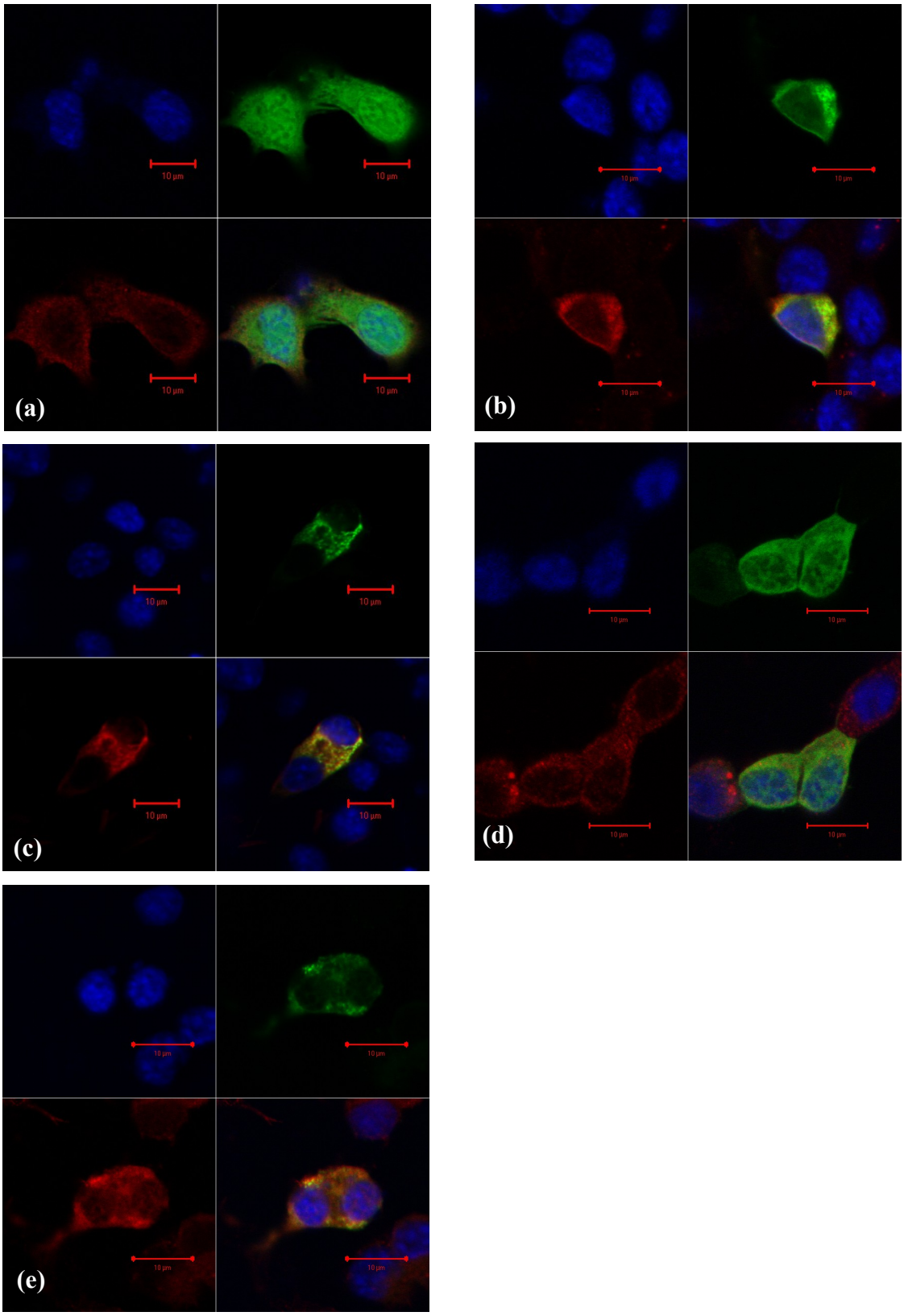

(a)
(b)
(c)
(d)
(e)

