REGULATION OF APOPTOSIS IN POSTMITOTIC CELLS

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ABSTRACT

Michelle I. Smith: Regulation of Apoptosis in Postmitotic Cells
(Under the direction of Dr. Mohanish Deshmukh)

Apoptosis is a genetically regulated evolutionarily conserved form of cell death. During the intrinsic pathway of apoptosis, the Bcl-2 family of proteins regulates the release of cytochrome *c* from the mitochondria. Cytosolic cytochrome *c* is then free to bind to the adaptor protein Apaf-1. This causes Apaf-1 to oligomerize and form what is known as the apoptosome complex. This apoptosome then recruits and activates proteases called caspases which cleave numerous cellular substrates ultimately killing the cell.

Postmitotic neurons exhibit increased resistance to apoptosis. By increasing the regulation of caspase activation, they ensure they can survive long term to serve their vital function for the lifetime of the organism. This is particularly important since the death of postmitotic cells is associated with various pathophysiological conditions. For example, apoptosis causes the destruction of skeletal muscle in some neuromuscular diseases and endoplasmic reticulum (ER) stress triggered apoptosis is the underlying cause of several neurodegenerative diseases. Here I investigated the pathway and regulation of apoptosis in postmitotic cells.

The mechanism by which ER stress induces neuronal apoptosis remains controversial. Here I identified the pathway of apoptosis carried out in neurons triggered to die by ER stress. Importantly, my results show that ER stress commits neurons to die prior to cytochrome *c* release and that this commitment requires Bax activation and JNK signaling.

Furthermore, I identify the importance of the apoptosome as the non-redundant caspase activation pathway to execute neuronal apoptosis in response to ER stress.

I have also examined the regulation of apoptosis in skeletal muscle. My results show that as skeletal muscle differentiate; they become resistant to apoptosis because of the ability of XIAP to regulate caspase activation. This increased resistance is due to a decrease in Apaf-1 expression which directly couples to the increased effectiveness of endogenous XIAP to block caspase activation and death.

The increased restriction of apoptosis in myotubes is similar to that observed in neurons and cardiomyocytes and is presumably important to ensure the long term survival of these postmitotic cells as they play a vital role in the physiology of organisms.

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TABLE OF CONTENTS

LIST OF FIGURES ix

LIST OF ABBREVIATIONS xii

CHAPTER ONE: INTRODUCTION AND BACKGROUND 1

1.1 Overview of Apoptosis 2

The role of apoptosis in development, homeostasis and disease 2

Discovery of the essential apoptotic players 3

Intrinsic pathway: Bcl-2 family regulation of cytochrome c release 4

Intrinsic pathway: Apoptosome formation and caspase activation 6

Regulators of the apoptosome and caspase activation:
Proteins and modifications 9

Regulators of the apoptosome and caspase activation:
Focus on IAPs and their inhibitors 13

1.2 ER Stress Induced Apoptosis 18

Induction of the unfolded protein response 18

Characterization of the ER stress induced apoptotic pathway 20

Caspase 12 pathway of ER stress induced apoptosis 23

The role of ER stress induced apoptosis in disease..28

1.3 Skeletal Muscle Apoptosis 30

Skeletal muscle differentiation 30

Apoptosis in myoblasts: Apaf-1 independent caspase activation 31

The role of apoptosis in skeletal muscle and muscle disease 32

1.4 Figures and Legends...35

CHAPTER TWO: ER STRESS INDUCED APOPTOSIS REQUIRES BAX FOR COMMITMENT AND APAF-1 FOR EXECUTION IN PRIMARY NEURONS...49

2.1 Abstract 50

2.2 Introduction 51

2.3 Results 54

Tunicamycin induced ER stress results in caspase dependent apoptosis in neurons 54

Neurons activate JNK signaling which is required for apoptosis in response to ER stress 54

BH3-only genes DP5 and Puma are upregulated independently of MLK signaling during ER stress induced neuronal apoptosis 56

Bax deficiency inhibits ER stress induced apoptosis in neurons, allowing for long term survival 57

ER stress commits neurons to die early, prior to cytochrome c release, and requires Bax and JNK activation 57

Apaf-1 is required for neurons to undergo ER stress induced apoptosis 59

2.4 Discussion 60

2.5 Materials and Methods...66

2.6 Figures and Legends 72

CHAPTER THREE: SKELETAL MUSCLE DIFFERENTIATION INVOKES ENDOGENOUS XIAP TO RESTRICT THE APOPTOTIC PATHWAY 92

3.1 Abstract 93

3.2 Introduction 94

3.3 Results 97

Myotubes develop resistance to cytochrome c-induced apoptosis upon differentiation 97

Myotube resistance to cytochrome c can be overcome with the exogenous addition of the IAP inhibitor Smac or genetic deletion of XIAP 98

Endogenous XIAP effectively restricts cytochrome c induced death in myotubes due to reduced Apaf-1 levels 99

Endogenous Smac can overcome XIAP inhibition in myotubes 100

3.4 Discussion 102

3.5 Materials and Methods 106

3.6 Figures and Legends 110

CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS 120

4.1 Summary of Findings 121

4.2 Discussion of the Major Findings and Future Directions 123

ER stress induced apoptosis requires Bax for commitment and Apaf-1 for execution in sympathetic neurons 123

Decreased apoptosome function with skeletal muscle differentiation enables strict XIAP regulation of apoptosis 129

Myoblasts utilize a novel alternative mitochondria mediated pathway to activate caspases 132

APPENDIX A: Sympathetic neurons upregulate Apaf-1 in response to ER stress: Mechanisms to become competent to die 137

APPENDIX B: Bax deficiency provides long term survival against ER stress, which may involve autophagy 142

APPENDIX C: Transcriptional regulation of decrease Apaf-1 expression in postmitotic myotubes 146

APPENDIX D: XIAP function may be overcome by Apaf-1 translational upregulation in response to DNA damage in myotubes 152

APPENDIX E: Mitochondria mediated, Apaf-1 independent pathway of caspase activation in primary myoblasts 160

REFERENCES....................166

LIST OF FIGURES

Figure 1.1: Pathway of programmed cell death in *C. elegans* and its mammalian homologs 35

Figure 1.2: Regulation of cytochrome *c* release from the mitochondria by the Bcl-2 family of proteins 37

Figure 1.3: The intrinsic pathway of apoptosis 39

Figure 1.4: Model for reduced caspase activation in neurons due to the coupling of reduced Apaf-1 levels to the increased effectiveness of XIAP 41

Figure 1.5: Signaling of the unfolded protein response 43

Figure 1.6: Pathway of ER stress induced caspase 12 mediated apoptosis 45

Figure 1.7: Skeletal muscle differentiation 47

Figure 2.1: Tunicamycin causes ER stress induced apoptosis in sympathetic neurons 72

Figure 2.2: JNK signaling is required for ER stress induced neuronal apoptosis 74

Figure 2.3: BH3-only proteins Puma and DP5 are transcriptionally upregulated independently of MLK signaling following ER stress in sympathetic neurons 76

Figure 2.4: Bax deficiency blocks ER stress induced apoptosis allowing for long term survival of sympathetic neurons 78

Figure 2.5: ER stress induced commitment to death occurs well before cytochrome *c* release and is blocked by Bax deficiency and by inhibiting JNK signaling 80

Figure 2.6: Apaf-1 deficient sympathetic neurons are able to release cytochrome *c* but do not undergo ER stress induced apoptosis 82

Supp. Figure 2.1: Tunicamycin causes ER stress induced apoptosis in sympathetic neurons as measured by MTT reduction. Thapsigargin induces ER stress induced apoptosis in sympathetic neurons which is inhibited by zVAD and CEP-11004 84

Supp. Figure 2.2: Tunicamycin causes cell death of MEFs which is not

inhibitable by CEP-11004..86

Supp. Figure 2.3: P53 deficiency does not block sympathetic neuronal apoptosis or the upregulation of BH3-only proteins Puma and DP5 after ER stress 88

Supp. Figure 2.4: Bax deficiency blocks thapsigargin induced apoptosis of sympathetic neurons 90

Figure 3.1: Myotubes develop gradual resistance to cytosolic cytochrome *c* induced apoptosis 110

Figure 3.2: Resistance to cytosolic cytochrome *c* is mediated by endogenous IAPs 112

Figure 3.3: Endogenous XIAP mediates cytosolic cytochrome *c* mediated death in myotubes..114

Figure 3.4: Apaf-1 levels are decreased in myotubes. Restoring Apaf-1 is sufficient to allow cytochrome *c* mediated death 116

Figure 3.5: tBid causes caspase inhibitable death in myotubes that requires endogenous Smac..118

App. Figure A1: ER stress induced sympathetic neurons maintain XIAP levels and transcriptionally upregulate Apaf-1..140

App. Figure B1: Prolonged ER stress causes changes in the ultrastructural features of Bax deficient sympathetic neurons..144

App. Figure C1: dC2C12 cell DNA is more methylated than mC2C12 cell DNA..148

App. Figure C2: HDAC inhibitors induce cytochrome *c* sensitivity in dC2C12 cells..150

App. Figure D1: Dose response of etoposide induced death in dC2C12 cells..154

App. Figure D2: Etoposide treatment permits cytochrome *c* to kill dC2C12 cells..156

App. Figure D3: XIAP levels remain constant but Apaf-1 protein levels increase with etoposide treatment of dC2C12 cells..158

App. Figure E1: Primary myoblasts are resistant to cytochrome *c*

and Smac induced death..162

App. Figure E2: Wildtype and Apaf-1 deficient primary myoblasts
are sensitive to tBid induced death 164

LIST OF ABBREVIATIONS

AB	amyloid beta
ALG-2	apoptosis-linked gene 2
Apaf-1	apoptotic protease-activating factor-1
ASK1	apoptosis signal-regulating kinase
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
CHOP	C/EBP homologous protein
dATP	deoxyadenosine triphosphate
dC2C12	differentiated C2C12 cells
DKO	double knockout
DOC	downstream of CHOP
eIF2α	eukaryotic translation initiation factor-1 alpha
ERAD	ER-associated degradation
ESR	ER stress response
IAP	inhibitors of apoptotic proteins
IMS	inter-membrane space
IP3R	inositol triphosphate receptor
IRE1	inositol-requiring enzyme 1
JIK	JNK inhibitory kinase
JNK	c-Jun N-terminal kinase
mC2C12	mitotic C2C12 cells

MEF	mouse embryonic fibroblast
MLK	mixed lineage kinase
MOM	mitochondrial outer membrane
NGF	nerve growth factor
PERK	PKR-like ER kinase
S1P	site-1 protease
S2P	site-2 protease
SMAC	second mitochondrial activator of caspases
TG	thapsigargin
TRAF1	TNF receptor-associated factor 1
TRAF2	TNF receptor-associated factor 2
TU	tunicamycin
UPR	unfolded protein response
UPS	ubiquitin proteasome system
VCP	valsoin-containing protein
XBP1	X-box-Binding Protein 1
XIAP	X-linked inhibitor of apoptotic proteins

CHAPTER ONE

INTRODUCTION AND BACKGROUND

1.1 Overview of Apoptosis

The role of apoptosis in development, homeostasis and disease

While there are many different types of cell death that a cell can undergo, apoptosis is a morphologically distinct and genetically regulated form of death that can be distinguished from all other forms. Due to its elegant genetic regulation, apoptosis has also been named programmed cell death and cell suicide. Apoptosis occurs in response to a variety of physiological or pathological stimuli.

The role of physiological apoptosis is most prominently seen during the development of an organism. One the best and clearest example of developmental apoptosis occurs during the development of nematode *Caenorhabditis elegans*. During development, exactly 131 of the original 1090 cells dies, leaving the organism with 959 cells (Danial and Korsmeyer, 2004). During the embryonic development of mammals, extensive apoptosis is responsible for organogenesis and the shaping of multicellular tissues. For example, predictions suggest that as many as 80% of neurons initially produced undergo apoptosis during development (Davies, 2003).

In addition to these roles in development, apoptosis is required for proper tissue homeostasis and plays a role in many pathological conditions. With respect to pathologies, both diminished apoptotic capacity and excessive apoptosis are to blame. The inability of cells to carry out apoptosis has been linked to the development of tumors and tumor resistance to chemotherapy. This diminished capacity may also be responsible for autoimmune syndromes. On the other hand, accelerated cell death has been linked to immunodeficiency, infertility and most notable degenerative diseases, especially those involving neurodegeneration (Danial and Korsmeyer, 2004; Lindholm et al., 2006).

Discovery of the essential apoptotic players

The original identification of the genetically regulated apoptotic pathway came from studies carried out in *C. elegans*. These ideas and elegant studies lead directly to Drenner, Horvitz and Sulston receiving the 2002 Nobel Prize. The use of *C. elegans* for delineating this pathway is particularly ideal since the nematode is a genetically tractable organism and the fate of all 1090 cells is well defined. Of the 1090 cells, the same 131 cells die during development reproducible between nematode to nematode. *C. elegans* with deficiency of either the *ced-3* or *ced-4* gene maintained their original 1090 cells (Ellis and Horvitz, 1986; Yuan and Horvitz, 1990). Further work delineated the pathway of this programmed cell death. In cells destined to die during development, there is transcriptional upregulation of *egl-1*. This results in the production of Egl-1 protein which directly binds to Ced-9. Under non-death promoting situations Ced-9 binds to Ced-4 and holds in inactive. The binding of Egl-1 to Ced-9 disrupts the Ced-9/Ced-4 interaction releasing the adaptor protein Ced-4. Free Ced-4 then interacts with and activates Ced-3, which is responsible for carrying out cell death (Fig. 1.1) (Danial and Korsmeyer, 2004; Vaux et al., 1994; Yuan, 2006). The identification of mammalian homologs for these proteins lead to the realization that the type of death experienced during *C. elegans* development is an evolutionary conserved cell death pathway.

ced-3 was identified to encode a protein homologous to caspase-1 (originally named interleukin-1β converting enzyme or ICE). Overexpression of either ced-3 or ICE in mammalian cells induced death (Yuan et al., 1993). In addition, Ced-9 protein proved to be the homolog of Bcl-2 (Hengartner and Horvitz, 1994), a mammalian oncogene already

shown at the time to have an inhibitory cell death function. The Ced-4 homolog, Apaf-1 was identified by the utilizing fractionation of mammalian cell extracts to reconstitute the activation of caspase 3 (Zou et al., 1997). These ground breaking experiments lead to the identification of three mammalian proteins required for cell death; Apaf-1, cytochrome *c* and caspase-9 (Liu et al., 1996; Zou et al., 1997). The identification of cytochrome *c* as a death inducing protein was particularly surprising as cytochrome *c* had only been associated with cellular energy production and is not required for cell death in *C. elegans*. Cytochrome *c* turned out to be required for caspase 9 to bind Apaf-1 resulting in the formation of a multimeric complex known as the apoptosome (discussed below) (Fig. 1.1).

Since the initial discoveries in *C. elegans* the field has identified that mammals have multiple caspases, multiple Bcl-2 family members and multiple Ced-4-like adaptor proteins. Their function and regulation will be the subject of the next few sections of this introduction.

Intrinsic pathway: Bcl-2 family regulation of cytochrome c release

The intrinsic pathway, whose key components include Bcl-2 proteins, cytochrome *c*, Apaf-1, caspase-9 and caspase-3, is the primary pathway of apoptosis. The intrinsic pathway can become activated by various stimuli such as growth factor withdrawal, DNA damage or lack of metabolic support. Regardless of the original stressor, ultimately signals impinge upon the mitochondria via Bcl-2 family members to cause cytochrome *c* release (Danial and Korsmeyer, 2004). The Bcl-2 family of proteins is the sensors which control cytochrome *c* release from the mitochondria and therefore they are the key regulators of apoptosis.

Bcl-2 proteins can be subdivided into three groups. The first contains the anti-apoptotic family members Bcl-2 and Bcl-xL, Bcl-w and Mcl-1. They all contain four

conserved, short Bcl-2 homology (BH) domains, BH1-BH4. Most of these family members are associated with the cytosolic surface of the mitochondrial outer membrane (MOM) and the endoplasmic reticulum (ER). Their mode of action is thought to involve sequestering pro-apoptotic Bcl-2 family members (Kaufmann and Hengartner, 2001; Willis and Adams, 2005).

The second group of Bcl-2 proteins contains only BH1-BH3 domains. This group is highly pro-apoptotic and includes Bax and Bak, the proteins directly responsible for releasing cytochrome *c*. Upon activation, Bax and Bak, which are cytosolic and loosely associated with membranes, undergo a conformational change and insertion into the MOM. This results in permeabilization of the MOM and release of cytochrome *c* and other proteins, from the inner membrane space (Kaufmann and Hengartner, 2001; Willis and Adams, 2005) .

The third group of family members is know as the BH3-only members, as they lack all but a single BH3 domain. This is the largest group comprised of Bid, Bad, Bim, Blk, Bmf, DP5/Hrk, Nix, Noxa and Puma amongst others. This group of proteins respond to a wide variety of apoptotic stimuli and can be activated by transcriptional upregulation, phosphorylation, cleavage and N-myristoylation (Kaufmann and Hengartner, 2001; Willis and Adams, 2005; Yuan, 2006). The BH3-only members are activated during apoptosis in a stress specific manner. For example, in sympathetic neurons, DNA damage causes the activation of both PUMA and Noxa but not Bim, whereas, nerve growth factor (NGF) withdrawal induces Bim and DP5 (Besirli et al., 2005; Eilers et al., 1998; Harris and Johnson, 2001).

How exactly these three groups of proteins interact is somewhat controversial. The main school of thought is that the antiapoptotic members (Bcl-2, Bcl-xL) bind directly to Bax

and Bak, sequestering them away form each other and membranes, preventing their activity. Upon a death stimulus, one or more BH3-only members become activated. Once activated, the BH3-only proteins can use two potential modes of action to activate Bax and Bak. First, they can bind the antiapoptotic Bcl-2 members, releasing their hold on Bax and Bak thus allowing active Bax and Bak to translocate to the mitochondria, oligomerize and release cytochrome *c*. Most BH3-only proteins appear to work in this fashion. Alternatively, some BH3-only proteins bind directly to Bax and Bak causing their activation. Bid, Bim and Puma are the most potent BH3-only proteins and appear to work by both modes (Willis and Adams, 2005) (Fig. 1.2).

Intrinsic pathway: Apoptosome formation and caspase activation

There are caspases responsible for cytokine maturation and others responsible for apoptosis. Caspases are a family of conserved cystine proteases that cleave their substrates on the carboxyl side of aspartate residues. The activation of caspases is the key signature of apoptosis. Caspases involved in apoptosis can be divided into two groups. The "executioner caspases" (caspases 3, 6 and 7) are responsible for directly cleaving a great number of diverse cellular substrates, ultimately leading to the demise of the cell. Executioner caspases become activated upon cleavage by active "initiator caspases". This group includes caspases 2, 8 and 9 and their activation is much more complex than cleavage alone (Bao and Shi, 2007; Shi, 2002). While initiator caspases do contain autocatalytic cleavage activity, cleavage may neither be necessary nor sufficient for activation. In fact, fully processed caspase 9 was found to have the same low level of catalytic activity as the unprocessed zymogen form of caspase 9 (Stennicke et al., 1999). Instead, these proteases become

activated on adaptor complexes. Caspase 2 gets activated on the PIDDosome, caspase 8 on the death-inducing signaling complex (DISC) and caspase 9 on the apoptosome (Bao and Shi, 2007). The formation of the apoptosome complex is critical to caspase 9 catalytic activity, as apoptosome bound caspase 9 shows activity three orders of magnitude higher than isolated caspase 9 (Rodriguez and Lazebnik, 1999).

Following the activation of the intrinsic pathway by cellular stressors, released cytochrome *c* binds and activates Apaf-1, the central component of the apoptosome. Apaf-1 contains three functional domains. The N-terminus contains the caspase activation and recruitment domain (CARD) which is responsible for binding caspase 9. The central region of Apaf-1 contains the nucleotide binding domain (NBD) and the oligomerization domain. The C-terminus contains 12-13 WD-40 repeats which bind to cytochrome *c*. In the absence of cytochrome *c*, Apaf-1 folds over on itself such that the CARD domain is auto-inhibited by intramolecular contact with two of its WD-40 repeats. This auto-inhibitor activity is confirmed by the finding that truncation of the WD-40 repeats of Apaf-1 results in constitutive binding and activation of caspase 9 even in absence of cytochrome *c* (Li et al., 1997) .

When cytochrome *c* binds the WD-40 domains of Apaf-1, the CARD domain is dislodged. Binding also causes monomeric Apaf-1 to hydrolyze dATP to dADP followed by the exchange of dADP for dATP. It is thought that this hydrolysis and exchanges produces a conformational change in Apaf-1 which allows it to oligomerize with other Apaf-1 monomers (Riedl et al., 2005). The result is the formation of a wheel shaped ~1.4 MDa complex heptamer, whose structure was determined by electron cyro-microscopy (Acehan et al., 2002; Yu et al., 2005). At the spokes of the wheel are the WD-40 repeats, while the

CARD and NBD make up the central hub. The now exposed CARD domain on each of the Apaf-1 molecules recruits and binds procaspase 9 via the CARD domain found in the prodomain of procaspase 9. Once on the apoptosome, procaspase 9 becomes activated and autocleaved (Bao and Shi, 2007).

The exact mechanism by which caspase 9 becomes activated remains elusive and controversial. However, based on structural and biochemical studies, several models have been suggested over the years. Original biochemistry approaches performed by several labs revealed that caspase 9 became cleaved upon induced oligomerization (MacCorkle et al., 1998; Muzio et al., 1998; Srinivasula et al., 1998; Yang et al., 1998a; Yang et al., 1998b). This led to the hypothesis that initiator caspases autoprocess themselves when they are in close proximity to each other, such as on the apoptosome. Gel filtration experiments furthered this "Induced Proximity Model" to include dimerization of caspase 9. Gel filtration experiments showed monomers of caspase 9 were as inactive as both the unprocessed zymogen and the full processed form. The active fraction contained the dimers (Renatus et al., 2001). As a result the "Proximity-driven dimerization model" was developed and suggests that the apoptosome recruits multiple molecules of procaspase 9 into close proximity of each other. This high local concentration of procaspase 9 favors dimerization and therefore activation. However, dimerization could not explain the full activation of caspase 9 as constitutive homodimers have much lower levels of catalytic activity than apoptosome associated caspase 9 (Chao et al., 2005). This led to the formation of the "Induced conformation model". This model still takes into account the two previous models but states that a conformational change must occur in the active site of apoptosome bound caspase 9. To date, there are several suggested ways in which this conformational change

could come about but the exact underlined mechanism remains elusive and necessitates continued study (Bao and Shi, 2007).

Regulators of the apoptosome and caspases activation: Proteins and modifications

Due to the importance of the apoptosome and subsequent caspase activation, it is not surprising that the cell has evolved several mechanisms to regulate their activity. The apoptosome and its components can be both negatively and positively regulated by post-translational modification, transcriptional control, binding proteins, small molecules, ions, nucleotides and amino acids, amongst others. In some pathological situations such as cancers, regulators have been usurped or additional forms of regulation have been developed to block apoptosis which serves to enhance the pathology.