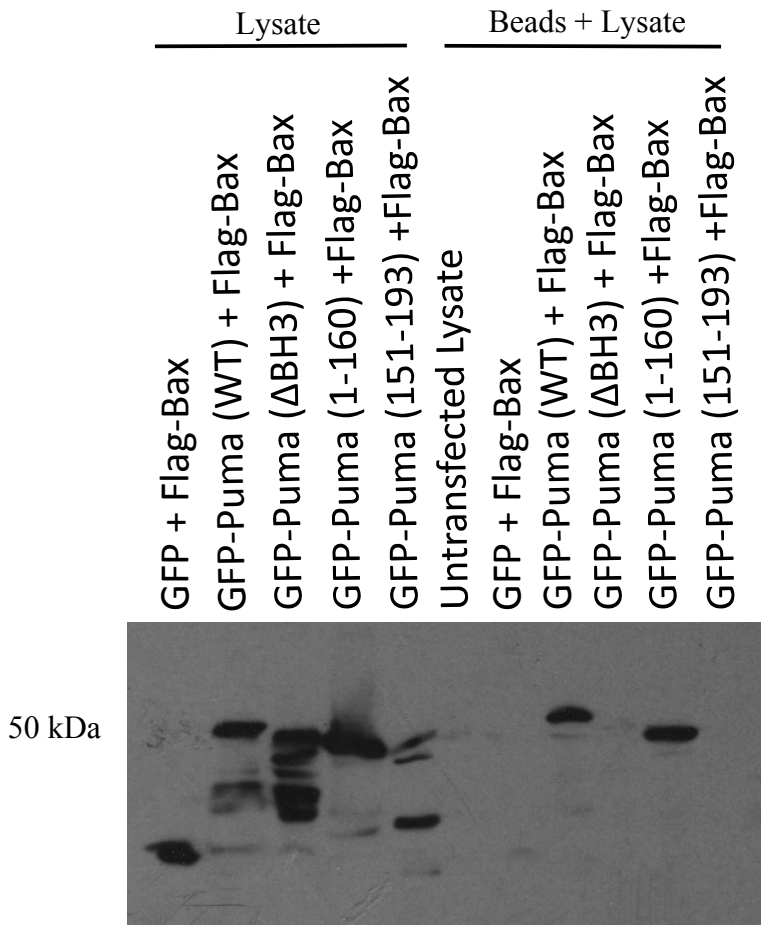
Figure 14 - Puma c-terminus is required for co-localization with Bcl-xl in N2A cells *in situ*. Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP, as well as Flag-Bcl-xl. Twenty-four hours post-transfection, cells were stained with Hoechst 33342, fixed, and immunofluorescently stained for Flag-Bcl-xl. Co-localization of the various GFP-Puma plasmids with Flag-Bcl-xl was assessed by confocal microscopy. Blue = Hoechst 33342; Green = GFP plasmids; Red = Flag-Bcl-xl. (a) GFP appears diffuse and does not appear to co-localize with Flag-Bcl-xl. (b) GFP-Puma (WT) appears punctate and appears to co-localize with Flag-Bcl-xl. (c) GFP-Puma (ΔBH3) appears punctate and appears to co-localize with Flag-Bcl-xl. (d) GFP-Puma (1-160) appears diffuse and does not appear to co-localize with Flag-Bcl-xl. (e) GFP-Puma (151-193) appears punctate and appears to co-localize with Flag-Bcl-xl. Representative images from three independent experiments are shown (n=3). Scale Bar = 10μm.

3.9 - Puma BH3-domain, but not Puma c-terminus, is required to bind to Bax *in vitro*.

We and others have previously demonstrated that, in addition to interacting with anti-apoptotic Bcl-2 family proteins, Puma can also interact with the pro-apoptotic member Bax *in vitro* (Steckley *et al* 2007, Cartron *et al.* 2004*).* This would suggest that Puma may also contribute to cell death through direct activation of Bax. Therefore, we next examined whether the Puma c-terminus was required for Puma to interact with Bax using a co-immunoprecipitation approach.

Differentiated Neuro2A cells were transfected with one of the various GFP-Puma mutants and Flag-tagged Bax. Twenty-four hours post-transfection, protein was collected and immunoprecipitated with Flag-beads. Samples were then run on SDS-PAGE and immunoblotted with GFP antibody to examine the potential of the various GFP-Puma mutants to bind to and immunoprecipitate with Flag-Bax.

Both GFP-Puma (WT) and GFP-Puma (1-160), the proteins with intact BH3 domains, were able to bind to Flag-Bax (Figure 15). However, the Puma proteins with intact c-termini but missing the BH3 domain (GFP-Puma (ΔBH3) and GFP-Puma (151-193)) were unable to bind to Flag-Bax (Figure 15). We did not detect non-specific binding between GFP and Flag Bax, or cell lysate and beads (Figure 15). These results suggest that the c-terminal region is not required for Puma to interact with Bax *in vitro.* However, it is possible that the c-terminal region could be required for co-localization and interaction of Puma with Bax in intact cells similar to the situation with Bcl-xl.



IP: Flag
IB: GFP

Figure 15 - Puma BH3-domain, but not Puma c-terminus, is required to bind to Bax *in vitro.* Differentiated Neuro2A cells were transfected with GFP-Puma (WT), GFP-Puma (ΔBH3), GFP-Puma (1-160), GFP-Puma (151-193), or GFP, as well as Flag-tagged Bax. Twenty-four hours post-transfection, protein was collected and immunoprecipitated with Flag-beads. An immunoblot was then performed on the samples using GFP-antibody to identify any co-precipitated GFP-Puma proteins. Representative blot from three independent experiments is shown (n=3).

Chapter 4 - Discussion

Our lab has previously shown the importance of Puma expression and mitochondrial-localization in neuronal apoptosis due to a variety of cell stressors, including: endoplasmic reticulum, trophic factor withdrawal, and oxidative stress (Ambacher *et al.* 2012; Galehdar *et al.* 2010; Steckley *et al.* 2007). Here, we have identified that a motif at the c-terminus of the Puma protein, but not its BH3 domain nor BH3 domain-mediated Bcl-2 family interactions, is responsible for its mitochondrial-localization. We have also shown that both this c-terminal mitochondrial-localization sequence and the BH3 domain of Puma are necessary for the apoptotic activity of the Puma protein.

4.1 - Identification of two important regions in the Puma protein

In order to explore the possibility of another region of importance in the Puma protein, we analyzed the Puma sequence using the MEta Server for protein Sequence Analysis (MESSA) software (Cong & Grishin 2012). Our analysis identified an amphipathic, alpha-helical domain at amino acids 137-150 in the protein (Figure 6). These features are consistent with the well-documented BH3 domain of Puma. Like all BH3-only members of the Bcl-2 family, Puma contains a BH3 domain responsible for its interactions with other Bcl-2 family proteins (Day *et al*. 2008; Reed *et al.* 1996; Petros *et al.* 2004). The BH3 domain of Puma is amphipathic and alpha-helical in structure (Day

et al. 2008; Nakano & Vousden 2001; Yu *et al.* 2001). It binds directly to anti-apoptotic proteins, such as Bcl-xl, and has been shown to bind all the anti-apoptotic proteins with similar strength (Chen *et al.* 2005; Day *et al*. 2008; Gautier *et al.* 2011; Kuwana 2005). The BH3 domain of Puma has also been shown to directly interact with the pro-apoptotic protein, Bax (Cartron *et al.* 2004; Du *et* al. 2011; Gallenne *et al.* 2009; Yee & Vousden 2008; Zhang *et al.* 2009; Steckley *et al.* 2007), although others have presented contrary evidence (Kuwana *et al.* 2005; Ming *et al.* 2006; Gautier *et al.* 2011; Willis *et al.* 2007). The sequence and structure of the BH3 domain in Puma are well-documented, although its ability to directly interact with the multi-domain pro-apoptotic Bcl-2 protein, Bax, and the physiological significance of this interaction are still in question.

In our analysis of the Puma sequence, we identified the BH3 domain of Puma in its anticipated location and with the same well-documented structural features we expected (Figure 6). We also identified a more novel region on the Puma protein: a putative mitochondrial-localization sequence at its c-terminus. In our analysis, we discovered a highly-conserved sequence that runs from approximately amino acids 165-185 at the c-terminus of the Puma protein (Figure 6). It is alpha-helical in structure and primarily consists of hydrophobic amino acids (Figure 6). This group of hydrophobic amino acids is flanked on either side by a few basic amino acids (Figure 6), similar to the trans-membrane domain of Bcl-xl identified by Kaufmann *et al.* (2003). In Bcl-xl, this region is responsible for targeting the protein specifically to the mitochondria (Kaufmann *et al.* 2003). Others have proposed that something in the c-terminal region of Puma may be responsible for localization of the protein, but the sequence and structure of this region

have not been well-defined (Nakano & Vousden 2001; Yee & Vousden 2008; Yu *et al.* 2001; Yu & Zhang 2009).