The most recognizable group of caspase inhibitors are the inhibitor of apoptosis (IAP) family of proteins (discussed below). Other protein inhibitors include the heat shock proteins, specifically Hsp27, Hsp70 and Hsp90. Hsp27 binds to cytochrome *c* and prevents its interaction with Apaf-1, preventing apoptosome formation all together (Bruey et al., 2000). Whereas, Hsp70 and Hsp90 bind directly to Apaf-1 and prevent its interaction with procaspase 9 (Beere et al., 2000; Pandey et al., 2000; Saleh et al., 2000). This mechanism is also used by the inhibitor APIP to prevent caspase activation (Cho et al., 2004). On the other hand, Aven binds Apaf-1 but prevents oligomerization and thus blocks apoptosome formation (Chau et al., 2000). Another protein inhibitor TUCAN works by utilizing its own CARD domain to bind to caspase 9 and prevent caspase activation (Pathan et al., 2001). There also exists a brain specific splice variant of caspase 9 named AIP. AIP is a dominant

negative and binds directly to Apaf-1, preventing full length procaspase 9 from binding and becoming activated (Cao et al., 2004).

In opposition to the inhibitors, several protein regulators act directly on the apoptosome components to increase caspase activation. Hepatocellular carcinoma antigen 66 (HCA66), binds directly to Apaf-1 causing increased caspase 9 recruitment to the apoptosome. Interestingly, decreased expression of HCA66 has been linked to cancer in people with neurofibromatosis type 1 (Piddubnyak et al., 2007). Like TUCAN, NAC also contains a CARD domain. In this case, NAC uses its CARD domain to interact with the CARD domain of Apaf-1 and promotes the apoptosome mediated activation of caspases (Chu et al., 2001). The protein Nucling uses a different mechanism than most of the other protein regulators. While it binds to Apaf-1/caspase-9 complexes, it causes the complex to translocate to the nucleus where it appears to be even more effective at activating caspase 9 (Sakai et al., 2004).

In addition to these direct protein regulators, two of the most recognizable indirect regulators include putative HLA-DR-associated protein (PHAP1) and porthymosin-α (ProT). PHAP1 enhances caspase 9 and caspase 3 activation on the apoptosome but how it carries out this activity is unknown (Jiang et al., 2003). The activity of PHAP1 has been exploited in some breast cancers. Breast cancer cell lines that show PHAP1 overexpression also show increased sensitivity to cytochrome *c* induced caspase activation (Schafer et al., 2006). ProT is an oncoprotein which negatively regulates caspase activation, by inhibiting the assembly of the apoptosome (Jiang et al., 2003).

Paradoxically, nucleotides are required for apoptosome formation, however, physiological concentration of nucleotides are actually inhibitory to apoptosome formation.

It has been proposed that this inhibition is the result of direct binding of nucleotides to cytochrome *c* (Chandra et al., 2006).

In addition to regulation by protein and small molecules, apoptosome activity is dramatically affected by posttranslational modifications of cytochrome *c*, Apaf-1 and caspase 9. Cytochrome *c* is synthesized in an Apo form with no heme group attached. While this Apo form is capable of binding to Apaf-1 it is ineffective at inducing Apaf-1 oligomerization and therefore does not activate caspases (Martin and Fearnhead, 2002; Martin et al., 2004). In order for cytochrome *c* to be active it must exist as a holoenzyme with the heme group attached. The heme gets attached to the synthesized cytochrome c in the mitochondria by heme lyase. In this respect, cytochrome *c* activity is regulated since the addition of the heme group does not occur until cytochrome *c* is in the mitochondria, as such newly synthesized cytochrome c in the cytosol has no death inducing activity (Martin and Fearnhead, 2002; Martin et al., 2004). In addition, the heme attached form of cytochrome *c* can be modified to make it more or less effective at inducing death. The heme group becomes nitrosylated in the mitochondria and inhibiting this event decreases its ability to activate caspases (Schonhoff et al., 2003). In addition to nitrosylation, cytochrome *c* is also subjected to reduction and oxidation. Reduced cytochrome *c* is inefficient at activating caspases (Schonhoff et al., 2003; Suto et al., 2005).

Caspase 9 is the subject of phosphorylation by several kinase pathways. All of these modifications are antiapoptotic and work by blocking the apoptosome mediated activation of caspases. The survival kinases, Akt and ERK, are thought to phosphorylate caspase 9 directly (Allan et al., 2003; Cardone et al., 1998; Tashker et al., 2002). However, the relevance of Akt phosphorylation is not entirely clear and the Akt phosphorylation sites are

not conserved in a number of mammals (Rodriguez et al., 2000). Caspase 9 is phosphorylated directly by protein kinase A (PKA) and the zeta form of atypical protein kinase C (PKCzeta) (Brady et al., 2005; Martin et al., 2005). Studies indicate it is not the direct PKA phosphorylation sites but rather the activity of an alternative PKA substrate that is responsible for preventing apoptosome formation (Martin et al., 2005).

Apaf-1 is a major target of regulation, especially in pathological conditions such as cancers. In chronic myelogenous leukemia (CML), the activity of constitutively active tyrosine kinase Bcr-Abl abolishes the ability of Apaf-1 to interact with caspase 9, preventing apoptosome formation and caspase activation (Deming et al., 2004).

Other cancers have prevented Apaf-1 from carrying out its caspase activating function by sequestering it away from the cytosol. In cell lines derived from Burkitt lymphomas, Apaf-1 is compartmentalized to the plasma membrane, potentially sequestered away by lipid rafts, leaving insufficient cytosolic levels to activate cytochrome *c* mediated apoptosis (Sun et al., 2005).

In addition, Apaf-1 is subjected to regulation at the transcriptional level by cancers and during physiological development. Both metastatic malignant melanomas and some leukemia's block apoptosis by using epigenetic silencing to completely shut down Apaf-1 expression. Methylation of the Apaf-1 promoter region is responsible for this silencing. As a result, treatment with DNA methylation inhibitors is sufficient to allow the reexpression of Apaf-1 and restore sensitivity to chemotherapeutics (Fu et al., 2003; Furukawa et al., 2005; Soengas et al., 2001).

Apaf-1 levels are also subject to transcriptional downregulation during differentiation and maturation of postmitotic cells (Chapter three) (Burgess et al., 1999; Potts et al., 2005;

Sanchis et al., 2003; Wright et al., 2004; Wright et al., 2007). This decrease makes postmitotic neurons, cardiomyocytes and myotubes, more refractory to cytochrome *c* induced caspase activation. The cause of the Apaf-1 downregulation seen in mature sympathetic neurons has been examined. When compared to younger developing neurons, the promoter region of Apaf-1 in mature neurons exhibits increased trimethylation of lysine 9 on histone 3 and decreased acetlyation of histone 3 (Wright et al., 2007). These modifications are associated with chromatin condensation and decreased transcription (Fischle et al., 2003).

Regulators of the apoptosome and caspases activation: Focus on IAPs and their inhibitors

Once a virus has infected a cell, it must interfere with the host apoptotic response to keep the host cell alive long enough to allow viral replication. Therefore, it was not surprising that the identification of IAPs came from studying viruses. The first IAP discovered, OpIAP, was discovered as a baculoviral gene product. It was capable of protecting insect cells from death and it enhanced viral replication (Kaiser et al., 1998). Since then many family members have been uncovered based on the presence of one to three baculoviral IAP repeats (BIR) domains. These BIR domains chelate zinc to form finger-like structures that bind to the surfaces of caspases, which allows the sequences between BIRs to block the catalytic grooves of caspases (Callus and Vaux, 2007).

Originally it was thought that several members of the IAP family had the ability to block caspase activity directly. However, most of this data came from *in vitro* studies using overexpressed proteins. It is now clear that most IAPs do not play a role in direct caspase inhibition but instead, carryout apoptosis independent functions. For example, Survivin is an IAP with one BIR domain. It is primarily expressed during fetal development and plays a

critical role during mitosis. At the beginning of mitosis, Survivin is affiliated with microtubules of the mitotic spindle and binds to proteins responsible for chromosomal segregation. Inhibiting Survivin results in failed cytokinesis (Lens et al., 2006). Another IAP family member, NAIP is involved in providing innate immunity following bacterial infection (Ting et al., 2008). Based on functional and structural data, XIAP appears to be the only direct caspase inhibitor in mammalian cells.

XIAP contains three N-terminal BIR domains and a C-terminal RING finger domain. The RING finger is an ubiquitin ligase which confers E3 activity on XIAP. This allows XIAP to autoubiquitinate and degrade itself and possibly its target substrates, caspases (Creagh et al., 2004; Morizane et al., 2005; Suzuki et al., 2001). However, the primary caspase inhibitory ability of XIAP is not through ubiquitination and degradation of caspases but by occlusion of the active site of caspases. Structural data has identified that XIAP uses a two site binding mechanism to inhibit caspase 9 and caspase 3 or 7. The first site is a conserved surface found in BIR2 and BIR3 of XIAP. This site binds proteins through an N-terminal exposed IAP-binding motif (IBM). An IBM is an interacting grove which can be found on the N-terminus of the small subunit of cleaved caspases as well as the IAP inhibitor protein, Smac. This anchoring occurs between BIR3 and caspase 9 and between BIR2 and caspase 3 or 7 (Eckelman et al., 2006).

The second site of interaction is responsible for directly binding and inhibiting the action of the caspase active site. In XIAP, this site is found in the flanking region of the BIR domain. The region directly following BIR3 is responsible for inhibiting caspase 9. This region forms a helix that packs against the dimer interface of caspase 9 causing caspase 9 to become monomerized and thus inactive (Shiozaki et al., 2003). Within this helix His343 is

critical for this inhibitory activity (Sun et al., 2000). In the case of the caspase 3 or 7 complex with BIR2, the peptide sequence directly preceding BIR2 (amino acids 140-156) stretches across and occupies the active site of caspase 3 or 7. Residues Leu141 and Asp148 are particularly important for this tight inhibition (Eckelman et al., 2006).

All of the structural data generated thus far has led to the understanding that, in order for an IAP to be able to block caspase activation, it must not only have an IBM-interacting groove but also a secondary inhibitory element. Based on these requirements, it is predicted that none of the other IAPs are caspase inhibitors. The two IAPs most structurally similar to XIAP are cIAP1 and cIAP2. These IAPs also contain three BIR domains and a RING finger and are able to bind caspases. However, both cIAPs lack the proper sequences flanking the BIR domains to produce caspase inhibition (Eckelman and Salvesen, 2006). Both are incapable of inhibiting caspases at physiological levels, but can block death to some degree when overexpressed. This is likely through indirect means such as binding IAP inhibitors to free up XIAP, targeting caspases for degradation through the ubiquitin proteasome system (UPS) or influencing NF-kB signaling (Callus and Vaux, 2007).

The importance of XIAP is most clearly seen in neurons. Unlike in mitotic cells, the microinjection of cytochrome *c* to intact neurons or its addition to neuronal cytosolic lysates does not induce caspase activation. This occurs because the levels of Apaf-1 are reduced resulting in less caspase activation, thus allowing endogenous XIAP to efficiently block caspase activation (Fig. 1.4) (Wright et al., 2004). When XIAP function is removed, by genetic deletion or inhibition, caspase activation occurs rapidly with the introduction of cytochrome *c*. Neurons undergoing developmental death in response to nerve growth factor

(NGF) withdrawal, actively and selectively degrade XIAP (become competent to die) before releasing cytochrome *c* (Potts et al., 2003).

There are two identified inhibitors of the IAP family of proteins, Smac/DIABLO and OMI/HtrA2. These are both IMS proteins that can be released from the mitochondria during apoptosis. Structural data has cast doubts on the ability of HtrA2 to inhibit IAPs (Li et al., 2002b). On the other hand, Smac was discovered based on its ability to facilitate caspase activation in cell free extracts, and is a very effective IAP inhibitor. As a dimer, Smac binds BIR2 of XIAP and prevents caspase 3 interaction, thereby allowing caspase 3 activation. As a monomer, Smac uses the four residues of its N-terminus (AVPI) to bind to the interacting groove of BIR3 and displaces caspase 9 from XIAP (Chai et al., 2000). It is of note that the AVPI peptide alone is sufficient to bind and inhibit XIAP *in vitro* (Chai et al., 2000). This finding led to the production of Smac mimetics and peptides for the treatment of cancer. These mimetics have been found to be useful in the treatment of some cancers in which IAPs are overexpressed. For example, XIAP is overexpressed in non-small cell lung carcinoma leaving the cells chemoresistant. The use of Smac peptides along with chemotherapeutics triggers apoptosome mediated apoptosis and induces tumor regression in mice with non-small cell lung carcinomas (Yang et al., 2003).

The structure and function of Smac has been well studied *in vitro* and in cancer models. However, the role of endogenous Smac is less understood since most cells do not need to inhibit endogenous XIAP or other IAP family members to undergo cytochrome *c* mediated apoptosis. The only cell type so far in which endogenous Smac is thought to play a role is myoblasts (Ho et al., 2007) (see below).

Over the last decade or so, our understanding of the core pathway of apoptosis had proceeded rapidly. We have gone from the identification of the pathway in *C. elegans* to the discovery of its human homologues and the development of cancer therapeutics. The next challenge will be to uncover how the core pathway is controlled in response to different stimuli and in different cell types.

1.2 ER Stress Induced Apoptosis

Induction of the unfolded protein response

The endoplasmic reticulum (ER) is site for the synthesis, folding and post-translational modification of proteins intended for endo/exocytotic pathway. The protein concentration in the ER at any given time is extremely high, approximately 100 mg/ml (Wu and Kaufman, 2006). As such, the ER is vital for various aspects of cell biology and physiology and its proper function must be maintained. The cell has developed a sophisticated and evolutionarily conserved mechanism for dealing with stress within the ER lumen. This is known as the ER stress response (ESR). The ESR becomes activated when the normal ER function is interrupted by disturbances such as accumulation of unfolded proteins, changes in the calcium homeostasis or redox changes in the ER lumen (Boyce and Yuan, 2006; Breckenridge et al., 2003; Wu and Kaufman, 2006).

There are two main branches to the ESR, ER-associated degradation (ERAD) and the unfolded protein response (UPR). ERAD is activated in order to remove and destroy the irreparably misfolded proteins within the ER lumen. During ERAD, misfolded proteins are shipped out of the ER lumen the same way they were translated in, through Sec61. They are then substrates for the UPS, becoming ubiquitinated and subsequently degraded by proteasomes which are closely associated with the ER membrane (Wu and Kaufman, 2006).

The main arm of the stress response, the UPR, serves to decrease general protein synthesis and to specifically increase chaperone proteins and other stress genes important to refolding the proteins already present in the ER lumen. There are three main protein sensors associated with the ER lumen which activate the UPR. These sensors, PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) are

all transmembrane proteins which are regulated by the ER lumen chaperone protein BiP/Grp78 (Wu and Kaufman, 2006).

BiP is a member of the heat shock protein (Hsp70) family and is one of the most highly represented ER chaperone proteins. In circumstances of normal ER function, BiP is found bound to the luminal domain of the three ER sensors, holding them in an inactive state. However, as unfolded or misfolded proteins build up within the ER lumen, BiP becomes sequestered away from the sensors to fold proteins in the lumen. This loss of BiP binding to the sensors permits their activation. Both PERK and IRE1 become activated by homooligomerization and transautophosphorylation (Wu and Kaufman, 2006).

Once activated, PERK phosphorylates the eukaryotic translation initiation factor-1 alpha (eIF2α), causing a block in translation initiation and global protein synthesis. It also activates the translation of specific mRNAs that are eIF2α phosphorylation dependent ER stress genes such as the transcription factor ATF 4. This serves to prevent new proteins from being translated into the ER lumen, giving the cell an opportunity and the tools necessary to deal with the already disruptive load of protein present (Harding et al., 2000).

Once IRE1 becomes autophosphorylated, it initiates its site-specific endoribonuclease (RNase) activity. The target of this RNase activity is a 26-base intron from X-box-binding protein 1 (XBP1). The intron removal causes a frameshift in the XBP1 mRNA resulting in a functional transactivation domain and a potent transcription factor. XBP1 protein induces the expression of itself and other ER stress-specific genes (Calfon et al., 2002).

ATF6 becomes activated in a different fashion. When BiP is titrated away, the Golgi localization signal sequence of ATF6 becomes exposed, allowing ATF6 to traffic to the cis-Golgi compartment. Once in the Golgi, it is cleaved by site-1 protease (S1P) and site-2

protease (S2P). The processed cytosolic N-terminal fragment of ATF6 translocates to the nucleus where, along with ATF4 and XBP1, it serves as a transcription factor to upregulate genes involved in protein refolding including chaperones and protein disulfide isomerases (Haze et al., 1999).

Taken together, in the face of ER stress the ESR serves to decrease protein synthesis, refold misfolded ER luminal proteins and degrade proteins that are beyond repair. This is all done in attempt to return to homeostasis within the ER lumen. Therefore, the ESR is a protective response whose initial actions promote cell survival. However, it is well reported that under conditions of prolonged stress these same responses induce programmed cell death.

Characterization of the ER stress induced apoptotic pathway

While the goal of the initial ESR is cytoprotective, prolonged ER stress results in apoptosis. The link between the ER and the apoptotic pathway remains one of the most complicated and important questions in this field. Studies have identified at least four possible links. It is possible that one or more of these potential pathways are necessary for activation of the apoptotic pathway and there could be cross talk between them. The first involves the activation of the proapoptotic Bcl-2 family members, Bax and Bak, localized to the ER. The second is the activation of an IRE1/TRAF/ASK pathway. Third is the upregulation of CHOP, an UPR target gene, which has proapoptotic activity. Finally, the activation of the ER localized caspase 12, which is the most extensively studied of all of the potential links.

The identification of the presence of the proapoptotic family members Bax and Bak at the ER (Nutt et al., 2002a; Nutt et al., 2002b; Oakes et al., 2003; Scorrano et al., 2003; Zong et al., 2003), has raised many interesting questions about the activation of apoptosis at the ER as well as potential crosstalk between the ER and mitochondria. The localization of Bax and Bak to the ER has a profound effect on ER Ca2+ stores and release. Bax/Bak double knockout (DKO) MEFs contain decreased levels of luminal Ca2+, the same effect is achieved when antiapoptotic Bcl-2 is overexpressed (Oakes et al., 2003; Scorrano et al., 2003; Zong et al., 2003). This decrease in ER Ca2+ stores is mediated by the ER Ca2+ receptor inositol trisphosphate receptor (IP3R). Bcl-2 physically interacts with IP3R leading to increased Ca2+ leak and increased permeability of the receptor (Oakes et al., 2003). Therefore the presence of Bax and Bak serve to maintain ER Ca2+ homeostasis by competitively binding to Bcl-2. A decrease in ER Ca2+ stores is cytoprotective because Ca2+ release from the ER, seen with multiple stressors, can result in the uptake of Ca2+ by the mitochondria leading to cytochrome *c* release (Nutt et al., 2002a). This suggests, there may exist multiple ways to initiate cytochrome *c* release, one being mitochondrial Ca2+ uptake and another being the canonical BH3-only initiated Bax/Bak pore formation at the mitochondria. In addition, one study found that ER targeted Bak induced the cleavage of ER localized caspase 12, likely due to the activation of calpains (see below) (Zong et al., 2003). Therefore, the Ca2+ regulating actions of Bax and Bak may play a role in activating caspases at the ER, prior to and independent from any caspase activation downstream of the mitochondria.

In addition to the endonuclease activity of IRE1, it also has signal transduction activities, which aid in the induction of ER stress induced apoptosis. ER stress causes the

activation of JNK signaling (Urano et al., 2000a), which is known to be lethal in primary neurons, due to its ability to cause the upregulation and activation of antiapoptotic BH3 only proteins (Ham et al., 2005). Upon ER stress, IRE1 becomes activated and recruits TNF receptor-associated factor 2 (TRAF2) to its cytosol domain (Urano et al., 2000b). In turn, TRAF2 directly interacts with apoptosis signal-regulating kinase (ASK1) resulting in the activation of a cascade that ultimately activates JNK (Nishitoh et al., 2002; Urano et al., 2000b). Importantly, the deletion of IRE1, ASK1 or the use of a dominant negative TRAF2, all result in decreased JNK signaling and decreased cell death in response to ER stress (Nishitoh et al., 2002; Urano et al., 2000b), suggesting that this pathway plays an important role in mediating ER stress induced apoptosis.

The transcription factor CHOP/GADD153 is a member of the CCAAT/enhancer binding proteins (C/EBPs) that was originally identified in a screen looking for genes induced by genotoxic stress and growth arrest (Luethy et al., 1990). However, CHOP expression is much more responsive to ER stress than DNA damage. CHOP is normally ubiquitously expressed at low levels in the cytosol but under conditions of ER stress it becomes highly upregulated and translocates to the nucleus. While the maximal induction of CHOP expression occurs when all three ER stress sensing signaling pathways are intact, the PERK/eIF2α is essential. The role for CHOP in mediating ER stress induced apoptosis comes from the finding that CHOP overexpression causes cell cycle arrest and/or apoptosis. Likewise, CHOP null MEFs have increased resistance to ER stress induced apoptosis. However, CHOP null mice show normal development, suggesting that some functional redundancy for the function of CHOP exists (Oyadomari and Mori, 2004). There are several potential ways in which CHOP may induce apoptosis. Two target genes of CHOP which

may participate include downstream of CHOP (DOC) 1 and DOC6. DOC1 is a stress inducible form of carbonic anhydrase VI, which serves to decrease the intracellular pH. DOC6 is a homologue of the actin binding proteins villin and gelsolin, which are thought to be responsible for the change in the actin cytoskeleton during apoptosis. In addition, CHOP disrupts the redox state of the cell by depleting cellular glutathione, leading to an increase in ROS. CHOP also affects several of the BCL-2 family of proteins which are more directly connected to the apoptotic pathway. CHOP overexpression causes a decrease in the antiapoptotic protein Bcl-2 while causing the proapoptotic family member Bax to translocate to the mitochondria (Oyadomari and Mori, 2004).

In addition to these pathways, the most extensively studied pathway by which ER stress could induce death is through caspases 12. The activation and downstream effects of caspases 12 will be reviewed extensively in the following section.

Caspase 12 pathway of ER stress induced apoptosis

Caspase 12 was first identified as an ER localized caspase in the year 2000. Murine caspase 12 has high amino acid sequence homology to the inflammatory murine caspases, caspase 1 (39%) and caspase 11 (38%) as well as human caspase 4 (48%) and caspase 5 (45%). Caspase 12 is ubiquitously expressed in mouse tissues and its overexpression induces apoptosis, that is inhibited by zVAD-fmk (Nakagawa et al., 2000). Due to the localization of caspase 12 on the outer ER membrane, it was postulated that caspase 12 would be well suited to initiate apoptosis at the ER in response to ER stress. Indeed, caspase 12 cleavage (and thus activity was inferred) occurs in PC12 cells and MEFs specifically in response to ER stress agents such as tunicamycin (TU) and not in W4 cells in response to non ER stress

agents such as cycloheximide or TNF (Nakagawa et al., 2000). While the caspase 12 knockout mice show no obvious developmental phenotype, *in vivo* studies showed a significant protection of renal epithelial cells and animal survival in response to TU injections. Caspase 12 deficient MEFs show resistance to ER stress induced death, as do cultured cortical neurons in response to AB incubation (Nakagawa et al., 2000). However, this resistance is only mild. In addition, a second caspase 12 null line of mice were made, MEFs from this mouse do not show resistance to ER stress induced apoptosis (Saleh et al., 2006). This leaves open the possibility that there may be other caspase 12 independent ways of carrying out ER stress induced apoptosis.