We and others have previously demonstrated that a variety of apoptotic stimuli induce Puma expression and localization to the mitochondrial outer membrane, and that ectopically expressed Puma protein also displays this characteristic localization (Nakano & Vousden, 2001; Steckley *et al.* 2007; Yee & Vousden 2008; Yu & Zhang 2009). Here, we have identified a highly-conserved, hydrophobic, alpha-helical region at the c-terminus of Puma (Figure 6). This region resembles the mitochondrial-localization region of Bcl-xl (Figure 6; Kaufmann *et al.* 2003) and likely has a similar function in the Puma protein.

Most of the Bcl-2 family members contain c-terminal membrane-localization sequences, and it is unlikely that the prevalence of membrane-localization sequences among the Bcl-2 family members is merely coincidental. Indeed, the mitochondrial outer membrane has been shown to play an essential role in many Bcl-2 family member interactions (Bogner *et al.* 2010; Jeong *et al.* 2004; Lalier *et al.* 2007; Lindsay, J. e*t al.* 2011). The cytoplasmic conformations of the Bcl-2 family members Bcl-xl and Bax discourage protein interactions and make it difficult for other proteins to interact with them until they change to their membrane-anchored conformation (Jeong *et al.* 2004; Lalier *et al.* 2007). Other BH3-only proteins, Bid and Bad, require interaction with membranes in order to interact with multi-domain pro-apoptotic proteins (Lindsay, J. e*t al.* 2011). If Puma is able to specifically localize to the mitochondria, it may aid in its

ability to interact with other Bcl-2 family members and may help to explain the efficacy of Puma as a pro-apoptotic protein.

4.2 – Puma mitochondrial localization is regulated by a c-terminal targeting region but not its BH3 domain or Bcl-2 family interactions

Our lab has previously shown that endogenous Puma localizes to the mitochondria in response to oxidative stress in neuronal cells (Steckley *et al.* 2007). Here, we have demonstrated that GFP-Puma (WT) displays a distinct cellular localization pattern (Figure 8) and that it co-localizes with both Mitotracker ® Red CMXRos stain and the mitochondrial protein, Tomm-20 (Figures 9 and 10). These results are in agreement with other studies that have shown that ectopically expressed Puma localizes to the mitochondria (Nakano & Vousden 2001; Yee & Vousden 2008; Yu *et al.* 2001; Yu & Zhang 2009). However, the region of the Puma protein responsible for its localization has not been well-defined in the literature.

In order to investigate the role of Bcl-2 family protein interactions in the localization of Puma, we synthesized a tat-peptide designed to target Puma's BH3 domain. The BH3 domain is well established as the site for the interactions between Puma and other Bcl-2 family members (Day *et al*. 2008; Reed *et al.* 1996; Petros *et al.* 2004). A peptide designed to mimic the Hα1 region of Bax has been shown to bind to the BH3 domain of Puma and interfere with the interaction between Puma and Bax (Cartron *et al.* 2004; Lalier, 2007). A mutation at amino acid residue 33 of this peptide from

aspartate to alanine has been shown to abrogate its binding to the Puma-BH3 domain (Cartron *et al.* 2004; Lalier *et al.* 2007). We synthesized Tat-fused Bax-Hα1 and Bax-Hα1(D33A) peptides to determine whether Puma interactions with other Bcl-2 family proteins are required for its mitochondrial localization. In our studies with these peptides, we found that Puma maintained its characteristic cellular localization even when its interactions with other Bcl-2 family members were blocked by the Bax-Hα1 tat-peptide (Figure 5). These results suggest that Bcl-2 family member interactions are not necessary for the mitochondrial-localization of Puma. This is in agreement with previous observations from our lab that Puma maintains its mitochondrial-localization in neurons cultured from Bax knockout mice (Steckley *et al.* 2007).

Consistent with these findings, we determined that the BH3 domain is not necessary for Puma's mitochondrial-localization. In our examination of the localization of the GFP-Puma mutants, we found that, although deletion of the BH3 domain did abrogate Puma's ability to bind to the Bcl-2 family members, Bcl-xl and Bax (Figures 13 and 15), it had no affect on the localization of Puma (Figure 8). The variant of GFP-Puma without its BH3 domain also retained its ability to co-localize with both Mitotracker ® Red CMXRos stain and Tomm-20 (Figures 9 and 10). Taken together, these experiments show that neither its BH3 domain, nor BH3 domain-mediated Bcl-2 family member interactions, are necessary for the mitochondrial-localization of Puma.

Instead, our results indicate that a distinct region at the c-terminus of Puma is responsible for its mitochondrial-localization. We found that GFP-Puma (1-160), the

variant missing its c-terminus, did not show the punctate localization characteristic of the Puma protein; instead it showed diffuse fluorescence, similar to the pattern seen in cells expressing the GFP plasmid (Figure 8). Although deletion of the c-terminus did not affect Puma's ability to bind to other Bcl-2 family members (Figures 13 and 15), it abrogated the co-localization of Puma with both Mitotracker ® Red CMXRos stain and Tomm-20 (Figures 9 and 10). In contrast, the GFP-Puma variant consisting of only the c-terminus of Puma, GFP-Puma (151-193), displayed a localization pattern consistent with wild-type GFP-Puma protein (Figure 8). Although this variant was unable to bind to Bcl-xl or Bax (Figures 13 and 15), it was able to co-localize with both Mitotracker ® Red CMXRos stain and Tomm-20 (Figures 9 and 10). These data confirm that the BH3 domain and BH3 domain-mediated Bcl-2 family member interactions are not necessary for the mitochondrial-localization of Puma; they indicate that instead, a distinct c-terminal region is responsible for Puma's localization. This is consistent with our earlier finding that Puma contains an important region at the c-terminus of the protein (Figure 6) and also consistent with some of the initial findings about the Puma protein. Upon its discovery, Nakano and Vousden (2001) looked at different transcriptional variants of Puma, including the truncated Puma-δ and Puma-γ, both of which are missing the c-terminus of the full-length protein. They found that these Puma variants were unable to induce apoptosis or localize to the mitochondria (Nakano & Vousden, 2001).

Although the majority of GFP-Puma puncti appear to co-localize with mitochondria, in both the Mitotracker ® Red CMXRos stain and Tomm -20 immunofluorescent staining, there appear to be some GFP-Puma puncti that are not c-

localized with mitochondria (Figures 9 and 10). Some of these puncti may be protein aggregates caused by over-expression of the proteins. Another explanation is that the c-terminus may not direct the Puma protein specifically to the mitochondrial membrane, but may be a more general membrane-localization sequence that also directs Puma to other cellular membranes, such as the endoplasmic reticulum or the lysosomal membranes. The localization sequences of some Bcl-2 family members, such as Bcl-2, have been shown to direct the protein to general membranes rather than specifically to the mitochondria (Kaufmann *et al.* 2003). However, the membrane-localization sequence at the c-terminus of Puma appears more similar to the mitochondrial-specific localization sequence seen in Bcl-xl (Figure 6; Kaufmann *et al.* 2003). Our lab has also previously demonstrated that endogenous Puma localizes to the mitochondria in neurons undergoing oxidative stress (Steckley *et al.* 2007). Altogether, these data suggest the at least the majority of Puma localizes to the mitochondria in neuronal apoptosis.

Because the mitochondrial outer membrane has been shown to play such a crucial role in the interactions of Bcl-2 family proteins (Bogner *et al.* 2010; Jeong *et al.* 2004; Lalier *et al.* 2007; Lindsay, J. e*t al.* 2011), the ability of the Puma c-terminus to localize the majority of Puma protein to the mitochondrial membrane might help to explain the efficacy of Puma as a pro-apoptotic protein. The fact that the GFP-Puma protein without an intact c-terminus appears unable to localize to the mitochondria may also explain its inability to induce apoptosis.