Since the discovery of caspase 12 most research on ER stress has focused on identifying how caspase 12 becomes activated and what the downstream mediators of caspase 12 activation might be. Investigators have uncovered three possible ways to activate caspase 12. Calpains, cytosolic Ca2+ activated neutral cytosine endopeptidases, become activated during Ca2+ homeostasis disturbances that occur during ER stress and contribute to pathogenesis. Studies utilizing cultured glia showed that ER stress agents, thapsigargin and brefeldin A as well as oxygen-glucose deprivation, induced the UPR and caspase 12 cleavage. In addition, the antiapoptotic protein Bcl-xL gets cleaved into a potentially proapoptotic form (Nakagawa and Yuan, 2000). Importantly, various calpain inhibitors but not caspase inhibitors, block the cleavage of both caspase 12 and Bcl-xL (Nakagawa and Yuan, 2000), suggesting that calpains become activated with ER stress and are responsible for cleaving procaspase 12 and Bcl-xL into apoptotic proteins. (Nakagawa 2000 JCB).

The other two suggested ways of activating caspase 12 involve the formation of a complex. As stated before, activated IRE1 recruits TRAF2 and ASK to activate JNK

signaling. Additional studies show that JIK, a human STE20-related serine/threonine kinase implicated in JNK signaling, is already bound to IRE1 and activation of the UPR recruits TRAF2 to this complex. JIK appears to increase JNK signaling through this complex by directly or indirectly phosphorylating TRAF2 and promoting the interaction between IRE1 and TRAF2. TRAF2 coimmunoprecipitates with caspase 12 causing increased procaspase 12 homodimerization. Under conditions of UPR activation the interaction between caspase 12 and TRAF2 is inhibited (Yoneda et al., 2001). This data suggests that upon UPR activation, JIK which is already bound to IRE1 would recruit TRAF2 which is bound to caspase 12. Once this complex is formed, caspase 12 becomes homodimerized and is activated in a manner akin to that of other initiator caspases (for example, the oligomerization of caspase 9 on the apoptosome). Importantly, distinct TRAF2 sub domains are required to activate caspase 12 and the JNK pathway suggesting that they are unrelated events that act in series to induce apoptosis (Yoneda et al., 2001).

Another alternative complex formation which could be responsible for activating caspase 12 comes from the finding that *in vitro* cleaved caspase 12 is unable to cleave caspase 9 in untreated extracts but is able to cleave in thapsigargin treated extracts. This suggests that active caspase 12 must work in combination with another ER stress inducing molecule to activate caspase 9 and cause death. Fractionation experiments identified microsomal proteins valsoin-containing protein (VCP) and apoptosis-linked gene 2 (ALG-2) as important for increasing caspase 9 activation (Rao et al., 2004). VCP is a member of the AAA (ATPases associated with diverse cellular activities) family of ATP-binding homo-oligomeric ATPase proteins, and is know to be important in ERAD. ALG-2 is a Ca2+ binding protein, shown to have proapoptotic activity during T-cell receptor, Fas-, and

glucocorticoid-induced death. Under conditions of ER stress both VCP and ALG-2 bind to caspases 12 and 9 and are required for the cleavage of caspases 9 and 3 (Rao et al., 2004). However, maximal cleavage was only found when thapsigargin treated microsomes were used, suggesting that while VCP and ALG-2 are necessary for cleavage and activation of caspase 12, some other components from the microsomes are used to maximally activate caspases during ER stress. Therefore, a third possible way to get caspase 12 activation could be through the formation of a complex involving caspase 12, VCP and ALG-2 as well other microsomal components which also recruits caspase 9 allowing for maximal caspase 12 and caspase 9 activation.

Once caspase 12 has become activated it appears to activate downstream caspases independently of the mitochondria and Apaf-1. This type of cytochrome *c*, Apaf-1 mediated caspase activation is seen in the extrinsic pathway of apoptosis where executioner caspase 3 becomes directly cleaved and activated by caspase 8 (Danial and Korsmeyer, 2004). Surprisingly, under conditions specific to ER stress, Sak2 cells (immortalized Apaf-1 deficient MEFs) show increased apoptotic morphologies which can be blocked by specific caspase 9 inhibitors but not caspase 8 inhibitors (Rao et al., 2002). In addition, studies utilizing in vitro translated proteins show that active caspase 12 can directly and specifically cleave caspase 9 (Morishima et al., 2002). Therefore, caspase 12 appears to directly cleave caspase 9 to cause its activation resulting in the cleavage of caspase 3 and death of the cell.

There are several major differences between the intrinsic pathway of apoptosis and the accepted caspase 12 pathway of ER stress induced apoptosis. The caspase 12 dogma does not utilize the mitochondria and therefore induces a cytochrome *c* independent death. As a result, it also does not utilize Apaf-1 in order to activate caspase 9. As such, caspase 9

activation by caspase 12 appears to be by cleavage and not by the induced proximity model. This is highly unusual since the cleaved caspase 9 is not very efficient whereas dimerized caspase 9 is thought to be very active at cleaving caspase 3 (Bao and Shi, 2007) .

It is worth noting that the studies so far have not completely ruled out a role for Apaf-1 in caspase activation during ER stress induced apoptosis. Indeed, the ability of genetic caspase 12 deletion to save MEFs against chemical inducers of ER stress is only marginally effective (Nakagawa et al., 2000). This suggests that there are alternatives to the caspase 12 pathway, which may be even more efficient. In addition, the role of human caspase 12 in ER stress induced apoptosis is controversial.

Human caspase 12 has acquired a frame shift mutation and premature stop codon that prevents the translated protein from having any caspase catalytic activity (Fischer et al., 2002). Subsequently, research has uncovered a role of human caspase 12 in sepsis, where a read-through single nucleotide polymorphism allows the production of a full-length procaspase 12 that plays a role in the inflammatory and innate immune system but not as an apoptotic caspase (Saleh et al., 2006; Saleh et al., 2004). On the other hand, human caspase 4 which shares 48% sequence homology with murine caspase 12 may be involved in human ER stress induced apoptosis. Like murine caspase 12, human caspase 4 is localized to the ER but also to the mitochondria, and is cleaved specifically in response to ER stress inducing agents. The overexpression of Bcl-2 did not prevent the cleavage of caspase 4, suggesting that despite the presence of caspase 4 at the mitochondria, it is primarily activated at the ER following stress. While Bcl-2 was not able to block caspase 4 cleavage, it did prevent morphological signs of death suggesting that in ER stress induced death at least in humans may utilize the mitochondria (Hitomi et al., 2004).

The role of ER stress induced apoptosis in disease

Several neurodegenerative diseases such as Parkinson's, Alzheimer's, ALS and numerous polyglutamine (polyQ) diseases, including Huntington's disease, are associated with ER stress. Neurons affected by these diseases show multiple markers of ER stress. These and other neurodegenerative disorders share common pathological characteristics in that they have an accumulation of insoluble or misfolded proteins within the cell. These aggregates damage neurons and cause neuronal death. It is believed that the underlying reason for cell death is due to the malfunction of the UPS leading to ER stress and ER stress induced apoptosis. The aggregates tie up chaperones, ubiquitin and other proteins involved in the UPS. The UPS is responsible for degradation of proteins from the cytosol, the ER and synaptic proteins. Disrupting the UPS causes more protein accumulation within the cell including within the ER lumen, due to a decrease in ERAD function, which is required to maintain normal ER homeostasis. As a result, ER stress is activated and without alleviation, the neuron undergoes apoptosis (Lindholm et al., 2006; Nishitoh et al., 2002; Oyadomari and Mori, 2004).

In addition to neurodegenerative disease, brain ischemia involves many characteristics that are strongly associated with ER stress including the deprivation of glucose, oxygen and ER Ca2+. In addition, protein aggregation in the ER is seen following focal brain ischemia/reperfusion. Direct evidence for ER stress in ischemia comes from the finding that CHOP mRNA increases following global cerebral ischemia and that CHOP-/- mice have a smaller infarct than wildtype mice subjected to bilateral carotid artery occlusion (Oyadomari and Mori, 2004).

Given our knowledge of ER stress involvement in these and other diseases, further understanding the pathway and regulation of ER stress induced apoptosis is vital to developing rational strategies to treat neuronal loss.

1.3 Skeletal Muscle Apoptosis

Skeletal muscle differentiation

Striated skeletal muscle is the largest tissue in the body and it is composed of bundles of contractile myofibers made up of postmitotic myotubes. During embryogenesis, somites give rise to embryonic myoblasts. These proliferating embryonic myoblasts can differentiate, give rise to satellite cells or undergo apoptosis (Schwartz, 2008; Scime and Rudnicki, 2006; Walsh and Perlman, 1997).

The differentiation process can be initiated by growth factor withdrawal. As a result, there is a decline in the levels of a protein known as inhibitor of differentiation (Id). This decline allows myogenic specific transcription factors to begin to express myogenic genes. At this point, the myoblasts are still proliferative. Subsequently, they express the cyclin-dependent kinase (cdk) inhibitor p21 and exit the cell cycle. Following this exit, myoblasts express contractile proteins and fuse to form long, multinucleated myotubes (Fig. 1.4) (Schwartz, 2008; Scime and Rudnicki, 2006; Walsh and Perlman, 1997).

Some embryonic myoblasts will give rise to satellite cells. These were dubbed satellite cells based on their location within the basal lamina between the basement membrane and the plasma membrane of individual myofibers. Satellite cells are quiescent but reenter the cell cycle and proliferate to give rise to adult myoblasts upon exposure to focal injury. Adult myoblasts undergo the same differentiation pathway undertaken by embryonic myoblasts to give rise to new fibers or fuse with existing damaged ones (Schwartz and Shah, 2005; Schwartz, 2008). Despite this regenerative potential, under conditions of continuous damage such as in muscular dystrophies and other diseases, satellite cells become exhausted and muscle repair is lost (Schwartz, 2008).

Embryonic myoblasts which do not differentiate or fail to express the appropriate survival proteins undergo apoptosis. A rather large population of myoblasts appears to undergo this developmental death. In C2C12 cells (myoblast like mouse cell line), 48 hours after removal of mitogens, 20 to 30 % of cell are undergoing apoptosis. One protein that provides resistance to developmental death is the cdk inhibitor p21. p21 becomes induced during differentiation and correlates with the resistance to differentiation induced apoptosis. Forcing expression of p21 or other cdk inhibitors is sufficient to block myoblasts apoptosis during differentiation (Wang and Walsh, 1996). This suggests that exiting the cell cycle confers increased apoptotic resistance to myoblasts. However, the mechanism by which p21 confers resistance remains unknown.

As skeletal muscle continues to develop, it becomes extremely refractory to apoptosis (Burgess et al., 1999; Schwartz, 2008). Despite this resistance, skeletal muscle does undergo apoptosis in pathological conditions including exposure to toxins and in certain muscular diseases (Schwartz, 2008; Tews, 2002) (see below).

Apoptosis in myoblasts: Apaf-1 independent caspase activation

Recently Zacksenhaus' lab reported a unique Apaf-1 independent pathway of caspase 9 activation in primary myoblasts. In response to cytotoxic agents, MEFs and other cells undergo the intrinsic pathway of apoptosis, utilizing the apoptosome. As such, Apaf-1 deficient MEFs are resistant to these same cytotoxic drugs because they can not activate caspase 9. However, Apaf-1 deficient myoblasts only experience marginal resistance to these drugs. Myoblasts do utilize caspase 9 to undergo apoptosis as the use of caspase 9 inhibitors or caspase 9 deficient myoblasts blocks death (Ho et al., 2004).

The reported mechanism is still being developed but it is clear that myoblasts express approximately half the level of XIAP as MEFs. In addition, Apaf-1 deficient myoblasts have increased amounts of Smac released into the cytosol following cytotoxic drug treatment (Ho et al., 2007). Therefore, upon apoptotic stimulation the ratio of cytosolic Smac to XIAP is much higher in Apaf-1 deficient myoblasts compared to MEFs. Genetically or pharmacologically inhibiting Smac activity along with Apaf-1 deficiency almost completely blocks caspase 9 activation and cell death. Death can be restored in the Apaf-1:Smac double knockout myoblasts by inhibiting XIAP function (Ho et al., 2007). This suggests that the resistance of these double knockouts is due to the accumulation of XIAP which can block the activity of caspase 9.

Importantly, mitochondria are involved even though Apaf-1 is not involved in this caspase 9 activating pathway. Blocking mitochondria permeabilization blocks death even when XIAP is being inhibited (Ho et al., 2007). This implies that XIAP inhibition is required but not sufficient for Apaf-1 independent activation of caspase 9. Overall the model suggests that myoblasts activate a mitochondria dependent Smac/XIAP pathway which exists to activate caspase 9 independently of Apaf-1.

The role of apoptosis in skeletal muscle and muscle disease

While the role for apoptosis during skeletal muscle differentiation is clearly observed, its role in postmitotic myotubes is less understood. The majority of cell death that occurs in skeletal muscle in response to toxic stimuli or in disease is necrotic. However, the uncovering of the apoptotic pathway and identifiable hallmarks of apoptosis, led investigators to examine various physiological and pathological conditions in muscle for the

involvement of apoptosis. Indeed, in some cases of skeletal muscle death, apoptosis is intimately involved. Multiple markers of apoptosis have been identified in models of muscular dystrophy (Tidball et al., 1995), in atrophied muscle (Allen et al., 1997), denervated muscle (Tews et al., 1997), metabolic myopathies (Aure et al., 2006; Ikezoe et al., 2002; Mirabella et al., 2000; Tews, 2005; Tews, 2006) and isolated myotubes treated with STS (McArdle et al., 1999).

Despite the identification of apoptosis in skeletal muscle, little progress has been made in uncovering its mechanism of action. It is clear that skeletal muscle is more resistant to apoptosis than its precursor myoblasts (Walsh and Perlman, 1997). There is some evidence that this increased resistance may be due in part to the downregulation of apoptotic components required to carry out apoptosis. One study examining healthy adult human muscle found that cytosolic lysates can not activate caspases in response to cytochrome *c*. This appears to be due to a complete loss of Apaf-1 protein expression and a severe decrease in Apaf-1 mRNA expression (Burgess et al., 1999). However, other studies have found Apaf-1 protein to be present in adult mouse and human skeletal muscle (Ikezoe et al., 2002; Ruest et al., 2002) .

There is also some dispute over the presence of caspase 3 protein in adult muscle. A study examining skeletal muscle found caspase 3 present during early postnatal development and during the regeneration of injured muscle but never in healthy adult skeletal muscle (Ruest et al., 2002). Other studies in mice and humans have shown the existence of caspase 3 protein in both disease and normal skeletal muscle (Burgess et al., 1999; Ikezoe et al., 2002). In addition, one study utilizing differentiated C2C12 cells showed not only the presence of caspase 3 protein but that it could be cleaved in response to cytotoxic drugs

(McArdle et al., 1999). The studies in differentiated C2C12 cells rule out the possibility that caspase 3 was coming from mitotic cells found in skeletal muscle tissue samples.

Overall, investigators have found that adult muscle still contains all of the components necessary to carry out the intrinsic pathway including proapoptotic and antiapoptotic Bcl-2 family members as well as Apaf-1 and caspases 9 and 3 (Burgess et al., 1999; McArdle et al., 1999; Ruest et al., 2002; Tews et al., 1997; Veal and Jackson, 1996). Therefore, the increased resistance to apoptosis seen in skeletal muscle is not simply due to a loss of the apoptotic pathway.

Instead, this increased resistance could be due to the increased regulation of the apoptotic pathway. This idea is supported by the identification of an apoptosis inhibitor protein known as apoptosis repressor interacting with CARD (ARC). ARC is selective expressed in postmitotic cells with particularly high expression in adult striated muscles. Its CARD domain interacts directly with caspase 2 and 8 (Koseki et al., 1998). In heart-derived H9C2 cells, ARC has been shown to inhibit cytochrome *c* release and protect mitochondrial function from ROS (Neuss et al., 2001). In addition, ARC colocalizes with mitochondria in muscle fibers suggesting that it has a role in inhibiting mitochondria mediated apoptosis (Li et al., 2002a). The identification of this apoptotic regulator suggests that much remains to be uncovered about the apoptotic pathway and its regulation in skeletal muscle.

For my thesis work, I have investigated the pathway and control of ER stress induced neuronal apoptosis. In addition, I have examined whether the increased regulation of apoptosis exhibited by neurons also occurs in other postmitotic cells, specifically myotubes.

1.4 Figures and Legends

Figure 1.1: Pathway of programmed cell death in *C. elegans* and its mammalian homologs

Developmental cues cause the transcriptional upregulation of EGL-1. EGL-1 (homology to proapoptotic BH3 only proteins) binds to CED-9 (homology to antiapoptotic Bcl-2) at the mitochondria and displaces it from its interaction with CED-4 (homology to Apaf-1). The newly freed CED-4 then translocates to the perinuclear region where it oligomerizes and binds CED-3 (homology to caspase 3). This results in CED-3 processing and activation which is ultimately responsible for cell death (Danial and Korsmeyer, 2004).

Figure 1.1

Figure adapted from Danial and Korsmeyer, Cell (2004)

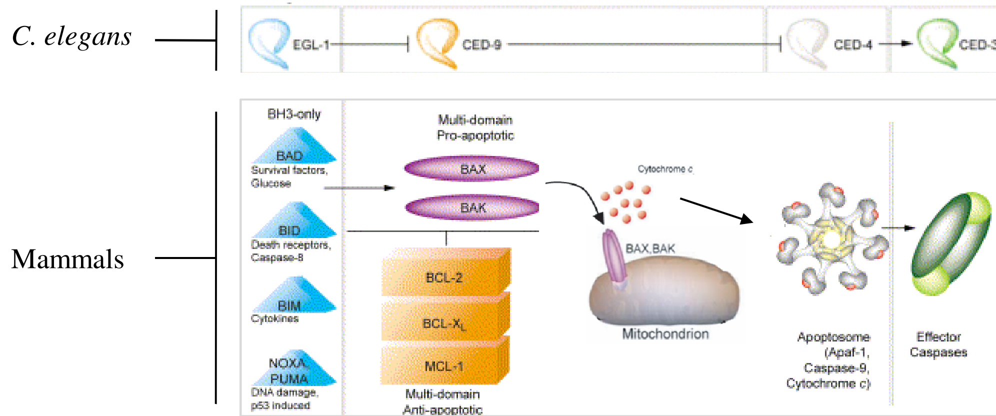


Figure 1.2: Regulation of cytochrome *c* release from the mitochondria by the Bcl-2 family of proteins

Proapoptotic BH3-only members of the Bcl-2 family of proteins become activated in response to various cellular stressors. They primarily act by directly activating the proapoptotic multidomain Bcl-2 family members, Bax and Bak, causing them to form channels in the mitochondria outer membrane, releasing cytochrome *c*. Alternatively, they can bind to the antiapoptotic members Bcl-2, Bcl-XL and Mcl-1 which are normally bound to and preventing the activation of Bax and Bak. By binding BH3-only proteins, antiapoptotic members are sequestered away from Bax and Bak thus allowing them to insert in the mitochondria and release cytochrome *c*.

Figure 1.2

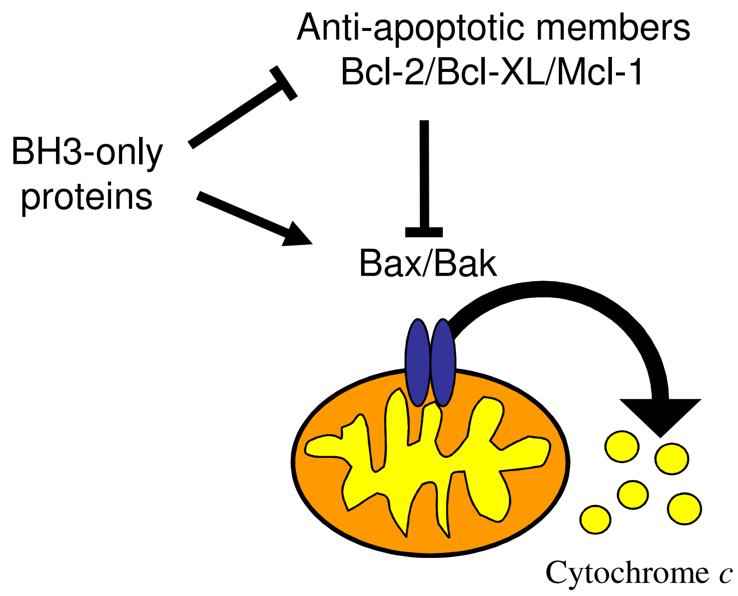


Figure 1.3: The intrinsic pathway of apoptosis

Cytochrome *c* is released from the mitochondria and binds to the adaptor protein Apaf-1 causing Apaf-1 to undergo a dATP-dependent conformational change. This results in the oligomerization of Apaf-1 to form a heptamer with exposed CARD domains at the center. The CARD domains recruit and bind procaspase 9 via the CARD domain of caspase 9. On the apoptosome, procaspase 9 becomes autoactivated and proceeds to cleave executioner caspases 3 and 7. Activated caspases are inhibited by the inhibitor of apoptosis protein XIAP. XIAP can be functionally inhibited by the release the IAP inhibitor Smac, from the mitochondria (Cain et al., 2002).