4.3 – Both the BH3 domain and c-terminal mitochondrial-localization region of Puma are required for Puma-mediated cell death and apoptosis

Although we did not find that the BH3 domain or BH3 domain-mediated Bcl-2 family interactions were important for the mitochondrial-localization of Puma, they do appear to be necessary for the full apoptotic function of the protein. Here we found that GFP-Puma (ΔBH3) and GFP-Puma (151-193), the variants unable to bind to the Bcl-2 family members, Bcl-xl and Bax (Figures 13 and 15), were unable to induce apoptosis (Figures 11 and 12). We also found that blocking the ability of Puma to bind to Bcl-2 family members with a tat-fused Bax-Hα1 peptide abrogated its apoptotic activity (Figure 5). In accordance with these results, we found that the BH3 domain of Puma is required for Puma-induced apoptosis. Deletion of the BH3 domain eliminated the protein's ability to induce apoptosis (Figures 11 and 12). Altogether, the results of these experiments suggest that Puma requires both its BH3 domain and BH3 domain-mediated Bcl-2 family member interactions in order to induce apoptosis. These conclusions are in agreement with others who have shown that Puma mediates apoptosis through its interactions with other Bcl-2 family members, and that the BH3 domain of Puma is the site of its interactions with both pro- and anti-apoptotic Bcl-2 family members (Cartron *et al.* 2004; Day *et al*. 2008; Du *et* al. 2011; Gallenne *et al.* 2009; Reed *et al.* 1996; Petros *et al.* 2004; Steckley *et al.* 2007; Yee & Vousden 2008; Zhang *et al.* 2009). Because the BH3-domain of Puma is believed to be the site of its interactions with other Bcl-2 family members, it makes sense for this domain to be of particular importance in the induction of apoptosis by Puma (Cartron *et al.* 2004; Day *et al*. 2008; Reed *et al.* 1996; Petros *et al.* 2004).

Our results suggest that Puma is able to activate Bax and promote apoptosis through both the indirect and the direct activation models. We have found that Puma is able both to bind to the anti-apoptotic Bcl-xl and to bind directly to Bax (Figures 13 and 15). This may help to reconcile the current controversy surrounding the activation of Bax by Puma. Many studies have shown that Puma can interact directly with Bax to activate it (Cartron *et al.* 2004; Du *et al.* 2011; Gallenne *et al.* 2009; Melino *et al.* 2004; Ren *et al.* 2010; Steckley *et al.* 2007; Yee & Vousden 2008; Zhang *et al.* 2009), while others have shown that apoptosis can occur without a direct interaction between Puma and Bax (Gautier *et al.* 2011; Kazi *et al.* 2011; Kuwana *et al.* 2005; Ming *et al.* 2006; Willis *et al.* 2007). Our results indicate that Puma is able to promote apoptosis through both models of Bax activation (Figures 13 and 15); perhaps Puma follows the indirect model if direct interaction with Bax is blocked in some way. Puma may even preferentially follow different models dependent on cell type or death stimulus. This flexibility or redundancy may help to explain the efficacy of Puma as a pro-apoptotic protein.

Interestingly, our results indicate that the BH3 domain and BH3 domain-mediated Bcl-2 family interactions are not the only requirements for Puma's full apoptotic activity. We examined the role of the c-terminus in the induction of apoptosis and found that the c-terminus of Puma is also required for the apoptotic function of the protein. GFP-Puma (1-160), the variant with an intact BH3 domain but missing the c-terminus, was unable to induce apoptosis (Figures 11 and 12). These results are consistent with other findings that the full-length Puma protein is required for its apoptotic activity (Nakano & Vousden 2001; Yu *et al.* 2003). If, as our earlier data indicates, this region is indeed responsible

for the cellular localization of the protein, then it appears that cellular localization plays an essential role in the apoptotic function of Puma. Most of the Bcl-2 family members contain c-terminal membrane-localization sequences, and it is unlikely that the prevalence of membrane-localization sequences among the Bcl-2 family members is merely coincidental. It is possible that these proteins localize to membranes in order to increase the chances of coming near enough to each other to interact. Additionally, some of the Bcl-2 family members, such as Bax, exist in a cytoplasmic conformation that might inhibit binding by other proteins (Lalier *et al.* 2007). Others, such as Bcl-xl, may form homodimers in the cytoplasm, possibly occupying their binding sites until they associate with a membrane (Jeong *et al.* 2004). These last two possibilities would require at least some of the Bcl-2 family members to associate with membranes before they could bind to other proteins. Indeed, the BH3-only proteins Bid and Bad require localization to the mitochondrial outer membrane in order to induce apoptosis (Bogner *et al.* 2010; Lindsay, J. e*t al.* 2011). Given the importance of membrane targeting among the other Bcl-2 family members, it is perhaps unsurprising that the c-terminal localization sequence of Puma is required for its apoptotic function.