Figure 1.3

Figure adapted from Cain, et al., Biochimie (2002)

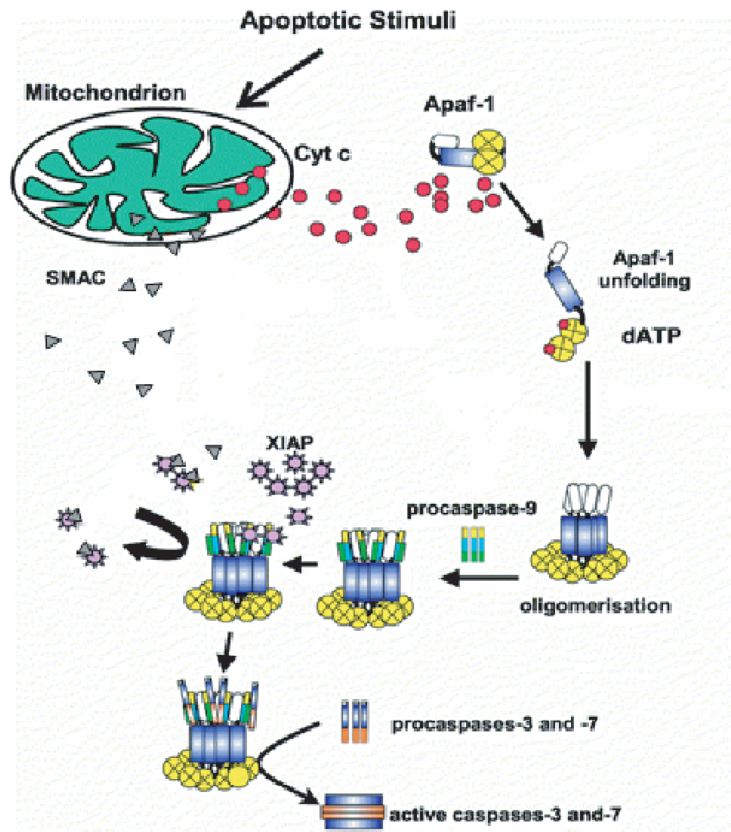


Figure 1.4: Model for reduced caspase activation in neurons due to the coupling of reduced Apaf-1 levels to the increased effectiveness of XIAP

Following cytochrome *c* release from the mitochondria, low levels of Apaf-1 present in neurons result in reduced apoptosome formation and caspase activation. As a consequence, endogenous XIAP levels are sufficient to effectively block this reduced level of caspase activation. However, in mitotic cells, high levels of Apaf-1 result in robust apoptosome formation and thus increased caspase activation that cannot be successfully inhibited by endogenous XIAP. As a consequence, these mitotic cells readily undergo apoptosis with cytosolic cytochrome *c* while postmitotic neurons do not.

Figure 1.4

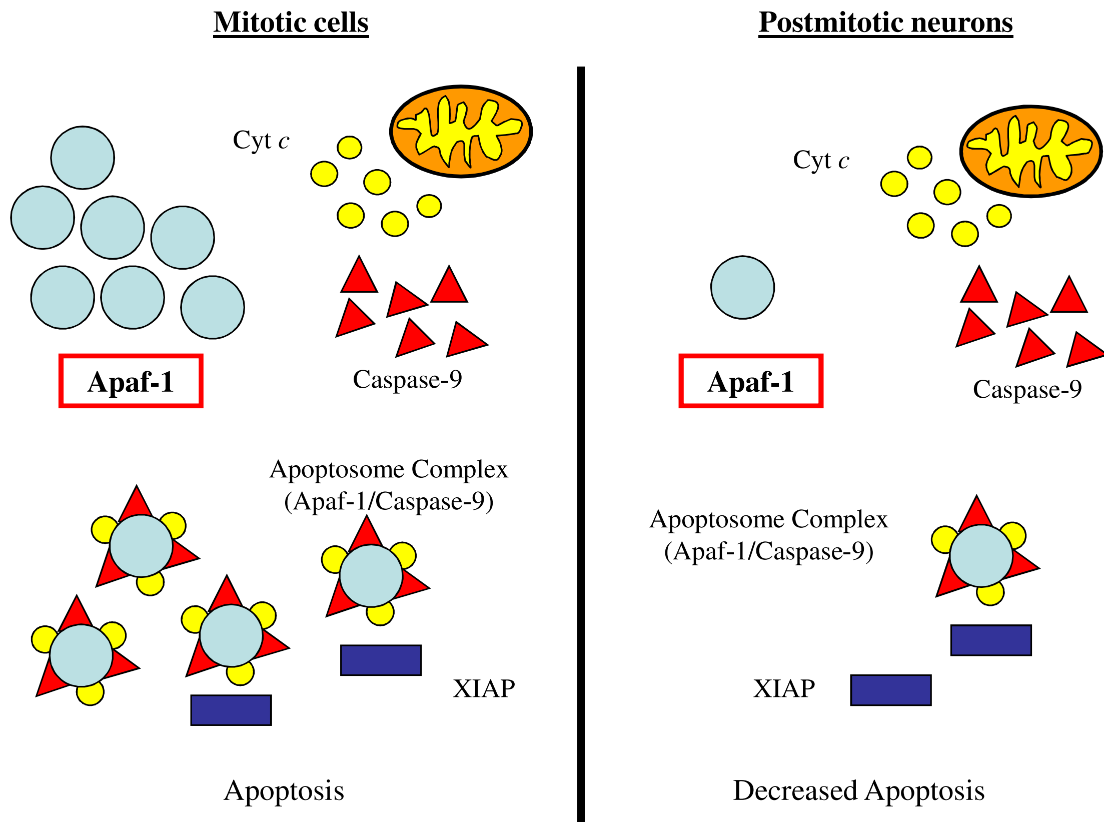


Figure 1.5: Signaling of the unfolded protein response

The three ER stress sensors, ATF6, PERK and IRE1, are bound and held inactive by the ER chaperone BiP. Upon accumulation of unfolded proteins in the ER lumen, BiP is sequestered away from the sensors allowing them to become activated. ATF6 transits to the Golgi where it is cleaved to release a transcription factor. PERK and IRE1 homodimerize and become autophosphorylated. Once active, PERK causes the phosphorylation and inactivation of eIF2α and general protein translation is attenuated. Active IRE1 is an endonuclease which cleaves the mRNA transcript of XBP-1 leading to the production of a potent transcription factor. The transcription factors generated by this response act in concert to transcribe ER chaperones and other genes involved in refolding proteins (Paschen and Mengesdorf, 2005).

Figure 1.5

Figure adapted from Paschen and Mengesdorf, Cell Calcium (2005)

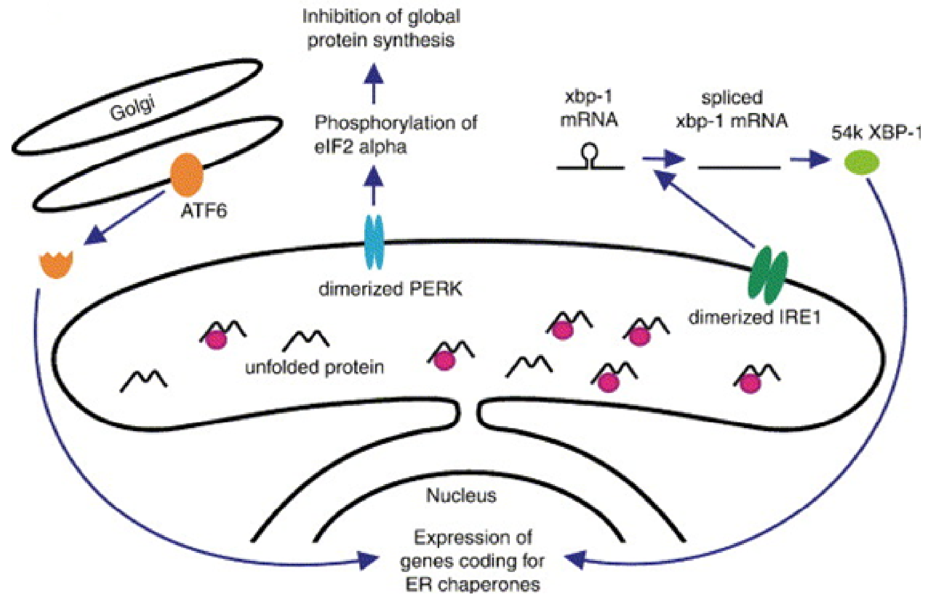


Figure 1.6: Pathway of ER stress induced caspase 12 mediated apoptosis

Prolonged ER stress causes activation of ER localized caspase 12. Active caspase 12 directly cleaves caspase 9 in the cytosol. Cleaved caspase 9 then cleaves executioner caspase 3 to cause the death of the cell.

Figure 1.6

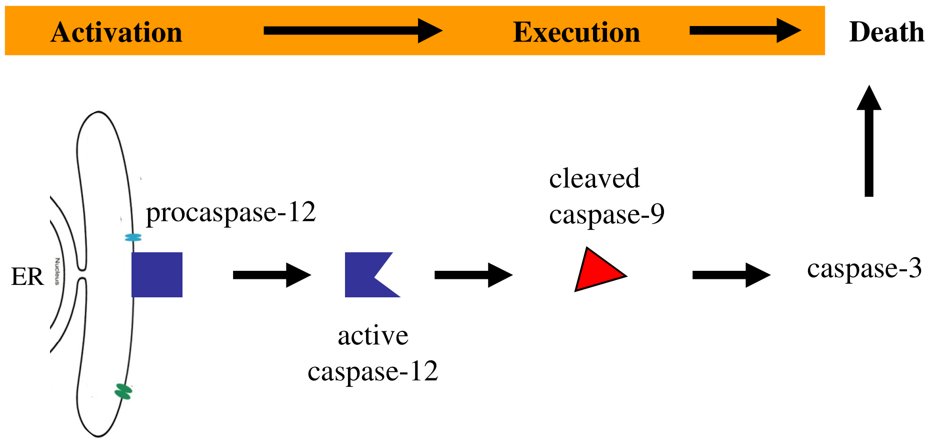
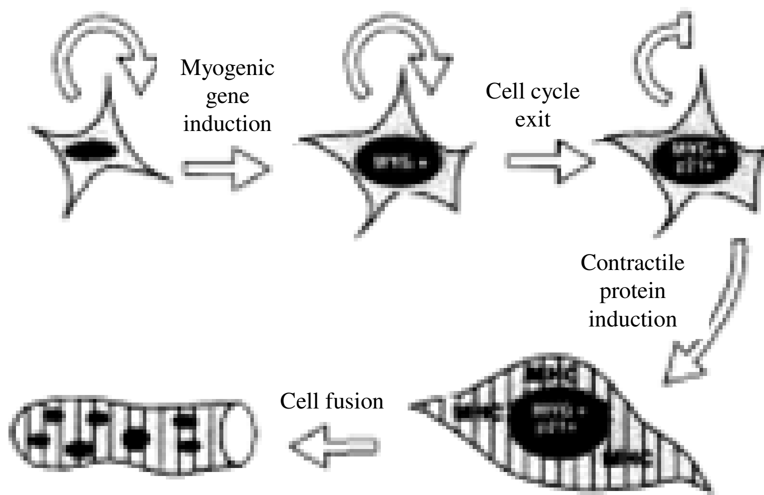


Figure 1.7: Skeletal muscle differentiation

Myoblasts exposed to certain environmental cues begin to express myogenic genes but maintain their proliferative potential. Subsequently, they permanently exit the cell cycle and go on to express contractile proteins. This is followed by myoblast fusion to form long multinucleated postmitotic myotubes (Walsh and Perlman, 1997).

Figure 1.7

Figure adapted from Walsh and Perlman, Current Opinion in Genetic and Development (1997)



CHAPTER TWO

ER STRESS INDUCED APOPTOSIS REQUIRES BAX FOR COMMITMENT AND APAF-1 FOR EXECUTION IN PRIMARY NEURONS

2.1 Abstract

Apoptosis triggered by endoplasmic reticulum (ER) stress is associated with various pathophysiological conditions including neurodegenerative diseases and ischemia. However, the mechanism by which ER stress induces neuronal apoptosis remains controversial. Here we identify the pathway of apoptosis carried out in sympathetic neurons triggered to die by ER stress inducing agent tunicamycin. We find that ER stress induces a neuronal apoptotic pathway which upregulates BH3-only genes DP5 and Puma. Importantly, we show that ER stress commits neurons to die prior to cytochrome *c* release and that this commitment requires Bax activation and JNK signaling. Furthermore, ER stress engages the mitochondrial pathway of death as neurons release cytochrome *c* and Apaf-1 deficiency is sufficient to block apoptosis. Our findings identify a critical function of Bax in committing neurons to ER stress induced apoptosis and clarify the importance of the apoptosome as the non-redundant caspase activation pathway to execute neuronal apoptosis in response to ER stress.

2.2 Introduction

Endoplasmic reticulum (ER) stress occurs when the ability of the ER to properly fold proteins is overwhelmed or compromised, resulting in the activation of the unfolded protein response (UPR) (Wu and Kaufman, 2006). Activation of UPR results in a global decrease in protein synthesis while increasing the production of ER chaperone proteins. However, under conditions of severe and prolonged ER stress, the UPR is unable to restore normal cellular function and apoptosis is triggered. Apoptosis induced by ER stress contributes to neuronal death seen in many neurodegenerative disorders as well as neuronal death following ischemia (Lindholm et al., 2006). Therefore, an understanding of the mechanism by which neurons undergo ER stress induced apoptosis has important clinical significance.

Caspase proteases are the critical executioners of apoptosis in mammalian cells (Denault and Salvesen, 2002). Many death stimuli converge on the mitochondria to activate caspases by the intrinsic pathway. In this pathway, activation of BH3-only proteins serves to activate proapoptotic Bcl-2 family members Bax and Bak, (Ham et al., 2005) which oligomerize in the mitochondrial membrane to induce the release of cytochrome *c* (Wang, 2001). Cytosolic cytochrome *c* binds to Apaf-1 to form the apoptosome complex where caspase 9 is activated. Active caspase 9 cleaves and actives executioner caspases, such as caspase 3, which then go on to cleave various target proteins resulting in the demise of the cell (Wang, 2001).

The mechanism by which apoptosis is induced in response to ER stress has been examined in multiple cell types. Bax and Bak are important in this pathway, as mouse embryonic fibroblasts (MEFs) doubly deficient in Bax and Bak are resistant to ER stress induced apoptosis.(Scorrano et al., 2003; Zong et al., 2003) In addition to the established

roles of Bax and Bak at the mitochondria, both are also found localized to the ER membrane and play an important role in Ca^{2+} regulation and release in response ER stress (Nutt et al., 2002a; Scorrano et al., 2003; Zong et al., 2003).

The discovery of an ER localized caspase, caspase 12, has brought into question the mechanism by which ER stress activates caspases to carry out apoptosis.(Nakagawa et al., 2000) *In vitro*, active caspase 12 is able to cleave caspase 9 directly, which subsequently activates caspase 3, potentially eliminating the requirement of the mitochondria and apoptosome to carry out ER stress induced apoptosis (Morishima et al., 2002; Rao et al., 2002). The lack of apoptosome involvement in ER stress induced apoptosis is consistent with the observation that Sak2 cells, which are deficient in Apaf-1, are capable of activating caspases when treated with ER stress inducing agents thapsigargin and brefeldin A (Rao et al., 2002). Likewise, C2C12 cells treated with tunicamycin or thapsigargin show caspase activation without any detectable release of cytochrome *c* (Morishima et al., 2002).

Although caspase 12 deficient cells show resistance to ER stress stimuli, this resistance is mild (Nakagawa et al., 2000). Additionally, human caspase 12 lacks protease activity, bringing into question the relative importance of the caspase 12 pathway in ER stress induced apoptosis (Fischer et al., 2002; Saleh et al., 2006). Recent studies suggest that the mitochondrial pathway may be significant in either inducing or amplifying ER stress induced apoptosis. First, in contrast to the data in C2C12 cells, cytochrome *c* is released in response to ER stress in a variety of other cell types (Di Sano et al., 2006; Reimertz et al., 2003). Second, a recent study found that the Apaf-1 deficient ETNA (murine embryonic telencephalic naïve) cell line and Apaf-1 deficient MEFs and embryonic cortical cells are resistant to ER stress induced apoptosis (Di Sano et al., 2006). The disparities between these

two sets of findings leave open the question as to whether mammalian cells, in particular primary postmitotic neurons, undergo apoptosis in a mitochondria and apoptosome dependent or independent manner in response to ER stress.

In this study we have examined the molecular pathway of ER stress induced apoptosis in primary sympathetic neurons. Our data show that neuronal commitment to death in response to ER stress occurs prior to cytochrome *c* release and is dependent on Bax activation and c-Jun N-terminal kinase (JNK) signaling. Importantly, we show clear evidence that, upon commitment, neurons engage the mitochondrial pathway and utilize the apoptosome as a non-redundant mechanism to induce apoptosis.

2.3 Results

Tunicamycin induced ER stress results in caspase dependent apoptosis in neurons

Tunicamycin, a N-glycosylation inhibitor, induces death in many cell types including sympathetic and hippocampal neurons (Chang and Korolev, 1996; Reimertz et al., 2003). Sympathetic neurons treated with 2.5 uM tunicamycin exhibited a robust increase in the levels of ER stress induced transcription factor CHOP and the ER resident chaperone BiP (Fig. 2.1A, B). Additionally XBP-1 became spliced, indicating that the ER stress sensor IRE1 was activated (Fig. 2.1B). Together, these results show that a vigorous ER stress response was being induced.

To determine the time course with which tunicamycin kills sympathetic neurons, neuronal viability was assessed, by cell morphology, at various timepoints following treatment. Following 36 hours of tunicamycin treatment, approximately 70% of neurons were still viable. However, by 60 hours virtually all neurons in the culture were dead (Fig. 2.1C, D). This method of measuring viability was confirmed by use of the MTT assay and correlates well with other cell survival assays such as trypan blue exclusion and staining with calcein AM (Supplemental Fig. 2.1A) (Potts et al., 2003). Importantly, tunicamycin induced neuronal death is caspase dependent as it was completely inhibited by the pan caspase inhibitor zVAD-fmk (50 uM) (Fig 2.1C, D). zVAD-fmk appeared to have no affect on the ER stress response itself as levels of BiP and XBP-1 splicing were unaffected by the addition of zVAD-fmk to tunicamycin treated cultures (Fig. 2.1B) These results show that tunicamycin induces ER stress and caspase dependent death in neurons.

Neurons activate JNK signaling which is required for apoptosis in response to ER stress

JNK is known to be activated following ER stress by the IRE1-TRAF2-ASK1 pathway (Nishitoh et al., 2002; Urano et al., 2000b). However, in sympathetic neurons, proapoptotic stimuli have been shown to activate JNK *via* mixed lineage kinases (MLK) (Maroney et al., 1999; Wang et al., 2005). In these neurons, MLK activation by Cdc42/Rac is thought to phosphorylate MEKK4/7 which subsequently phosphorylates and activates JNK signaling (Mota et al., 2001; Xu et al., 2001). JNK activation leads to the upregulation, phosphorylation and activation of the transcription factor c-Jun which is essential for sympathetic neuronal apoptosis in response to NGF withdrawal (Estus et al., 1994; Palmada et al., 2002). We examined whether the JNK pathway was activated in sympathetic neurons in response to ER stress by using immunohistochemistry to assess the phosphorylation state of c-Jun. While untreated neurons showed virtually no phospho-c-Jun staining, by 18 hours neurons treated with tunicamycin showed high levels of nuclear localized phosphorylated c-Jun, which peaked by 24 hours (76%) and were still elevated at 36 hours (Fig. 2.2A).

To determine if the MLK-mediated JNK pathway was required for ER stress induced apoptosis we treated sympathetic neurons with tunicamycin in the presence of a MLK inhibitor, CEP-11004 (Murakata et al., 2002; Wang et al., 2005). Following 48 hours of treatment, at a time when only about 10% of the tunicamycin treated neurons were alive, greater than 85% of the tunicamycin and CEP-11004 treated neurons remained viable (Fig. 2.2B). CEP-11004 was also protective against thapsigargin induced neuronal death (Supplemental Fig. 2.1B). CEP-11004 did not affect the ER stress response, as mRNA levels of BiP and XBP-1 splicing were unaffected by the addition of CEP-11004 to tunicamycin treated cultures (Fig. 2.2C). However, CEP-11004 was fully capable of blocking the phosphorylation of c-Jun in tunicamycin treated neurons (Fig. 2.2D). These results indicate

that the MLK-mediated JNK pathway is activated during ER stress induced neuronal apoptosis and its inhibition is sufficient to block or prolong death.

BH3-only genes DP5 and Puma are upregulated independently of MLK signaling during ER stress induced neuronal apoptosis

BH3-only proteins are responsible for binding to and activating proapoptotic Bax and Bak in response to diverse apoptotic stimuli (Ham et al., 2005). BH3-only proteins themselves can be activated by transcriptional or posttranslational mechanisms. For example, following NGF withdrawal in sympathetic neurons, BH3-only proteins Bim, DP5, and Puma become transcriptionally upregulated in part *via* the JNK pathway (Fig. 2.3C, D) (Ham et al., 2005).

We examined whether BH3-only proteins were transcriptionally upregulated in primary sympathetic neurons treated with tunicamycin. Our results show that both DP5 and Puma are transcriptionally induced as early as 12 hours by ER stress while levels of Noxa and Bim were not significantly altered (Fig. 2.3A, B).

We next examined whether MLK signaling was important for the transcriptional upregulation of these BH3-only genes in ER stress induced neuronal apoptosis. Surprisingly, CEP-11004 addition did not block the transcriptional increase in DP5 and Puma seen after tunicamycin treatment in these neurons (Fig. 2.3C, D). These results indicate that the mechanism by which BH3-only genes are transcriptionally upregulated during ER stress induced apoptosis is MLK independent.

Bax deficiency inhibits ER stress induced apoptosis in neurons, allowing for long term survival

Bax/Bak double knockout MEFs have been shown to be highly resistant to many apoptotic insults including ER stress (Scorrano et al., 2003; Zong et al., 2003). Even though Bax and Bak act redundantly to induce apoptosis in fibroblasts, sympathetic neurons do not express full length Bak (Sun et al., 2001). As a result, Bax deficient sympathetic neurons are resistant to death following NGF deprivation and DNA damage (Besirli et al., 2003; Deckwerth et al., 1996). Therefore, we examined whether Bax deficiency alone was capable of preventing ER stress induced apoptosis in sympathetic neurons. Our results show that Bax deficient neurons remained viable long after the wildtype neurons had undergone apoptosis in response to both tunicamycin and thapsigargin (Fig 2.4A, B, Supplemental Fig 2.4).

To determine whether Bax deficiency was simply delaying neuronal death, we maintained the Bax deficient neurons with or without tunicamycin for eleven days. Remarkably, Bax deficient neurons exhibited decreased cell volume but no morphological signs of apoptosis even with prolonged exposure to tunicamycin (Fig. 2.4C). This resistance to ER stress did not appear to be due to decreased activation of IRE1, as reported for Bax/Bak deficient MEFs (Hetz et al., 2006), as Bax deficient neurons were still able to splice the IRE1 target, XBP-1 (Fig. 2.4D). Together, these results show that Bax is essential for ER stress induced apoptosis in neurons and that in the absence of Bax, tunicamycin treated neurons can survive long term.

ER stress commits neurons to die early, prior to cytochrome c release, and requires Bax and JNK activation

Commitment to death is the point in the apoptotic pathway at which neurons can no longer recover and eventually die, even with the removal of the apoptotic stimuli. Defining the point of commitment is important from a therapeutic perspective as long term neuronal survival is expected if death is blocked upstream of commitment, whereas blocking downstream of commitment will only serve to delay death. In NGF deprived neurons, commitment to death occurs coincident with the time of cytochrome *c* release (Deshmukh et al., 2000). Our data show that the ER stress induced time course of cytochrome *c* release corresponded very well to the time of death (Fig. 2.5A) (Deshmukh et al., 2000). However, unlike NGF deprivation, tunicamycin treated neurons were committed to die significantly prior to cytochrome *c* release. For example, following 24 hours of tunicamycin treatment while only 20% of neurons had released cytochrome *c,* greater than 80% of neurons had already become committed to die (Fig. 2.5A).

To narrow down the point of commitment in ER stress induced apoptosis, we examined commitment in Bax deficient neurons. If commitment occurs upstream of Bax activation, then the Bax deficient neurons are expected to eventually undergo cell death in this assay. Our results show that in the absence of Bax, sympathetic neurons do not become committed to die in response to ER stress. Even after 120 hours of tunicamycin treatment, the Bax deficient neurons failed to be committed to death (Fig. 2.5B). These results identify a critical role of Bax for commitment to death in neurons undergoing ER stress induced apoptosis.

A recent report determined that Bax and Bak at the ER are critical for JNK activation following ER stress in fibroblasts (Hetz et al., 2006). Therefore, we examined the importance of JNK signaling in neuronal commitment to death in ER stress. Sympathetic

neurons were treated with tunicamycin for 12, 24, and 36 hrs following which the drug was washed out and either fresh media alone or fresh media containing the MLK inhibitor CEP-11004 was added during the recovery period. While CEP-11004 addition 12 hours after tunicamycin treatment was still capable of inhibiting the commitment to death, by 24 hours after tunicamycin treatment CEP-11004 could no longer inhibit commitment in the majority of neurons (Fig. 2.5C). Similar results were also obtained with the JNK inhibitor SP600125 (Fig. 2.5D). These results point to the importance of JNK signaling dependent events, which occur between 12 and 24 hours following tunicamycin treatment, in committing neurons to ER stress induced apoptosis.

Apaf-1 is required for neurons to undergo ER stress induced apoptosis

While the role of the Apaf-1-dependent apoptosome pathway is well established in many models of apoptosis, its specific importance in the ER stress induced apoptotic pathway is controversial (Breckenridge et al., 2003). To determine if the cytochrome *c* mediated apoptosome pathway is required to carry out ER stress induced neuronal apoptosis, we examined the effects of ER stress on sympathetic neurons isolated from Apaf-1 deficient mice. First, we ensured that Apaf-1 deficient neurons were capable of releasing cytochrome *c* in response to tunicamycin. Our results show that by 48 hours cytochrome *c* was released from mitochondria in both wildtype and Apaf-1 deficient neurons in response to tunicamycin treatment (Fig. 2.6A, B).

Next we examined whether Apaf-1 was required for ER stress induced apoptosis in sympathetic neurons. In contrast to wildtype neurons which were dead by 48 hours, Apaf-1 deficient neurons remained alive even at 96 hours of tunicamycin treatment (Fig. 2.6C).

These results identify an essential requirement of Apaf-1 in neurons undergoing apoptosis in response to tunicamycin.

Together, these results demonstrate that ER stress engages Bax-dependent cytochrome *c* release and requires the apoptosome pathway of caspase activation to carry out neuronal apoptosis.

2.4 Discussion

In this study we utilized the model of primary sympathetic neurons to examine the molecular pathway engaged by ER stress induced apoptosis in postmitotic neurons. First, we report that ER stress induced neuronal apoptosis requires JNK signaling. Tunicamycin induced the phosphorylation and upregulation of c-Jun in neurons and blocking this pathway with the MLK inhibitor CEP-11004 or the JNK inhibitor SP600125 prevented apoptosis (Fig. 2.2, 2.3, 2.5). Second, we identify Puma and DP5 as two BH3-only genes that are transcriptionally activated in response to ER stress (Fig. 2.3). This increase in Puma and DP5 occurred even in the presence of CEP-11004 (Fig. 2.3). Third, in response to ER stress, neurons release cytochrome *c* (Fig. 2.5A). However, neurons became committed to die long before the point of cytochrome *c* release and that this commitment requires both Bax activation and JNK signaling (Fig. 2.5). Thus, Bax deletion alone is able to block ER stress induced apoptosis long term in both cell survival and commitment studies (Fig. 2.4, 2.5B, Supplemental Fig. 2.4). Finally, we show that Apaf-1 deficient neurons are remarkably resistant to ER stress induced apoptosis (Fig. 2.6C). These results identify an essential and non redundant role of the Apaf-1 dependent apoptosome pathway in mediating ER stress induced apoptosis in neurons.

ER stress induces the activation of JNK signaling and the transcriptional upregulation of BH3-only proteins during neuronal apoptosis

Our results show that tunicamycin induced an early and sustained increase in JNK dependent phosphorylation of c-Jun in neurons (Fig. 2.2A, D). JNK signaling could be activated at the ER membrane through IRE1-TRAF2-ASK1 pathway or through the MLK

pathway that is known to become activated in these sympathetic neurons after NGF deprivation (Maroney et al., 1999; Nishitoh et al., 2002; Urano et al., 2000b; Wang et al., 2005). Studies utilizing ASK1-deficient mice have also demonstrated the importance of ASK1 in ER stress induced apoptosis in telencephalic neurons (Nishitoh et al., 2002). We find that an increase in JNK signaling was required for ER stress induced apoptosis in sympathetic neurons as the addition of SP600125 (JNK inhibitor) or CEP-11004 (MLK inhibitor) blocked apoptosis (Fig. 2.2B, 2.5C, D). Interestingly, CEP-11004 addition did not protect against tunicamycin induced death in primary fibroblasts (Supplemental Fig. 2.2). These results point to the importance of MLK signaling mediating JNK activation in sympathetic neurons undergoing apoptosis with ER stress, just as seen after NGF deprivation. Therefore, it is likely that both these pathways contribute to JNK activation during ER stress induced apoptosis in neurons.

JNK signaling is important in other models of neuronal apoptosis as well (Harding et al., 2001; Maroney et al., 1999; Palmada et al., 2002). For example, during NGF deprivation induced apoptosis, JNK signaling appears to be important for the transcriptional upregulation of BH3-only proteins DP5/Harakiri and Bim (Ham et al., 2005). We found DP5 and Puma levels were induced; however, Bim and Noxa levels did not change significantly after tunicamycin treatment in neurons (Fig. 2.3A, B). Recent studies with DP5 (Imaizumi et al., 2004) and Puma (Wyttenbach and Tolkovsky, 2006) knockout mice have shown that these BH3-only proteins contribute to apoptosis in sympathetic neurons. Importantly, addition of the MLK inhibitor CEP-11004, which blocks the upregulation and phosphorylation of c-Jun, did not block the increase in DP5 and Puma after ER stress (Fig. 2.3C, D). These results are

consistent with the observation that BH3-only proteins can be activated via JNK dependent and JNK independent pathways during sympathetic neuronal apoptosis (Ham et al., 2005).

Our results showing the transcriptional upregulation of Puma following ER stress are in agreement with other studies (Luo et al., 2005; Reimertz et al., 2003). While Puma was initially identified as a p53 regulated BH3-only gene, p53 deficient neurons were still able to upregulate Puma and did not show any resistance to ER stress induced apoptosis (Supplemental Fig. 2.3). Thus, while transcription factors c-Jun and p53 do not appear to be important for ER stress induced DP5 and Puma upregulation, this upregulation could be mediated by alternative transcription factors such FOXO3a, which is known to be activated in neurons in response to other apoptotic stimuli (Ham et al., 2005).

ER stress commits neurons to die prior to cytochrome c release and requires Bax

Neurons exposed to ER stress become committed to die long before they release cytochrome *c* (Fig. 2.5A). These results are in contrast to NGF deprivation, in which commitment to death occurs coincident with the mitochondrial release of cytochrome *c* (Deshmukh et al., 2000). In this respect, ER stress appears similar to DNA damage which also commits neurons to die early, prior to cytochrome *c* release (Besirli et al., 2003). As DNA damage commits neurons to die prior to Bax function at the mitochondria, Bax deficient neurons still become committed to die (Besirli et al., 2003). In striking contrast, Bax deficient neurons exposed to ER stress fail to become committed to die (Fig. 2.5B). These results show that during ER stress, Bax activation is an absolute requirement for neuronal commitment to death. While Bax function at the mitochondria is well characterized, Bax has also been localized to the ER in fibroblasts and cell lines (Nutt et al.,

2002a; Scorrano et al., 2003; Zong et al., 2003). Therefore, this difference in the requirement of Bax for neuronal commitment to death in response to ER stress but not DNA damage could be indicative of a critical function of Bax at the ER in neurons.

The Bax deficient neurons that are unable to be committed to die in the presence of tunicamycin exhibit no morphological signs of degeneration even after 11 days of sustained exposure to tunicamycin (Fig. 2.4C). These neurons are smaller than untreated neurons presumably due to the global inhibition of protein synthesis that is seen after ER stress in mammalian cells (Fig. 2.4C) (Wu and Kaufman, 2006). While it is surprising that these neurons can survive long term under these conditions, it is possible that they do so by utilizing autophagy which is known to provide energy in conditions of nutrient deprivation (Levine and Yuan, 2005).

The Apaf-1 dependent apoptosome pathway is required for neurons to undergo ER stress induced apoptosis

There is significant debate about the importance of caspase 12 and the role of an Apaf-1 independent pathway in ER stress induced apoptosis. Others have proposed that caspase 12, which is activated at the ER, directly cleaves and activates caspase 9 which is then free to go on to cleave and activate caspase 3 (Morishima et al., 2002; Rao et al., 2002). These findings imply that the apoptosomal components and therefore the apoptosome is not required for ER stress induced apoptosis.

Here we show that in response to ER stress, sympathetic neurons release cytochrome *c* and that Apaf-1 is required for apoptosis (Fig. 2.6). Thus, ER stress induced apoptosis is not able to bypass the apoptosome, nor does the apoptosome act simply as a feedback

amplification loop following caspase activation by an apoptosome independent pathway. These finding are consistent with a recent study focusing on ETNA cells (neuronal precursor cell line) which also found the apoptosome pathway to be key to ER stress activated apoptosis (Di Sano et al., 2006). Additionally, we find that a zVAD-fmk inhibitable caspase, such as caspase 12, (Nakagawa et al., 2000) does not act upstream of the mitochondrial release of cytochrome *c* during ER stress induced apoptosis, as addition of zVAD-fmk did not block cytochrome *c* release in response to tunicamycin treatment (Fig. 2.5A). In agreement with this finding, recent studies have indicated that caspases 12 is a downstream target of caspases 3 (Shiraishi et al., 2006) and that caspases 12 and human caspase 4 are dispensable for ER stress induced apoptosis (Obeng and Boise, 2005). Therefore, caspase 12 is not likely to play an essential role as a link between the ER and mitochondria to release cytochrome *c* in sympathetic neurons.

Together, the data presented in this study identify key components of the ER stress induced apoptotic pathway in neurons. In particular, these results demonstrate the essential and non-redundant function of the Apaf-1 dependent mitochondrial pathway in this model. Our results also point to Bax inhibition as a viable strategy for preventing neuronal death in pathological models of ER stress, as it would block the commitment to death point preventing not only apoptosis but other forms of death, allowing for long term survival.

2.5 Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich or Fisher Scientific, unless otherwise stated. Collagenase and trypsin were purchased from Worthington Biochemical Corporation. zVAD-fmk purchased from Enzyme Systems Products (MP Biomedicals). CEP-11004 was a kind gift from Cephalon Inc. (Frazer, PA). Aphidicolin was purchased from Sci-Quest-AG Scientific. Protease inhibitor cocktail was purchased from Roche.

Sympathetic neuronal and fibroblast cultures

Primary sympathetic neurons were isolated from superior cervical ganglia of postnatal day zero to one (P0-P1) mice as previously described for rats (Deshmukh et al., 2000). Briefly, dissected ganglia were treated with 1 mg/ml collagenase, followed by 2.5 mg/ml trypsin for 30 minutes each at 37°C. Ganglia were triturated and dissociated and plated on collagen coated dishes at a density of 60,000 cells per well for protein lysates or RNA extraction and 10,000 cells per well for survival counts and immunofluorescence experiments. Neurons were maintained in media containing MEM with Earle's salts supplemented with 50 ng/ml NGF, 10% FBS, 2 mM glutamine, 100 ug/ml penicillin, 100 ug/ml streptomycin, 20 uM fluorodeoxyuridine, 20 uM uridine and 3.3 ug/ml aphidicolin. Experiments were preformed on cells 4-5 days after plating. For NGF deprivation, cultures were rinsed twice with medium lacking NGF followed by addition of goat anti-NGF neutralizing antibody to this NGF-free medium. Other conditions required the addition of 2.5 uM tunicamycin, 50 uM zVAD-fmk, or 3 uM CEP-11004. In all of our studies we used a dosage of 2.5 uM tunicamycin. This dosage was based on a dose response we performed

(data not shown) and falls well within the range used in various studies of cultured cells including sympathetic neurons (Chang and Korolev, 1996; Reimertz et al., 2003).

Primary mouse embryonic fibroblasts (MEFs) were isolated from P0 mice. The dorsal skin was removed and treated with 1 mg/ml collagenase, and then with 2.5 mg/ml trypsin for 1 h each at 37°C. Tissue was then triturated and the dissociated cells were plated in DEM supplemented with 10% FBS, 100 ug/ml penicillin and 100 ug/ml streptomycin.

ICR outbreed mice (Harlan Sprague Dawley) were used for all experiments except those involving Bax deficient, Apaf-1 deficient and p53 deficient sympathetic neurons. The genetic background of Bax deficient, Apaf-1 deficient and p53 deficient mice is C57BL/6; wildtype littermates were used as controls in these experiments. We note that the time course of tunicamycin induced death of sympathetic neurons isolated from the C57BL/6 genetic background is faster than in sympathetic neurons isolated from the ICR background. Bax deficient mice were kindly provided by Dr. Stanley Korsmeyer and the Apaf-1 deficient mice were generated by Joachim Herz (UT Southwestern) and were kindly provided by the laboratory of Dr. Susan Ackerman (Jackson Laboratories). p53 deficient mice were purchased from Jackson Laboratories (Maine). Our methods for genotyping Bax deficient mice has been described previously (Deckwerth et al., 1996). The specific primers used to genotype Apaf-1 or p53 deficient mice are shown below:

Apaf-1: Forward WT: 5´ GCCTGCCATCCCATAGATGGT 3´

Forward KO: 5´ GATTGGGAAGACAATAGCAGG 3´

Reverse (common): 5´ CAGCAAGGCCTTTACCTGTTG 3´

WT: 900 base pairs; Apaf-1 KO: 600 base pairs

p53: Forward WT: 5′ ATAGGTCGGCGGTTCAT 3′

Reverse WT: 5′ CCCGAGTATCTGGAAGACAG 3′

Forward KO: 5′ TCCTCGTGCTTTACGGTATC 3′

Reverse KO: 5′ TATACTCAGAGCCGACCT 3′

WT: 600 base pairs; p53 KO: 280 base pairs

Western blots

Western Blots were preformed as previously described (Potts et al., 2003). Primary antibodies were as follows: anti-CHOP/GADD-153 (sc-7351, Santa Cruz Biotechnology Inc); anti-alpha tubulin (T9062, Sigma); anti-phospho-c-Jun (Ser 63) (9261, Cell Signaling Technology); anti-lactate dehydrogenase (200-1173, Rockland Immunochemicals Inc.). Mouse HRP conjugated secondary antibody was purchased from Pierce Chemical Co. Western blots were developed using the ECL-Plus detection system (Amersham Biosciences) and protein levels were quantified by scanning blots on a Typhoon scanner (Amersham Biosciences) and analyzed with ImageQuant software (Amersham Biosciences).

Quantitation of neuronal survival

Sympathetic neuronal survival after any treatment was assessed by counting clearly identifiable neurons with large and phase-bright cell bodies, whereas dead neurons atrophied and degenerated. All surviving cells in the culture were counted and expressed as a percent of the number of cells in the 0 hour condition. This method of assessing neuronal survival

correlates well with other cell survival assays such as trypan blue exclusion and staining with calcein AM (Potts et al., 2003).

In order to determine the commitment of neurons to die, sympathetic neuronal survival, after varying lengths of tunicamycin treatment, was assessed by determining the percentage of cells that could be rescued by washing out the tunicamycin at those times. Sympathetic neurons plated in multiple dishes were treated with tunicamycin in the presence of NGF for the indicated times, rinsed three times, and incubated in fresh NGF containing, tunicamycin-free media with or without CEP-11004 or SP600125 as indicated. Five to seven days after tunicamycin washout, the rescued neurons were clearly identifiable with large and phase-bright cell bodies, whereas the non rescued neurons atrophied and degenerated. All rescued cells in the dish were counted and expressed as a percent of the number of cells in the 0 hour condition.

Rate of MTT Reduction

Culture media was removed and 500 uL of 0.4 mg/ml 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in Leibovitz's L-15 medium containing 10% FBS was added to the neurons for 15 min at 37ºC. The MTT solution was aspirated and cells were lysed into 200 uL dimethylsulfoxide. The amount of MTT formazan was quantitated by determining its absorbance at 490 nM in a Bio-Tek Instruments Inc. ELx808 Ultra Microplate Reader. The same procedure was followed for MEFs except that MTT was in DMEM containing 10% FBS.

Immunofluorescence analysis

Immunofluorescence analysis on sympathetic neuronal cultures was performed as described previously (Deshmukh et al., 2000). Cultures that were treated with NGF deprivation or tunicamycin addition were maintained in the presence of the pan caspase inhibitor 50 uM zVAD-fmk to prevent cell loss. Primary antibodies were as follows: anti-cytochrome *c* (556432, BD Biosciences) and anti-phospho-c-Jun (Ser 63) (9261, Cell Signaling). Secondary antibodies used were anti-mouse Cy3-conjugated (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and anti-rabbit Alexa 488-conjugated (Molecular Probes Inc., Eugene, OR). Nuclei were stained with the nuclear dye bisbenzimide (Hoechst 33258; Molecular Probes Inc., Eugene, OR).

Quantitative RT-PCR analysis

Our method of quantitative RT-PCR analysis is a modification of a previously published protocol, (Estus et al., 1994) where we substituted the radioactivity-based detection method with a fluorescence-based detection technique. Briefly, RNA was isolated from sympathetic neuron cultures with Trizol (Invitrogen) using the manufacturers protocol. Equal amounts of the RNA isolated at specific times after the specified treatment was converted into cDNA with SuperScript II Reverse Transcriptase (Invitrogen). Two uL of cDNA was the template in a PCR using the following primer pairs:

<u>BimEL:</u> Forward 5′ GGT AAT CCC GAC GGC GAA GGG AC 3′; Reverse 5′ AAG AGA AAT ACC CAC TGG AGG ACC 3′

<u>CHOP:</u> Forward 5′ TCA CAC GCA CAT CCC AAA GC 3′; Reverse 5′ TCA GCT AGC TGT GCC ACT TTC C 3′

c-Jun: Forward 5′ AAT GGG CAC ATC ACC ACT ACA C 3′; Reverse 5′ TGC TCG TCG GTC ACG TTC T 3′

Dp5: Forward 5′ AGA CCC AGC CCG GAC CGA GCA A 3′; Reverse 5′ CTC TCT CTG TAG CTG GAC CTC 3′

GAPDH: Forward 5′ CCA TGG AGA AGG CTG GGG 3′; Reverse 5′ CAA AGT TGT CAT GGA TGA CC 3′

Noxa: Forward 5′ GAA CGC GCC AGT GAA CCC AA 3′; Reverse 5′ CTT TGT CTC CAA TCC TCC GG 3′

Puma: Forward 5′ CCT CAG CCC TCC CTG TCA CCA G 3′; Reverse 5′ CCG CCG CTC GTA CTG CGC GTT G 3′

Primers and PCR protocol for XBP-1 were previously described (Harding et al., 2005). Primers for BiP were previously described (Nishitoh et al., 2002). Preliminary experiments with sympathetic neuronal cultures validated that the RT-PCR technique was linear with respect to the amount of input RNA used for RT and with respect to the amount of cDNA used for PCR in these experiments. No product was amplified when water was used as input for a PCR reaction. Results were repeated in at least three independent RNA preparations. Levels were quantified using SYBR Green I Nucleic Acid Gel Stain (Molecular Probes Inc., Eugene, OR) and scanning blots on a Typhoon scanner (Amersham Biosciences) and analyzed with ImageQuant software (Amersham Biosciences).

2.6 Figures and Legends

Figure 2.1: Tunicamycin causes ER stress induced apoptosis in sympathetic neurons Cultures of sympathetic neurons were untreated (NGF), treated with 2.5 uM tunicamycin (NGF + TU) or treated with 2.5 uM tunicamycin and 50 uM zVAD-fmk (NGF + TU + zVAD). **(A)** Levels of CHOP, a marker of ER stress, were examined by Western blot of whole cell lysate from sympathetic neurons collected 36 or 48 hours after 2.5 uM tunicamycin treatment or untreated neurons. Tubulin serves as a loading control. **(B)** mRNA levels of BiP and the spliced form of XBP-1 (U- unspliced, S- spliced), both indicators of ER stress, were examined by RT-PCR after 24 hours of treatment. Cultures were also treated with an alternate ER stress inducer 10 uM thapsigargin (NGF + TG). GAPDH serves as a control. Data are representative of n≥2 separate experiments per timepoint. **(C)** Photographs were taken 60 hours following treatment. **(D)** Survival of sympathetic neurons was accessed by cell morphology at the indicated time points. Data are means ± SEM from n≥3 separate experiments per timepoint.

Figure 2.1

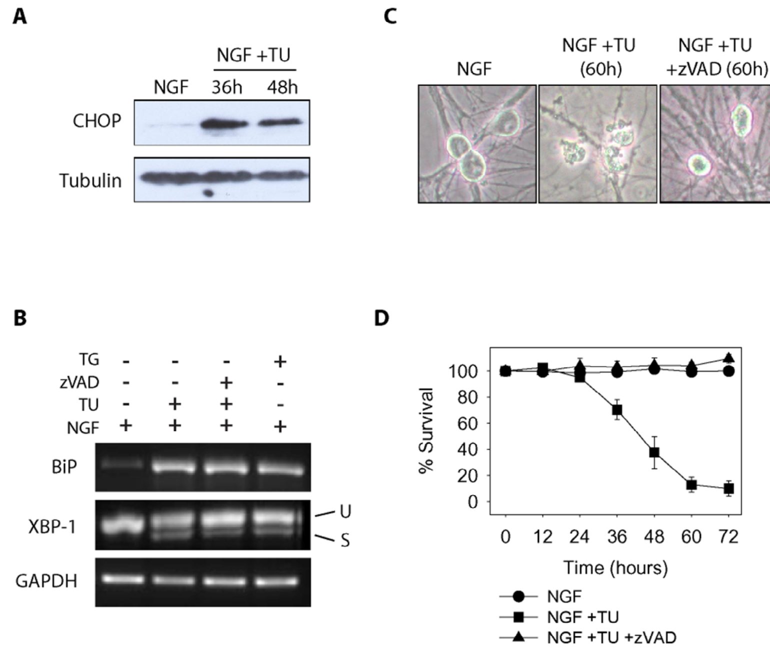


Figure 2.2: **JNK signaling is required for ER stress induced neuronal apoptosis**

(A) Presence of active, phosphorylated c-Jun was examined by immunofluorescence in sympathetic neurons that were untreated (NGF) or treated with 2.5 uM tunicamycin for 18, 24 or 36 hours (NGF + TU). Cultures deprived of NGF for 24 hours (-NGF) served as a positive control for c-Jun phosphorylation. Neurons were also stained with Hoechst to detect nuclei and the percentage of neurons with phospho-c-Jun was quantified as the percentage of nuclei which also had phospho-c-Jun positive staining. Data are means ± SEM from $n \geq 3$ separate experiments per treatment. **(B)** Sympathetic neurons were either untreated (NGF), treated with 2.5 uM tunicamycin (NGF + TU) or treated with 2.5 uM tunicamycin and 3 uM CEP-11004 (NGF + TU + CEP-11004), a MLK inhibitor. Survival was accessed by cell morphology at the indicated times. Data are means ± SEM from $n \geq 3$ separate experiments per timepoint. **(C)** mRNA levels of BiP and the spliced forms of XBP-1 (U- unspliced, S- spliced), both indicators of ER stress, were examined by RT-PCR after 24 hours of treatment. GAPDH serves as a control. Data are representative of $n \geq 2$ separate experiments per timepoint.