Overall, our findings demonstrate that neither the BH3 domain, nor the c-terminal mitochondrial-localization region of Puma are sufficient to induce apoptosis without the other. GFP-Puma (1-160) does not localize to the mitochondrial outer membrane (Figures 9 and 10) and is unable to induce apoptosis (Figures 11 and 12), even though its intact BH3 domain is able to bind to Bcl-xl and Bax *in vitro* (Figures 13 and 15). On the other hand, GFP-Puma (ΔBH3) cannot bind to Bcl-xl or Bax (Figures 13 and 15) and is

unable to induce apoptosis (Figures 11 and 12), even though its intact c-terminus localizes the protein to the mitochondria (Figures 9 and 10).

One possible explanation for the necessity of both regions of the Puma protein is that they both play a role in the ability of Puma to interact with Bcl-2 family members *in situ.* This is perhaps most obvious in the case of Puma's interaction with Bcl-xl. Unlike the other anti-apoptotic Bcl-2 family members, Bcl-xl localizes specifically to the mitochondria (Kaufmann *et al.* 2003; Lindsay *et al.* 2011). It is arguably one of the most important anti-apoptotic proteins and has also been shown to play a particularly important role in neuronal apoptosis (Motoyama *et al.* 1995; Ming e*t al.* 2006). The BH3-domain of Puma is understandably important for binding to Bcl-xl, as it is the domain generally accepted to be the location of this interaction (Day *et al*. 2008; Reed *et al.* 1996; Petros *et al.* 2004). When we examined the ability of the various GFP-Puma proteins to bind to Bcl-xl in homogenous cell lysate, we found that the BH3 domain, but not the c-terminus of Puma was required to bind to Bcl-xl (Figure 13). In these conditions, where the cellular contents are homogenously mixed, cellular localization does not affect protein interactions with the same importance as in intact cells. However, when we examined the ability of the GFP-Puma variants to co-localize with Bcl-xl *in situ*, we found that the c-terminus of Puma was essential for co-localization of the two proteins (Figure 14). In combination, these results indicate that the BH3 domain of Puma is the location of the bond between Puma and other Bcl-2 family members, while the c-terminus is responsible for ensuring the cellular co-localization of the proteins necessary for their interaction *in situ*. Whether this co-localization is important solely because the proteins must be near to

each other to interact, or if it is also vital that Puma interacts with the mitochondrial membrane in order for the interactions with other Bcl-2 family members to take place, requires further study. A large quantity of evidence is accumulating for the importance of membranes in the interactions among various Bcl-2 family members, including Bid, Bad, Bax, and Bcl-xl (Bogner et al. 2010; Lalier, L. et al. 2007, Lovell, J.F. et al. 2008; Roucou et al. 2002; Yethon et al. 2003), so it would not be surprising if the interactions between Puma and the mitochondrial membrane aided its interactions with other Bcl-2 family members as well.

Another possible explanation for the importance of the c-terminus is that Puma may bind to the pro-apoptotic protein, Bax, in the cytoplasm and then bring it to the mitochondria where it is able to continue the apoptotic process. In this scenario, the mitochondrial-localization region of Puma would be required for both proteins to translocate to the mitochondria. This explanation is supported by data previously shown by our lab that Puma was required for Bax to localize at the mitochondria in neuronal apoptosis (Steckley *et al.* 2007). In the present study, we did not find that the c-terminus of Puma was required to bind to Bax in homogenous cellular lysate. However, similar results were seen in the co-immunoprecipitation experiment with Puma and Bcl-xl, and these results were later explained upon examination of the co-localization of the proteins *in situ*. Since we and other have shown that Bax localizes to the mitochondrial outer membrane (Antonsson *et al.* 2000; Carton *et al.* 2003; Gardia *et al.* 2004; Lalier *et al.* 2007; Nechustan *et al.* 1999; Steckley *et al.* 2007), it is likely that, as with Pumas ability to bind to Bcl-xl, the c-terminus of Puma is not required to bind Bax in cellular lysate but

is required to bind Bax in intact cells where cellular localization is of greater importance. Future studies could use fluorescent or bioluminescent resonance energy transfer (FRET or BRET) to better examine the ability of Puma to functionally interact with Bax, Bcl-xl, and other Bcl-2 family members *in situ.*