Figure 2.2

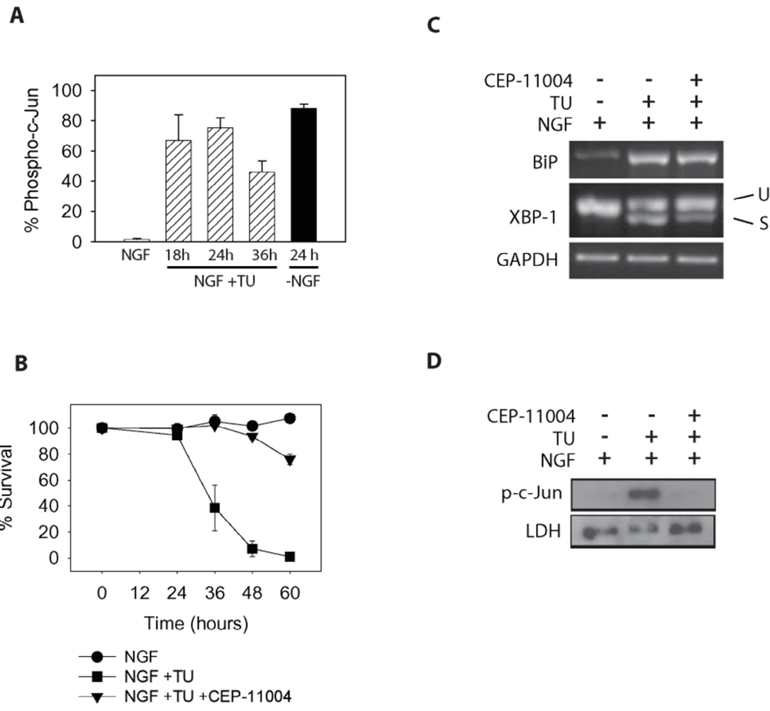


Figure 2.3: BH3-only proteins Puma and DP5 are transcriptionally upregulated independently of MLK signaling following ER stress in sympathetic neurons

(A) Cultured sympathetic neurons were untreated (NGF), treated with tunicamycin for 12, 18, or 24 hours (NGF+ TU) or deprived of NGF for 18 hours (-NGF) prior to collection of total RNA. All cultures were treated with 50 uM zVAD-fmk to prevent cell loss. RT-PCR was carried out with primers for the indicated proteins. CHOP levels were examined to ensure ER stress was being induced. GAPDH serves as a loading control. **(B)** Quantification of (A) normalized to GAPDH levels. Data are means ± SEM from n≥3 separate experiments per treatment. **(C)** Cultured sympathetic neurons were untreated (NGF), treated with 2.5 uM tunicamycin for 18 hours in the absence (NGF + TU) or presence of 3 uM CEP-11004 (NGF +TU+CEP11004) or deprived of NGF for 18 hours in the absence (-NGF) or presence of 3 uM CEP-11004 (-NGF + CEP11004) prior to collection of total RNA. All cultures were treated with 50 uM zVAD-fmk to prevent cell loss. RT-PCR was carried out with primers for the indicated proteins. CHOP levels were examined to ensure ER stress was being induced. GAPDH serves as a loading control. **(D)** Quantification of (C) normalized to GAPDH levels. Data are means ± SEM from n≥3 separate experiments per treatment.

Figure 2.3

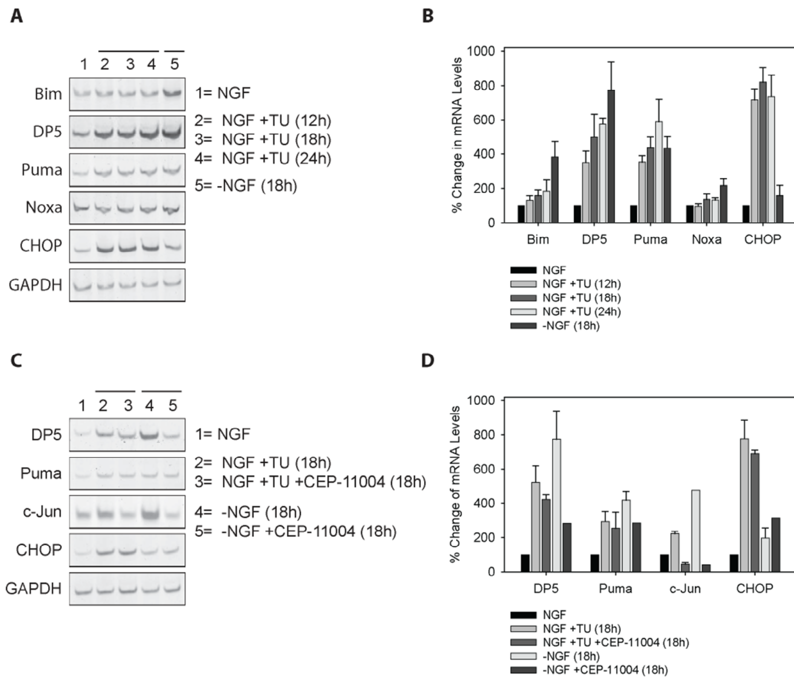


Figure 2.4: Bax deficiency blocks ER stress induced apoptosis allowing for long term survival of sympathetic neurons

(A) Sympathetic neurons from Bax deficient mice (Bax-/-) and their wildtype littermates (Bax+/+) were untreated (NGF) or treated with 2.5 uM tunicamycin (NGF + TU). Neuronal survival was determined by cell morphology at each of the indicated timepoints. Data are means ± SEM from n≥3 separate experiments per timepoint. **(B)** Photographs of wildtype and Bax deficient sympathetic neurons treated with 2.5 uM tunicamycin for 48 hours. **(C)** Photographs of wildtype (Bax+/+) and Bax deficient (Bax-/-) sympathetic neurons untreated or treated with 2.5 uM tunicamycin for 11 days. Fresh media containing tunicamycin was replenished every three days to ensure a sustained ER stress response. Photographs were taken at the same magnification. **(D)** Sympathetic neurons from Bax deficient (Bax-/-) and wildtype (Bax+/+) mice were untreated (NGF) or treated with 2.5uM tunicamycin (NGF + TU) for 24 hours. Using RT-PCR, the spliced form of XBP-1 (U- unspliced, S- spliced) was examined as an indicator of IRE1 activity, and level of BiP was examined as a marker of ER stress. GAPDH serves as a control. Data are representative of n≥2 separate experiments per timepoint.

Figure 2.4

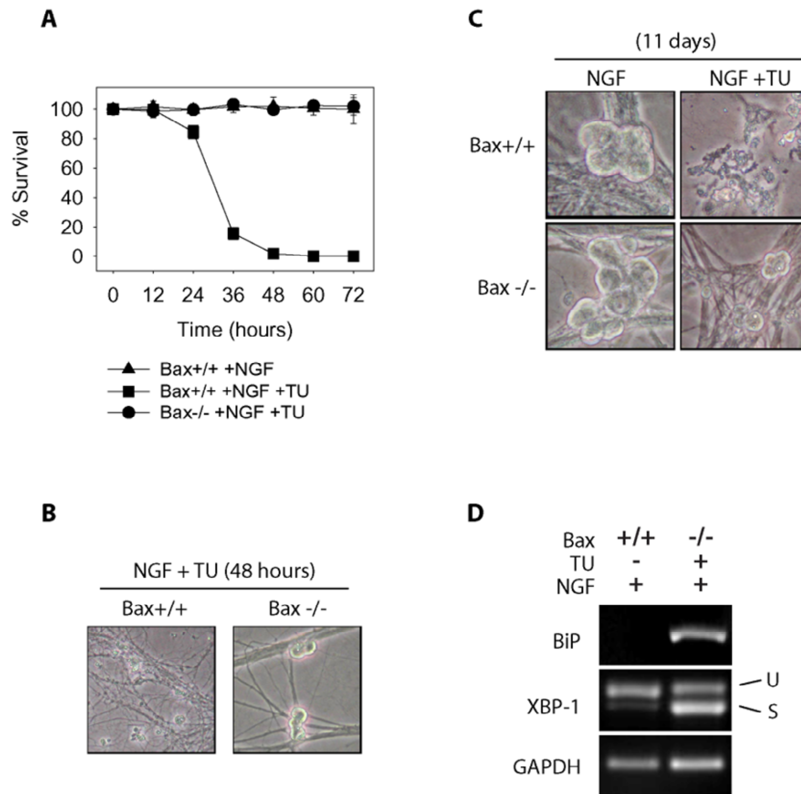


Figure 2.5: ER stress induced commitment to death occurs well before cytochrome *c* release and is blocked by Bax deficiency and by inhibiting JNK signaling

(A) Sympathetic neurons were treated with 2.5 uM tunicamycin and the death, release of cytochrome *c* and commitment to die were quantified. Cell morphology was assessed to determine the percent of death at the indicated time points. Cytochrome *c* release was determined by immunofluorescence. These immunofluorescence studies were done on cultures treated with 50 uM zVAD-fmk to prevent cell loss. Neurons were also stained with Hoechst to detect nuclei and the percentage of neurons which had released cytochrome *c* was measured as a percentage of cells which had nuclei but lacked intact punctate cytoplasmic cytochrome *c* staining. Commitment to die studies were carried out as indicated and explained in the results and materials and method sections. The percent of neurons committed to die was determined as the percent of the cells lost after the recovery period (5 to 7 days) relative to those present prior to the treatment period (length of the treatment period is as indicated on the graph). **(B)** Sympathetic neurons isolated from Bax deficient mice (Bax-/-) or their wildtype littermates (Bax+/+) were exposed to 2.5 uM tunicamycin and commitment was determined as described in (A). **(C)** Sympathetic neuronal cultures were exposed to 2.5 uM tunicamycin and commitment studies were carried out as described in (A) with the exception that neurons were either saved with NGF containing media or NGF containing media with 3 uM CEP-11004. **(D)** Sympathetic neuronal cultures were exposed to 2.5 uM tunicamycin and commitment studies were carried out as described in (A) with the exception that neurons were either saved with NGF containing media or NGF containing media with 10 uM SP600125. Data are means ± SEM from $n \geq 2$ separate experiments per timepoint.

Figure 2.5

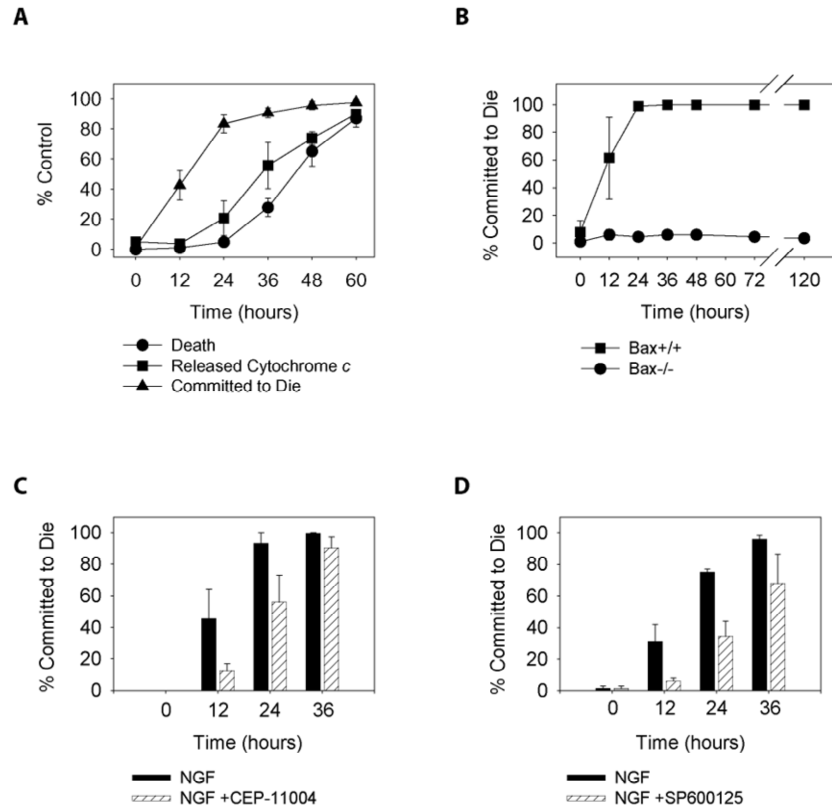
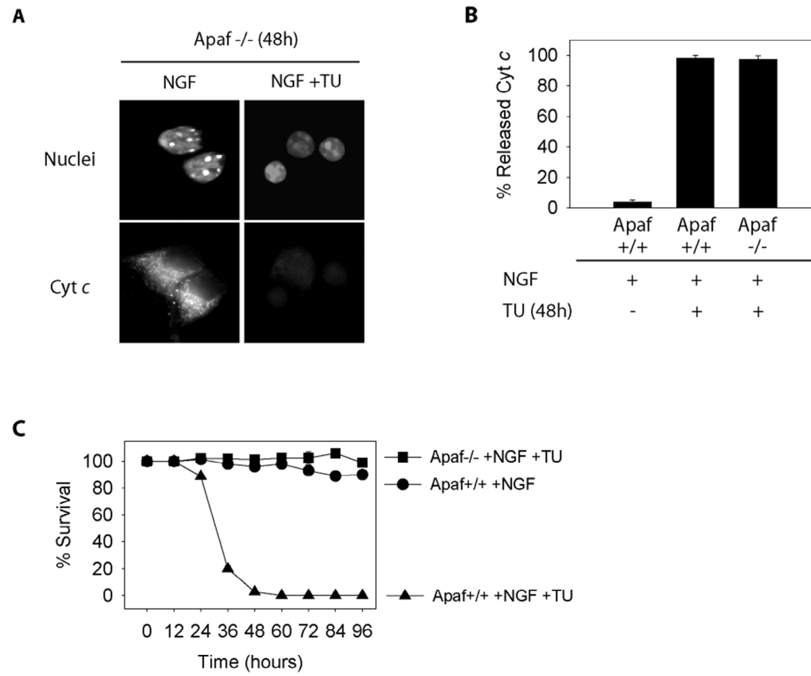


Figure 2.6: Apaf-1 deficient sympathetic neurons are able to release cytochrome *c* but do not undergo ER stress induced apoptosis

(A) Apaf-1 deficient sympathetic neurons (Apaf -/-) were untreated (NGF) or treated with 2.5 uM tunicamycin (NGF + TU) for 48 hours. Neurons were also stained with Hoechst to detect nuclei. **(B)** Apaf-1 deficient sympathetic neurons (Apaf -/-) and their wildtype littermates (Apaf +/+) were untreated (NGF) or treated with 2.5 uM tunicamycin (NGF + TU) for 48 hours in the presence of 50 uM zVAD-fmk. Neurons were also stained with Hoechst 3358 to detect nuclei and the percentage of neurons which had released cytochrome *c* was measured as those which had nuclei but lacked intact punctuate cytoplasmic cytochrome *c* staining. Data are means ± SEM from n≥3 separate experiments per treatment. **(C)** Apaf-1 deficient sympathetic neurons (Apaf -/-) and their wildtype littermates (Apaf +/+) were untreated (NGF) or treated with 2.5 uM tunicamycin (NGF + TU). Cell survival was accessed by morphology at the indicated timepoints. Data are means ± SEM from n≥3 separate experiments per timepoint.

Figure 2.6

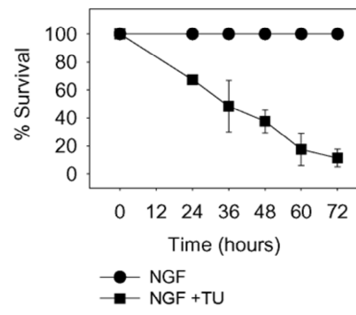


Supplemental Figure 2.1: Tunicamycin causes ER stress induced apoptosis in sympathetic neurons as measured by MTT reduction. Thapsigargin induces ER stress induced apoptosis in sympathetic neurons which is inhibited by zVAD and CEP-11004

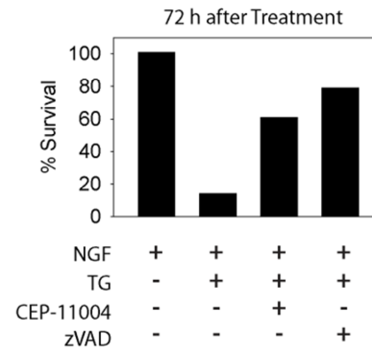
(A) Sympathetic neurons were untreated (NGF) or treated with 2.5 uM tunicamycin (NGF + TU). Neuronal survival was determined by MTT reduction at each of the indicated timepoints. Data are means ± from n≥2 separate experiments per timepoint. **(B)** Cultures of sympathetic neurons were untreated (NGF), treated with 10 uM thapsigargin (TG), 10 uM thapsigargin and 3 uM CEP-11004, or 10 uM thapsigargin and 50 uM zVAD-fmk (zVAD). Survival of sympathetic neurons was accessed by cell morphology at 72 hours following treatment. Data are representative of n≥2 separate experiments per condition.

Supp. Figure 2.1

A



B

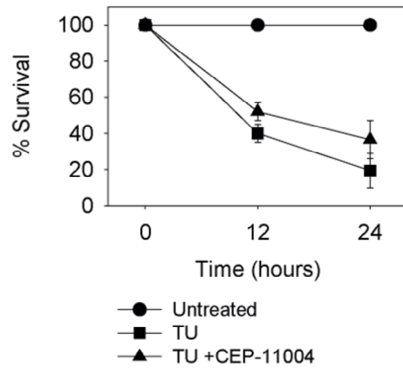


Supplemental Figure 2.2: Tunicamycin causes cell death of MEFs which is not inhibitable by CEP-11004

MEFs were untreated, treated with 2.5 uM tunicamycin (TU) or treated with 2.5 uM tunicamycin with 3 uM CEP-11004 (TU + CEP11004). **(A)** Survival was determined by MTT reduction at each of the indicated timepoints. Data are means ± from n≥2 separate experiments per timepoint. **(B)** Photographs were taken 24 hours following treatment.

Supp. Figure 2.2

A



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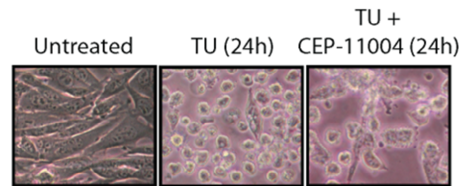
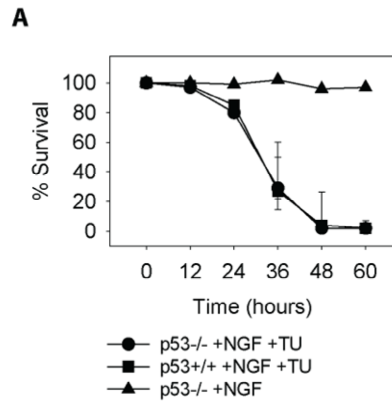
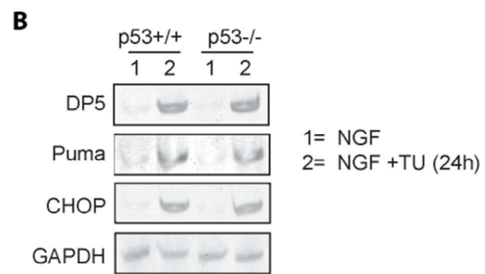


Supplemental Figure 2.3: P53 deficiency does not block sympathetic neuronal apoptosis or the upregulation of BH3-only proteins Puma and DP5 after ER stress

Sympathetic neurons from p53 deficient mice (p53-/-) and their wildtype littermates (p53+/+) were untreated (NGF) or treated with 2.5 uM tunicamycin (NGF + TU). **(A)** Neuronal survival was determined by cell morphology at each of the indicated timepoints. Data are means ± SEM from $n \geq 3$ separate experiments per timepoint. **(B)** RT-PCR was carried out with primers for the indicated proteins. CHOP levels were examined to ensure ER stress was being induced. GAPDH serves as a loading control. Data are representative of $n \geq 2$ separate experiments per treatment.

Supp. Figure 2.3

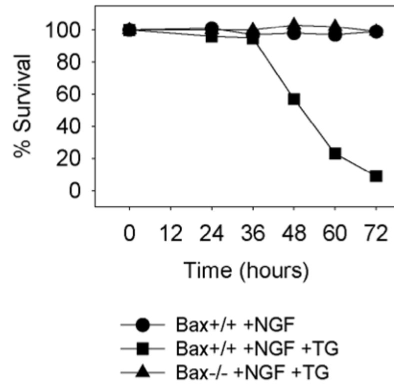




Supplemental Figure 2.4: Bax deficiency blocks thapsigargin induced apoptosis of sympathetic neurons

(A) Sympathetic neurons from Bax deficient mice (Bax-/-) and their wildtype littermates (Bax+/+) were untreated (NGF) or treated with 10 uM thapsigargin (NGF + TG). Neuronal survival was determined by cell morphology at each of the indicated timepoints.

Supp. Figure 2.4



CHAPTER THREE

SKELETAL MUSCLE DIFFERENTIATION EVOKES ENDOGENOUS XIAP TO RESTRICT THE APOPTOTIC PATHWAY

3.1 Abstract

Myotube apoptosis occurs normally during muscle development and aging but it can lead to destruction of skeletal muscle in neuromuscular diseases. Therefore, understanding how myotube apoptosis is regulated is important for developing novel strategies for treatment of muscle loss. We investigated the regulation of apoptosis in skeletal muscle and report a striking increase in resistance to apoptosis following differentiation. We find mitotic C2C12 cells (myoblast-like cells) are sensitive to cytosolic cytochrome *c* microinjection. However, differentiated C2C12 cells (myotube-like cells) and primary myotubes are markedly resistant. This resistance is due to endogenous X-linked inhibitor of apoptotic proteins (XIAP). Importantly, the selective difference in the ability of XIAP to block myotube but not myoblast apoptosis is not due to a change in XIAP but rather a decrease in Apaf-1 expression. This decrease in Apaf-1 links XIAP to caspase activation and death. Our findings show that in order for myotubes to die they must either degrade XIAP, functionally inactivate XIAP or upregulate Apaf-1. Importantly, we identify a role for endogenous Smac in overcoming XIAP to allow myotube death. Our results show that as skeletal muscle differentiate; they become resistant to apoptosis because of the ability of XIAP to regulate caspase activation. The increased restriction of apoptosis in myotubes is presumably important to ensure the long term survival of these postmitotic cells as they play a vital role in the physiology of organisms.

3.2 Introduction

Skeletal muscle is a highly specialized tissue that is unique in its structure and development. Individual myotubes that comprise skeletal muscle are derived from mitotic myoblasts which, under the right environmental cues begin to express myogenic markers, exit the cell cycle and fuse to form long multinucleated myotubes (Walsh and Perlman, 1997). While the molecular details of this differentiation process are well understood, very little is known about whether fundamental biological processes such as apoptosis are altered during this process of differentiation. Understanding this phenomenon is important because, following the developmental period, muscle loss can have deleterious effects. For example, conditions such as muscular dystrophies, neurogenic muscular atrophy and mitochondrial myopathies result in skeletal muscle death involving apoptosis (Tews, 2002).

Apoptosis is a genetically regulated, evolutionarily conserved form of cell death. It is characterized by the activation of caspase proteases that cleave numerous substrates within the cell to cause the demise of the cell (Fuentes-Prior and Salvesen, 2004). In the intrinsic pathway of apoptosis which can be activated by various stressors such as growth factor withdrawal, ER stress and DNA damage, signaling pathways converge upon the proapoptotic proteins Bax and Bak. This causes their activation and translocation to the mitochondria where they release cytochrome *c* from the intermembrane space. Once free in the cytosol, cytochrome *c* binds to the adapter protein Apaf-1. This binding induces a conformational change in Apaf-1 in such a way that Apaf-1 oligomerizes as well as binds to procaspase 9 to form the apoptosome complex. Once on the apoptosome, caspase 9 becomes active and cleaves procaspase 3 into its active form. Active caspase 3 is known as the executioner

caspase because it cleaves various proteins ultimately leading to the death of the cell (Hengartner, 2000; Wang, 2001).

Recent reports indicate that myoblasts utilize an alternative mechanism of activating caspases (Ho and Zacksenhaus, 2004). Activation of caspases 9 in these cells occurs independently of Apaf-1 but still requires release of endogenous Smac from the mitochondria (Ho et al., 2004; Ho et al., 2007). Smac is a mitochondrial intermembrane space protein which acts as an inhibitor of an antiapoptotic family of proteins known as the Inhibitor of Apoptotic Proteins (IAPs) (Chai et al., 2000; Du et al., 2000). IAPs regulate apoptosis by binding to and inhibiting caspases (Eckelman et al., 2006). Despite our knowledge of the structure and function of Smac, a critical role for endogenous Smac in regulating apoptosis has not been discovered in other primary cells. Importantly, what happens to this pathway upon differentiation of myoblasts into myotubes is unknown. Skeletal muscles are known to become more resistant to apoptosis upon differentiation (Sandri and Carraro, 1999). However, as most of the studies examining skeletal muscle apoptosis have focused on whole tissue, the mechanism for this increased resistance has not been clearly identified at the cellular level.

In this study, we investigated how isolated myotubes regulate their caspase activation following differentiation. We report that myotubes exhibit an increase in their resistance to apoptosis relative to their mitotic precursor cells. While mitotic C2C12 cells (mC2C12) die with the introduction of cytochrome *c* into their cytosol, differentiated C2C12 (dC12C12) cells and primary myotubes do not. This increased resistance is due to endogenous XIAP. We show that endogenous XIAP is able to selectively block caspase activation in myotubes not because its levels are increased in myotubes but rather because the level of Apaf-1 is

dramatically decreased. Importantly, our studies identify endogenous Smac as having a vital role in overcoming this XIAP inhibition in myotubes.

3.3 Results

Myotubes develop resistance to cytochrome c-induced apoptosis upon differentiation

To determine whether the cytochrome *c*-mediated caspase activation pathway becomes restricted with skeletal muscle differentiation, we microinjected cytochrome *c* into myoblast-like C2C12 (mC2C12) cells, differentiated C2C12 cells (dC2C12) and primary myotubes. mC2C12 cells were very sensitive to bovine cytochrome *c* with almost a complete loss of injected cells within one hour (Fig. 3.1a, b). Yeast cytochrome *c* serves as an ideal control because unlike mammalian cytochrome *c*, it is not capable of binding to Apaf-1, and therefore, cannot activate the apoptosome (Ellerby et al., 1997). As anticipated, mC2C12 cells injected with yeast cytochrome *c* did not die, indicating that microinjection alone was not killing these cells (Fig. 3.1a, b). In striking contrast to the mC2C12 cells, C2C12 cells that had been differentiated for nine days (dC2C12) did not undergo death when injected with bovine or yeast cytochrome *c* (Fig. 3.1a, b). To examine primary cells, we isolated murine myoblasts. While the small size of primary myoblasts made them technically difficult to microinject, we were able to differentiate them in culture for 14 days into myotubes and inject these myotubes. Just as seen with the dC2C12 cells, primary myotubes were markedly resistant to bovine cytochrome *c* (Fig. 3.1a). Together, this data indicated that while mC2C12 cells were sensitive to cytochrome *c* induced apoptosis, post-mitotic dC2C12 cells and primary myotubes developed resistance.

During differentiation, myoblasts exit the cell cycle and fuse to form myotubes (Walsh and Perlman, 1997). To determine at what point myotubes gained resistance to cytosolic cytochrome *c*, we injected dC2C12 cells and primary myotubes at different days of differentiation. Following six days of differentiation, C2C12 cells still remained sensitive to

cytosolic cytochrome *c* injections, showing complete apoptosis one hour after injection. However, by seven days of differentiation they had developed some resistance as over 50 % of cells survived three hours following injection. By nine days of differentiation, almost all cells survived cytochrome *c* injection (Fig. 3.1a, b, c). Differentiating primary myotubes also showed a gradual resistance to cytochrome *c*. Four days into the differentiation process only 30 % survived one hour post cytochrome *c* injection. However, by 11 days this survival increased to about 60 % and almost complete protection was seen by 14 days (Fig. 3.1d). Injecting yeast cytochrome *c* did not induce death at any stage of differentiation. These data suggest that as myotubes differentiate they gradually develop resistance to cytochrome *c* and loose their ability to undergo apoptosis in response to cytochrome *c*.

Myotube resistance to cytochrome c can be overcome with the exogenous addition of the IAP inhibitor Smac or genetic deletion of XIAP

Resistance to cytochrome *c* has been seen in primary neurons and cardiomyocytes, where it has been linked to the function of endogenous XIAP (Potts et al., 2005; Potts et al., 2003; Wright et al., 2004). To determine if IAPs also play a role in the myotube resistance to cytochrome *c*, we injected both dC2C12 cells and primary myotubes with the IAP inhibitor Smac. When cytochrome *c* and Smac were coinjected into dC2C12 cells or primary myotubes, it resulted in rapid and complete death (Fig. 3.2). Control injections with cytochrome *c* or Smac alone did not induce much death in these cells. To ensure that it was the IAP inhibiting function of Smac that was responsible for this action, we also injected cytochrome *c* into dC2C12 cells with a mutant form of Smac (MVPI-Smac) carrying a single point mutation that does not allow it to bind and inhibit IAPs (Chai et al., 2000). In contrast

to wildtype Smac (AVPI-Smac), coinjection of cytochrome *c* and mutant MVPI-Smac did not result in any significant cell death (Fig. 3.2a).

These Smac injection experiments suggested that IAPs were in fact responsible for inhibiting cytochrome *c* induced death in myotubes. Since XIAP has been shown to be the most effective IAP at inhibiting apoptosis (Eckelman et al., 2006), we tested whether endogenous XIAP was responsible for restricting myotube apoptosis. Myoblasts were isolated from XIAP-deficient mice and differentiated in culture for 14 days prior to injection with cytochrome *c*. In contrast to wildtype myotubes, XIAP-deficient myotubes were strikingly sensitive to cytochrome *c* and underwent apoptosis by two hours following injection (Fig. 3.1a, 3.3). XIAP-deficient myotubes injected with yeast cytochrome *c*, as a control, did not die. Taken together, these data suggest that myotubes are unable to undergo apoptosis in response to cytochrome *c* due to the strict control of caspase activation by endogenous XIAP.

Endogenous XIAP effectively restricts cytochrome c induced death in myotubes due to reduced Apaf-1 levels

XIAP is a ubiquitously expressed protein (Duckett et al., 1996). To determine why postmitotic myotubes have selectively developed this XIAP brake in apoptosis, we looked at the level of XIAP in these mitotic and postmitotic cells. We found XIAP levels to be the same in mC2C12 versus dC2C12 and primary myoblasts versus primary myotubes (Fig. 3.4a, b). However, when we examined the level of other apoptotic proteins we found that Apaf-1 levels were decreased in both dC2C12 cells and primary myotubes relative to their mitotic precursors (Fig. 3.4a, b). This decrease in Apaf-1 appears to be at the transcriptional level as

the mRNA levels are decreased in primary myotubes relative to myoblasts (Fig. 3.4c). These results lead us to examine whether Apaf-1 was limiting for caspase activation in myotubes. To test this, we injected plasmids for Apaf-1 and GFP in dC2C12 cells. Twenty four hours following injections, GFP expressing cells were injected with cytosolic cytochrome *c*. Expression of Apaf-1 alone in dC2C12 cells did not induce death and the cells remained resistant to control injection of yeast cytochrome *c*. In contrast, injection of bovine cytochrome *c* was able to induce death in the Apaf-1 overexpressing dC2C12 cells. Cells injected with vector and GFP showed significantly less death with bovine cytochrome *c* (Fig. 3.4d). Thus, expressing Apaf-1 was sufficient to allow cytochrome *c*-mediated death in myotubes. Together, these results suggest that the decreased levels of Apaf-1 in myotubes results in limited caspase activation, thus allowing endogenous XIAP to effectively protect against cytochrome *c*-mediated death. Consistent with this model, our results show that increasing Apaf-1 levels overcame this XIAP inhibition and rendered the myotubes sensitive to cytochrome *c* injections.

Endogenous Smac can overcome XIAP inhibition in myotubes

Despite this increase in resistance to apoptosis, myotubes undergo apoptosis during development (Sandri and Carraro, 1999) and in response to pathological stimuli (Tews, 2002). Our experiments suggest that in order for myotubes to undergo apoptosis, they would not only have to release cytochrome *c* but also overcome the function of XIAP in order to become competent to die. This could occur by decreasing XIAP levels (Fig. 3.3), upregulating of Apaf-1 (Fig. 3.4d) or by functional inactivation of XIAP (Fig. 3.2). There are several known potential IAP inhibitors in cells, two of which, HtrA2 and Smac, are

localized to the mitochondria. As structural studies have cast doubts on the IAP inhibitory activity of HtrA2 (Li et al., 2002b), we focused on Smac. Our data shows that excess exogenous Smac is able to permit cytochrome *c*-mediated apoptosis in myotubes (Fig. 3.2), but whether the release of *endogenous* Smac is capable of doing so is unknown.

To examine the importance of endogenous Smac in inhibiting XIAP and permitting apoptosis, we took advantage of the proapoptotic Bcl-2 family member tBid in order to release endogenous cytochrome *c*, Smac and other factors from the mitochondria (Van Loo et al., 2002). Plasmids for tBid and GFP were injected into dC2C12 cells and primary myotubes and survival was assessed 24 hours later. Unlike cytochrome *c* injection, tBid expression induced potent death in these cells. This death was apoptotic as it was blocked with the pancaspase inhibitor Q-VD-OPH (Fig. 3.5). These data suggest that tBid is able to release cytochrome *c* and presumably other mitochondrial factor(s) to permit a caspase-mediated apoptotic death in myotubes.

To directly determine if endogenous Smac was responsible for overcoming the function of XIAP in this tBid-mediated death, we isolated myoblasts from Smac-deficient mice. Once differentiated, we injected tBid and GFP into these myotubes. Unlike wildtype myotubes, Smac-deficient myotubes were completely resistant to tBid induced death (Fig. 3.5c). These results identify an important role for endogenous Smac in myotubes and imply that if Smac were released from the mitochondria this would be sufficient to overcome the XIAP brake and allow cytochrome *c* to induce myotube apoptosis.

3.4 Discussion

Together, the data presented in this study identify that postmitotic myotubes have increased suppression of their apoptotic pathway relative to their mitotic precursors. Differentiated C2C12 cells and primary myotubes were both resistant to cytosolic cytochrome *c* due to the activity of endogenous XIAP (Fig. 3.1, 3.2, 3.3). However, myotubes inhibit caspase activation not by *increasing* XIAP levels but rather by *decreasing* Apaf-1 (Fig. 3.4a, b, c). Importantly, overexpressing Apaf-1 alone in dC2C12 cells, is sufficient to allow cytosolic cytochrome *c* injection to kill dC2C12 cells (Fig. 3.4d). Therefore, an Apaf-1 reduction is sufficient to set up this differential resistance between mitotic precursors and myotubes. It is noteworthy to mention that a previous study found that human skeletal muscle cytosol completely lacks Apaf-1 and therefore is refractory to cytochrome *c*-mediated caspase activation (Burgess et al., 1999). This difference could be attributed to the different model systems or the age of the skeletal muscle used, suggesting that skeletal muscle continue to reduce Apaf-1 expression with age.

Based on this data, we propose that following cytochrome *c* release from the mitochondria, the low levels of Apaf-1 present in myotubes result in reduced apoptosome formation and caspase activation. As a consequence, endogenous XIAP is sufficient to effectively block this reduced level of caspase activation. However, in mC2C12 cells, high levels of Apaf-1 result in robust apoptosome formation and thus increased caspase activation that cannot be successfully inhibited by endogenous XIAP. As a consequence, these mitotic cells readily undergo apoptosis with cytosolic cytochrome *c* while myotubes do not.

This increased resistance to apoptosis employed by myotubes is strikingly similar to that found in other postmitotic cells, specifically neurons and cardiomyocytes (Potts et al.,

2005; Potts et al., 2003; Wright et al., 2004). These findings suggest that regardless of the function or phenotype of the cell, postmitotic cells share the same features in restricting their ability to undergo apoptosis. By requiring not only cytochrome *c* release but also inhibition of XIAP function in order to activate caspases, endogenous XIAP serves as a safety brake to death. For example, if the mitochondria accidentally release cytochrome *c*, the presence of XIAP would block caspase activation and therefore prevent these cells from undergoing unwanted apoptosis. Arguably, increased resistance to caspase activation is critical to these postmitotic cells because of their inability to replicate and their limited regenerative potential. These postmitotic cells also serve vital functions that require their presence for the lifetime of the organism. On the other hand, not having this resistance to apoptosis in mitotic cells is beneficial to the organism as mitotic cells can give rise to cancer. Indeed, the mechanisms by which the apoptotic pathway is inactivated in many cancers are similar to those seen in postmitotic cells. For example, several melanomas restrict their Apaf-1 expression at the transcriptional level in order to block apoptosis (Fu et al., 2003; Furukawa et al., 2005; Soengas et al., 2001). In addition, many chemoresistant cancers have been found to utilize XIAP to effectively block caspase activation (Beauparlant and Shore, 2003).

Despite the ability of myotubes to restrict apoptosis there are certainly circumstances in which myotubes activate caspases and die. Caspase-mediated death has been found during development and to some extent in pathological conditions including muscular dystrophies, neurogenic muscular atrophy and mitochondrial myopathies (Tews, 2002). Based on our findings, death stimuli that activate caspases in myotubes would not only have to cause the release of cytochrome *c* but also inhibit XIAP. Here we identified three ways in which this could occur. First, as XIAP-deficient myotubes are completely sensitive to cytosolic

cytochrome *c* (Fig. 3.3), a stimulus which is able to degrade or cleave XIAP and release cytochrome *c* would be able to kill myotubes. In fact, selective XIAP degradation has been seen in neurons undergoing apoptosis in response to nerve growth factor withdrawal (Potts et al., 2003). Second, dC2C12 cells overexpressing Apaf-1 became sensitive to cytochrome *c*-mediated death (Fig. 3.4d). Therefore, a death stimulus which increases Apaf-1 expression would be able to overcome XIAP inhibition. Supporting this idea, it has been shown that skeletal muscle undergoing caspase activation due to metabolic deficiencies with ATP-depletion and oxidative stress show an increase in Apaf-1 levels (Ikezoe et al., 2002). Third, XIAP could also be functionally inhibited in response to a death stimulus. This could occur through several different mechanisms including an inactivating posttranslational modification of XIAP or via an endogenous inhibitor of XIAP. Here we show that releasing endogenous Smac from the mitochondria is indeed sufficient to overcome XIAP and allow cytochrome c to activate caspases (Fig. 3.5c).

While the role of Smac as an IAP inhibitor has been extensively characterized *in vitro* (Chai et al., 2000; Du et al., 2000), the role of endogenous Smac has remained elusive since most cells do not need to inhibit XIAP to die (Harlin et al., 2001; Okada et al., 2002). Here we show that endogenous Smac is required for myotube death initiated by the permeabilization of the outer mitochondrial membrane (Fig. 3.5). While neurons engage the XIAP brake just like myotubes, one striking contrast between these two postmitotic cells is that neurons do not appear to utilize endogenous Smac to overcome XIAP (Potts et al., 2003). Interestingly, other than myotubes, the only cell type in which a role for endogenous Smac has been identified is their precursor, myoblasts (Ho et al., 2007). This raises the

intriguing possibility that endogenous Smac may play a vital role in this particular myogenic cell lineage but not in other cell types.

A potential significance of this XIAP brake can be seen in mitochondrial encephalomyopathies, a group of heterogeneous disorders due to mutations in either mitochondrial DNA or nuclear genes. These mutations lead to mitochondrial abnormalities which ultimately result in a decrease in ATP synthesis and increased oxidative stress. Due to the heterogeneous nature of these deficiencies even within the same patient, some fibers appear healthy, some appear to suspend apoptosis and others seem to die with active caspase 3 immunoreactivity (Aure et al., 2006; Ikezoe et al., 2002; Mirabella et al., 2000). Many fibers from mitochondrial encephalomyopathies show clear Bax upregulation and cytochrome *c* release. However, the number of fibers that show TUNEL staining is much lower (Ikezoe et al., 2002), suggesting that in these fibers cytochrome *c* is released but cannot undergo caspase-mediated death. Our data would predict that in these fibers, it is XIAP that is able to prevent apoptosis despite the mitochondrial release of cytochrome *c*. A potential role of endogenous Smac can be seen *in vivo* as well, in patients with neurogenic muscular atrophy. In this condition, where patients experience apoptotic muscle fiber loss, there is an upregulation of multiple IAPs, including XIAP, but also Smac. In addition, Smac appears to become released into the sarcoplasm (Tews et al., 2008). Our findings would suggest that this release of Smac would be able to overcome XIAP and allow the affected muscle fibers to die.

3.5 Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich or Fisher Scientific, unless otherwise stated. Q-VD-OPH was purchased from MP Biomedicals. Protease inhibitor cocktail was purchased from Roche. XIAP-deficient mice were obtained from Dr. Craig B. Thompson (University of Pennsylvania). Smac-deficient mice were obtained from Dr. Tak W. Mak (University of Toronto). Our procedure for genotyping these mice was previously described (Potts et al., 2003).

C2C12 cell line and primary cultures

C2C12 cell line was maintained in DMEM containing 20 % FBS, 100 mg/ml penicillin and 100 mg/ml streptomycin. When C2C12 cultures reached 70-90 % confluency they were differentiated by changing the media to differentiation medium consisting of DMEM supplemented with 5% horse serum, 100 mg/ml penicillin and 100 mg/ml streptomycin. Satellite cell derived primary myoblasts were isolated from lower hind limb muscle from mice ranging in age from two to four weeks old as described previously (Megeney et al., 1996). The primary cultures were maintained on collagen-coated dishes in Ham's F10 supplemented with 20 % FBS, 2.5 ng/ml bFGF, 100 mg/ml penicillin, 100 mg/ml streptomycin, and 0.002 % Fungizone. The medium was changed every other day and cultures were differentiated with the addition of differentiation media when they reached 60-70 % confluency. All experiments were performed using primary cultures that had undergone between four and twelve passages. All experiments were performed on dC2C12 cells

following 9 days of differentiation and on primary myotubes following 14 days of differentiation unless otherwise indicated.

Microinjection

Cells were plated on 35mm dishes and microinjected with needles pulled on a Flaming-Brown horizontal micropipette puller (Sutter Instruments) using a Narashigi micromanipulator mounted on a Leica inverted florescent microscope. Between 25 and 100 cells were injected in each experiment. The microinjection buffer contained 100 mM KCl and 10 mM KP_i, pH 7.4. For injections involving plasmid DNA, cells were injected and allowed 24 hours to express plasmid DNA prior to experimentation. DNA microinjections contained 50 ng/µl enhanced GFP (Clontech) and 200 ng/µl of indicated plasmid. Cytochrome *c* microinjections contained 5 mg/ml rhodamine dextran to mark injected cells and 25 ug/ul cytochrome *c*. Where indicated, 1 mg/ml recombinant Smac protein was injected along with 25 ug/uL cytochrome *c*. Following injections, viable cells were identified as rhodamine positive and intact. Data shown are mean ±SEM of three independent experiments.

Quantitation of cell survival

Cell survival after any treatment was assessed by counting clearly identifiable cells with intact morphology, whereas dead cells atrophied and degenerated. All surviving cells in the culture were counted and expressed as a percent of the number of cells in the 0 hour condition. This method of assessing survival correlates well with other cell survival assays such as trypan blue exclusion and staining with calcein AM (Potts et al., 2003).

Western Blots

Western Blots were performed as previously described (Potts et al., 2003). Primary antibodies were as follows: anti-Apaf-1 (Alexis), anti-XIAP (MBL) and anti-alpha tubulin (Sigma). Mouse/rabbit/goat HRP conjugated secondary antibodies were purchased form Pierce Chemical Co. Western blots were developed using the ECL-Plus detection system (Amersham Biosciences).

Quantitative RT-PCR analysis

Our method of quantitative RT-PCR analysis is a modification of a previously published protocol (Estus et al., 1994), where we substituted the radioactivity-based detection method with a fluorescence-based detection technique. Briefly, RNA was isolated from cells with DNeasy kit (Qiagen) using the manufacturer's protocol. Equal amounts of the RNA isolated at specific times after the specified treatment was converted into cDNA with SuperScript II Reverse Transcriptase (Invitrogen). One uL of cDNA was the template in a PCR using the following primer pairs:

<u>APAF-1:</u> Forward 5' GAG GCA CAA TGG ATG CAA AGG 3'; Reverse 5' GGC TGC TCG TTG ATA TTG AGT GG 3'

<u>GAPDH:</u> Forward 5' CCA TGG AGA AGG CTG GGG 3'; Reverse 5' CAA AGT TGT CAT GGA TGA CC 3'

` Preliminary experiments validated that the RT-PCR technique was linear with respect to the amount of input RNA used for RT and with respect to the amount of cDNA used for PCR in these experiments. No product was amplified when water was used as input for a PCR reaction. Results were repeated in at least three independent RNA preparations. Levels

were quantified using SYBR Green I Nucleic Acid Gel Stain (Molecular Probes Inc., Eugene, OR) and scanning blots on a Typhoon scanner (Amersham Biosciences).