Chapter 5 – Summary and Conclusions

In this work we have identified two key regions of Puma: its BH3 domain and its c-terminal mitochondrial-localization region. We have found both of these regions to be necessary for interactions with other Bcl-2 family members and for full apoptotic function of the protein. We have also elucidated several factors that help to explain the efficacy of Puma as a pro-apoptotic protein: its ability to localize to the mitochondrial membrane, a key membrane in Bcl-2 family-mediated apoptosis; its ability to co-localize and interact with the important anti-apoptotic protein, Bcl-xl; and its ability to activate Bax through both the indirect and the direct models. The efficacy of Puma as a pro-apoptotic protein appears to rely on both an intact BH3 domain and c-terminal mitochondrial-localization region.

We believe that further investigation of these two regions on the Puma protein will yield even more information about the unique apoptotic efficacy of Puma, and that further understanding of this key protein will contribute to our understanding of Bcl-2 family regulated apoptosis. Since the intrinsic, Bcl-2 family regulated, apoptotic pathway has been shown to play an important role in many chronic and acute neuronal pathologies (Engel *et al.* 2011; Higgins *et al.* 2010; Kotipatruni *et al.* 2011; Nitatori *et al.* 1995; Portera-Cailliau *et al.* 1995; Steckley *et al.* 2007; Vila & Przedborski 2003), knowledge of critical regions on the Puma protein may also provide targets for the treatment of these conditions.

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Appendices

Table 1 - Restriction Enzyme Digest

Reagents	
Reagent	Volume (µl)
Enzyme 1	1.00
Enzyme 2	1.00
DNA	5.00
10x NEBuffer	5.00
Dnase- Rnase-free water	38.00
Total	50.00
Restriction Enzymes	
Plasmids	**Enzymes**
GFP-Puma (WT and ΔBH3)	Kpn I and Bam H1
GFP-Puma (1-160 and 151-193)	Eco RI and Sal I

Table 2 - Ligation

Reagents	
Reagent	Volume (µl)
Vector	1.0
Insert	5.0
Ligase Buffer	2.0
T4 DNA Ligase	1.0
Dnase- Rnase-free water	11.0
Total	20.0
Ligation Buffers	
Plasmids	**Buffer**
GFP-Puma (WT and ΔBH3)	NEBuffer 2 (New England Biolabs #B7002S)
GFP-Puma (1-160 and 151-193)	NEBuffer 3 (New England Biolabs # B7003S)

Table 3 – Polymerase Chain Reaction Cloning

Qiagen Reagents for PCR Cloning	
Reagent	Volume (µl)
Buffer	5.0
Mg	4.0
dNTP	4.0
Q	10.0
Forward primer	4.0
Reverse primer	4.0
Qiagen Taq	1.0
High Fidelity Phusion Taq	0.5
DNA	1.0
Dnase- Rnase-free water	17.5
Total	50.0

Cycling Conditions
1. 94°C for 4 minutes 2. 94°C for 15 seconds 3. 55°C for 15 seconds 4. 68°C for 30 seconds 5. Repeat #2-4 for 35 cycles 6. 72°C for 10 minutes 7. 10°C indefinitely

Primers		
Plasmid	**5'-sense-3'**	**3'-antisense-5'**
Puma (1-160)	GATCGAATTCTTGATGGCCC GCGCACGC	CTAGCTGCAGCTGCTCCTCT GGTCTCCG
Puma (151-193)	CATCGAATTCCAGTACGAGC GGCGGAGA	CTAGGTCGACCTAATTGGGC TCCATCTC

Table 4 – Summary of plasmid properties.

Plasmid	Localize at mitochondria	Apoptotic	IP with Bcl-xl	Co-localize with Bcl-xl	IP with Bax
GFP	-	-	-	-	-
GFP-Puma (WT)	+	+	+	+	+
GFP-Puma (ΔBH3)	+	-	-	+	-
GFP-Puma (1-160)	-	-	+	-	+
GFP-Puma (151-193)	+	-	-	+	-

Table 5 – Peptide sequences.

Peptide	Sequence
Bax-Hα1	Ac-YGRKKRRQRRRSEQIMKTGAFLLQGFIQDRA-NH2
Bax-D33A	Ac-YGRKKRRQRRRSEQIMKTGAFLLQGFIQARA-NH2

Curriculum Vitae

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