Image Acquisition and Processing

All images were acquired by a Hamamatsu ORCA-ER digital B/W CCD camera mounted on a Leica inverted fluorescence microscope (DMIRE 2). The image acquisition software was Metamorph version 5.0 (Universal Imaging Corporation). Images were scaled down and cropped in Adobe Photoshop to prepare the final figures.

3.6 Figures and Legends

Figure 3.1: Myotubes develop gradual resistance to cytosolic cytochrome *c* induced apoptosis

(A) mC2C12 cells, dC2C12 cells differentiated for 9 days and primary myotubes differentiated for 14 days, were injected with either yeast or bovine cytochrome *c* and rhodamine dextran. **(B)** Photographs of mC2C12 and dC2C12 cells one hour following injection with yeast or bovine cytochrome *c* and rhodamine dextran. Arrows point to inject cells. Scale bars represent 50 um. **(C)** C2C12 cells differentiated for 6, 7 or 9 days (dd = days differentiated) were injected with either yeast or bovine cytochrome *c* and rhodamine dextran. **(D)** Primary myotubes differentiated for 3, 8, 11 or 14 days were injected with either yeast or bovine cytochrome *c* and rhodamine dextran. Cell survival was assessed by morphology at the indicated times following injection. Data are the mean ±SEM of $n \geq 3$ separate experiments per time point.

Figure 3.1

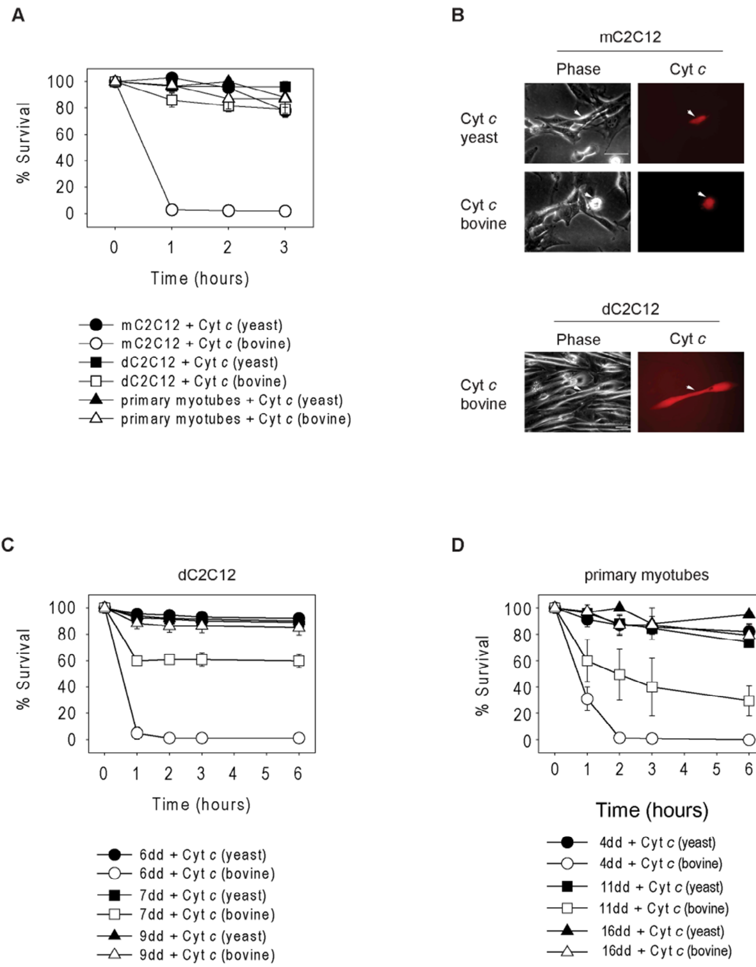


Figure 3.2: Resistance to cytosolic cytochrome *c* is mediated by endogenous IAPs

(A) dC2C12 cells were injected with rhodamine dextran and either bovine cytochrome *c*, wildtype AVPI-Smac, mutant MVPI-Smac or AVPI-Smac and cytochrome *c* together. **(B)** Primary myotubes were injected with rhodamine dextran and bovine cytochrome *c* or AVPI-Smac and bovine cytochrome *c*. Cell survival was assessed by morphology at the indicated times following injection. Data are the mean±SEM of $n \geq 3$ separate experiments per time point.

Figure 3.2

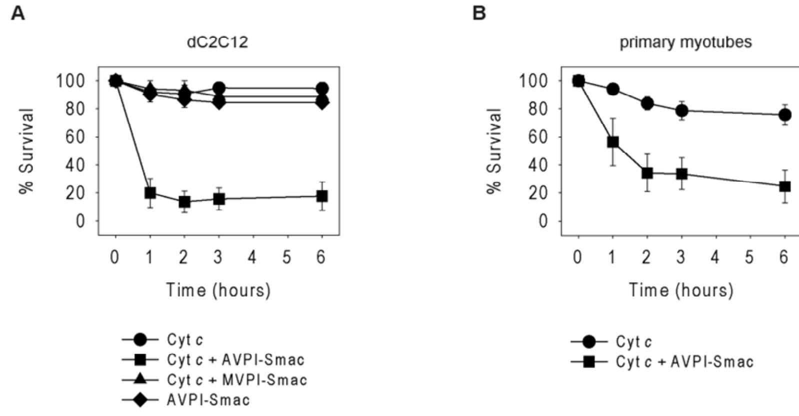


Figure 3.3: Endogenous XIAP mediates cytosolic cytochrome *c* mediated death in myotubes

XIAP-deficient (XIAP-/-) myotubes were injected with rhodamine dextran and either bovine or yeast cytochrome *c*. Cell survival was assessed by morphology at the indicated times following injection. Data are the mean±SEM of n≥3 separate experiments per time point.

Figure 3.3

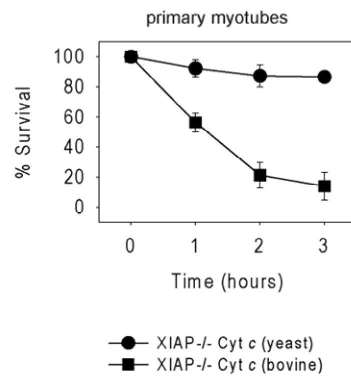


Figure 3.4: Apaf-1 levels are decreased in myotubes. Restoring Apaf-1 is sufficient to allow cytochrome *c* mediated death

(A) Levels of the indicated apoptotic proteins were examined by Western blot of whole cell lysate from mC2C12 and dC2C12 cells. Tubulin serves as a loading control. **(B)** Levels of the indicated apoptotic proteins were examined by Western blot of whole cell lysate from primary myoblasts and primary myotubes. **(C)** RT-PCR was carried out with primers for the indicated mRNA using RNA from primary myoblasts and myotubes. GAPDH serves as a control. **(D)** dC2C12 cells were injected with plasmids for Apaf-1 (Apaf) or empty vector as well as GFP. 24h following injection, GPF positive cells were injected with rhodamine dextran and either yeast or bovine cytochrome *c*. Cell survival was assessed by morphology at the indicated times following cytochrome *c* injection. Data are the mean±SEM of n≥3 separate experiments per time point.

Figure 3.4

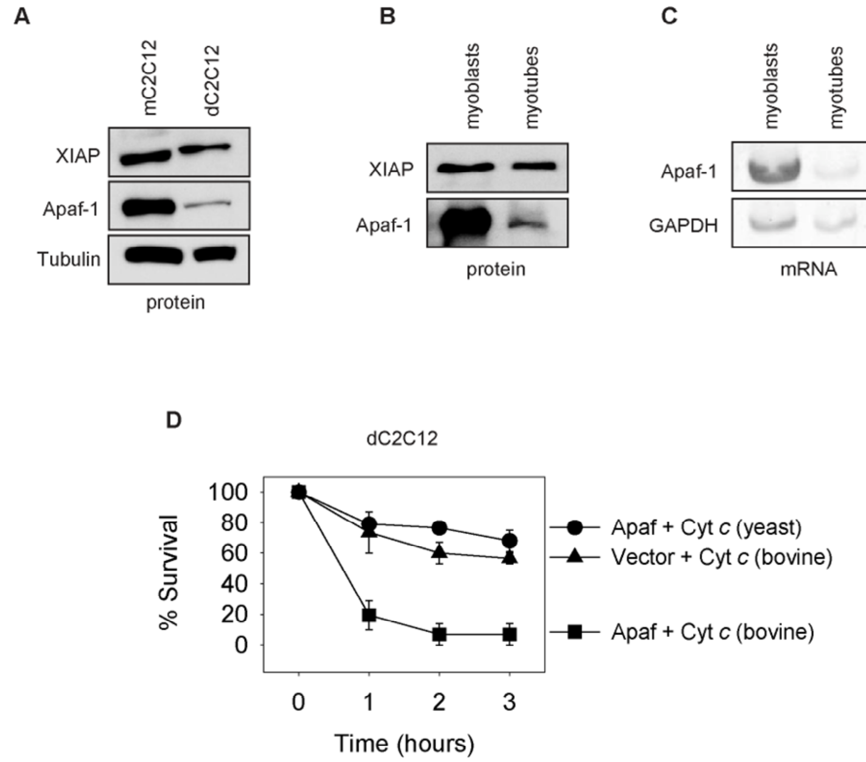
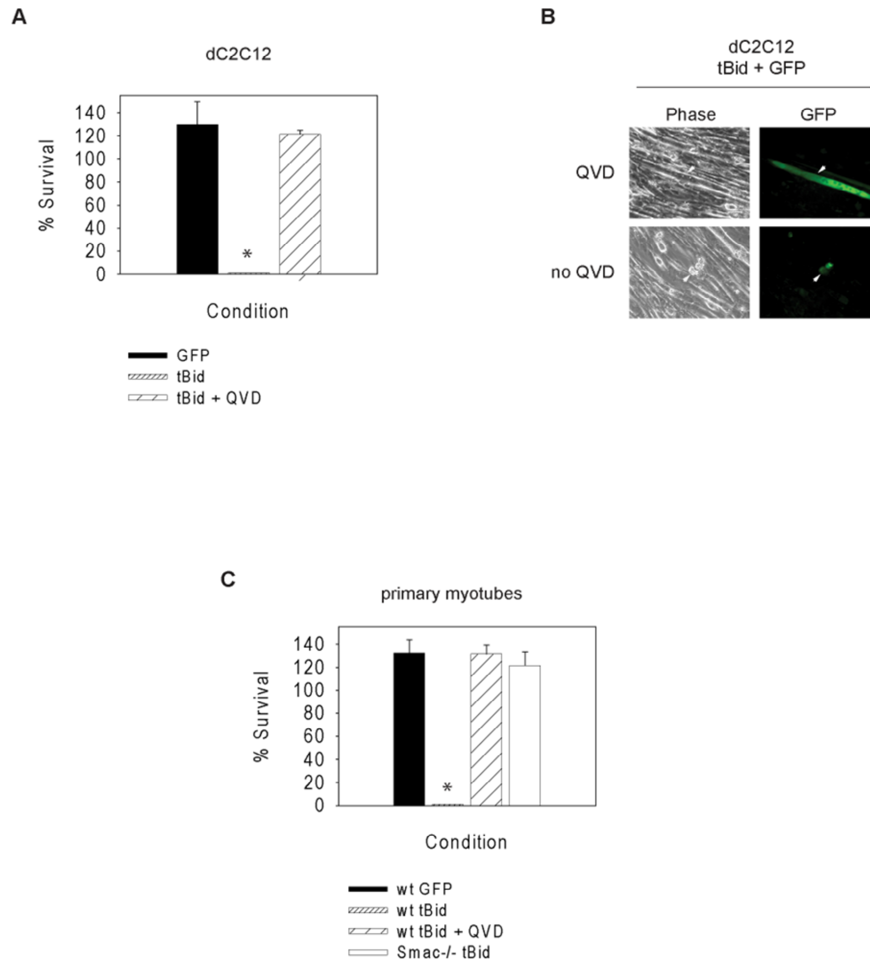


Figure 3.5: tBid causes caspase inhibitable death in myotubes that requires endogenous Smac

(A) dC2C12 were injected with plasmids for tBid or empty vector as well as GFP in the presence or absence of Q-VD-OPH (QVD). GFP expressing cells were counted 8h and 24h following injection. Percent survival was expressed as the percent of cells at 8h that remained alive at 24h following injection. Cell survival was assessed by morphology. Asterisk indicates that the actual survival was 0 %. **(B)** Photographs of mC2C12 dC2C12 cells eight hours following injection with tBid and GFP in the presence or absence of Q-VD-OPH (QVD). Arrows point to inject cells. Scale bars represent 50 um. **(C)** Wild type (wt) and Smac-deficient (Smac-/-) primary myotubes were injected and assessment of survival was the same as in (A). Asterisk indicates that the actual survival was 0 %. Data are the mean ±SEM of $n \geq 3$ separate experiments per time point.

Figure 3.5



CHAPTER FOUR

DISCUSSION AND FUTURE DIRECTION

4.1 Summary of Findings

ER stress induced apoptosis requires Bax for commitment and Apaf-1 for execution in sympathetic neurons

1. Tunicamycin causes ER stress induced apoptosis in sympathetic neurons.
2. JNK signaling is required for ER stress induced neuronal apoptosis.
3. BH3-only proteins, Puma and DP5, are transcriptionally upregulated independently of MLK signaling following ER stress in sympathetic neurons.
4. Bax deficiency blocks ER stress induced apoptosis allowing for long term survival of sympathetic neurons.
5. ER stress induced commitment to death occurs well before cytochrome *c* release and is blocked by Bax deficiency and inhibition of JNK signaling.
6. Apaf-1 deficient sympathetic neurons are able to release cytochrome *c* but do not undergo ER stress induced apoptosis.

Decreased apoptosome function with skeletal muscle differentiation enables strict XIAP regulation of apoptosis

1. Cytosolic cytochrome *c* is sufficient to induce caspase activation and apoptosis in mC2C12 cells but not in dC2C12 or primary myotubes.
2. Resistance develops gradually with increased differentiation.
3. The increased resistance to cytochrome *c* in myotubes is mediated by endogenous IAPs, specifically XIAP.

4. The ability of XIAP to selectively regulate death in myotubes but not in mC2C12 is not due to a change in XIAP expression but due to a specific decrease in Apaf-1 expression in myotubes.
5. Levels of Apaf-1 decrease in differentiating myotubes, allowing endogenous levels of XIAP to effectively regulate apoptosis.
6. Cytochrome *c* can induce apoptosis in myotubes if the function of XIAP is overcome by functional inactivation, selective degradation or an increase in Apaf-1 levels.

Myoblasts utilize a novel alternative mitochondria mediated pathway to activate caspases

1. Cytochrome *c* is not sufficient to induce caspase activation in primary myoblasts.
2. Unlike myotubes, the resistance of myoblasts to cytochrome *c* is not due to the function of XIAP.
3. Apoptosis of myoblasts occurs in response to mitochondria permeabilization.
4. Mitochondria mediated myoblasts apoptosis does not require Apaf-1.

4.2 Discussion of the Major Findings and Future Directions

ER stress induced apoptosis requires Bax for commitment and Apaf-1 for execution in sympathetic neurons

ER stress has been implicated as the underlying cause of the death of many neurodegenerative disorders (Lindholm et al., 2006; Oyadomari and Mori, 2004). As a result, the pathway of ER stress induced apoptosis has been the subject of much study. The identification of an ER specific caspase, caspase 12, led to the model of a mitochondria and Apaf-1 independent pathway of caspase activation (Breckenridge et al., 2003). However, there are several problems with this proposed pathway. First, two caspase 12 deficient lines of mice have been generated and MEFs from only one of the lines shows resistance to ER stress inducing agents (Nakagawa et al., 2000; Saleh et al., 2006). In addition, the caspase 12 deficient MEFs that do show resistance only exhibit mild protection (Nakagawa et al., 2000). Second, the human caspase 12 has acquired a point mutation which makes it unable to carry out its protease function (Fischer et al., 2002). Third, the model developed suggests that active caspase 12 cleaves caspases 9 directly in order to activate caspase 9 (Morishima et al., 2002). However, cleaved caspase 9 does not show catalytic activity. Rather, caspase 9 shows high catalytic activity when in an oligomerized form, which requires the function of a scaffold protein, such as Apaf-1 (Bao and Shi, 2007).

Here we examined the pathway of caspase activation in primary sympathetic neurons in response to ER stress. We did not find evidence of caspase 12 mediated death but rather sympathetic neurons utilized the mitochondria mediated intrinsic pathway of apoptosis. This pathway requires JNK signaling, as both a MLK inhibitor (CEP-11004) and a JNK inhibitor

(SP600125) were able to prevent ER stress induced apoptosis (Fig. 2.2, 2.3, 2.5). However, JNK signaling was not responsible for the upregulation of the BH3-only proteins, DP5 and Puma, seen in neurons treated with tunicamycin (Fig. 2.3). Following their upregulation, the BH3-only members act through Bax, to release cytochrome *c* (Fig. 2.6). However, ER stress commits neurons to die before cytochrome *c* release. This commitment step requires both Bax activity and JNK signaling (Fig. 2.5). Consistent with the requirement of Bax for commitment, Bax deficiency allows neurons to survive long term even in the continued presence of ER stress (Fig. 2.4, Supp. Fig. 2.4). Most importantly, Apaf-1 was required for sympathetic neurons to undergo ER stress induced apoptosis (Fig. 2.6). These results identified an essential and non-redundant role of the Apaf-1 dependent apoptosome pathway in mediating ER stress induced neuronal apoptosis.

How do sympathetic neurons become committed to die during ER stress induced apoptosis?

Neurons exposed to ER stress become committed to die long before they release cytochrome *c* (Fig. 2.5). This result is in contrast to NGF deprivation, in which commitment to death occurs coincident with the mitochondrial release of cytochrome *c* (Deshmukh et al., 2000). In this respect, ER stress appears similar to DNA damage, which also commits neurons to die early, prior to cytochrome *c* release (Besirli et al., 2003). However, this is one interesting difference in the commitment points of death between DNA damage and ER stress. As a result of DNA damage, Bax deficient neurons still become committed to die (Besirli et al., 2003). In striking contrast, Bax deficient neurons exposed to ER stress fail to become committed to die (Fig. 2.5). These results show that during ER stress, Bax activation is an absolute requirement for neuronal commitment to death.

While Bax function at the mitochondria is well characterized, Bax has also been localized to the ER in fibroblasts and cell lines (Nutt et al., 2002a; Scorrano et al., 2003; Zong et al., 2003). Therefore, this difference in the requirement of Bax for neuronal commitment to death in response to ER stress, but not DNA damage, could be indicative of a critical function of Bax at the ER. Bax localized to the ER is thought to be responsible for maintaining Ca2+ homeostasis. Bax/Bak DKO cells have lower levels of luminal Ca2+, which is cytoprotective (Nutt et al., 2002a; Scorrano et al., 2003). Therefore, it is possible that the release of Ca2+ from the ER is the actual commitment step for neurons to undergo ER stress induced apoptosis. Future experiments could be designed to specifically localize Bax to the ER in Bax deficient neurons. As a result, ER luminal Ca2+ levels would be restored but cytochrome *c* could not be released. If commitment studies showed death of these neurons it this would imply that the function of Bax at the ER is directly responsible for committing the cells to die.

Bax deficient neurons exhibit no morphological signs of degeneration even after 11 days of sustained exposure to tunicamycin (Fig. 2.4). These neurons are smaller than untreated neurons presumably due to the global inhibition of protein synthesis that occurs as part of the ER stress response (Fig. 2.4) (Wu and Kaufman, 2006). Electron microscopy performed on Bax deficient neurons sustained for 11 days with tunicamycin showed a change in their ultrastructure. The ER of these treated neurons was vastly dilated. There was also the presence of large vacuoles with electron dense matter (App. Fig. B1). While it is surprising that these neurons can survive long term under these conditions, it is possible that they do so by utilizing autophagy, which is known to provide energy in conditions of nutrient deprivation (Levine and Yuan, 2005) and following ER stress (Ferraro et al., 2008).

To further explore this possibility experiments designed to uncover the existence and importance of autophagy in maintaining survival could be preformed. To examine existence, formation of autophagosomes can be examined by the conversion of microtubule-associated protein 1 light-chain (LC3)-I to the lipidated form LC3-II by Western blot or by the aggregation of LC3-II *in vivo*. If autophagy is aiding in this long term survival, using chemical inhibitors or knocking down vital autophagy genes would be expected to cause ER stress treated Bax deficient neurons to die.

How is the function of XIAP overcome to allow neuronal death during ER stress?

In chapter two, I established that sympathetic neurons utilize the intrinsic pathway of apoptosis in response to ER stress, not the canonical caspase 12 pathway. Previously published work uncovered the existence of increased resistance to caspase activation in sympathetic neurons undergoing the intrinsic pathway. Unlike mitotic cells, neurons require not only cytochrome *c* release but they also relief of XIAP function in order to activate caspases (Potts et al., 2003; Wright et al., 2004). In response to nerve growth factor deprivation or DNA damage, sympathetic neurons selectively degrade XIAP or upregulate Apaf-1, respectively (Potts et al., 2003; Vaughn and Deshmukh, 2007). Increasing Apaf-1 levels allows for increased levels of caspase activation, which overwhelms the caspases inhibitory activity of endogenous XIAP and allows death to be carried out (Vaughn and Deshmukh, 2007; Wright et al., 2004). Alternatively, XIAP can be inhibited by a factor such as the endogenous IAP inhibitor Smac.

Unpublished work found in Appendix A shows that XIAP levels do not change but rather, Apaf-1 levels increase at the transcriptional level in response to lethal doses of ER

stress (App. Fig. A1). This suggests that Apaf-1 upregulation is responsible for overcoming XIAP function, whether this upregulation is necessary or sufficient has not been established. Studies using cycloheximide are unlikely to be useful since they would not only block Apaf-1 upregulation but also block the protein production required to induce the UPR and thus, alleviate the stressor. In addition, experiments using Apaf-1 deficient sympathetic neurons would not be meaningful. As Apaf-1 is required for the death, its absence would block death regardless of whether an upregulation of Apaf-1 was required or not (Fig 2.6). These experiments have also not ruled out the possibility that XIAP is functionally inhibited upon ER stress. One possible candidate for this job is Smac, an endogenous inhibitor of IAPs. Future experiments will test if Smac deficient sympathetic neurons are sensitive to ER stress.

Implications for neurodegeneration

Apoptosis has been found to be responsible for the loss of neurons in various neurodegenerative disorders as well as following ischemia and reperfusion. Importantly, the initiating trigger of neuronal apoptosis in these conditions is ER stress (Lindholm et al., 2006; Oyadomari and Mori, 2004). Therefore, understanding the ER stress induced apoptotic pathway is critical for developing rational therapeutic strategies to ameliorate neurodegeneration.

In chapter two, I established that ER stress induced neuronal apoptosis utilizes the mitochondria mediated pathway and not a mitochondria independent caspase 12 pathway, as previously suggested. As a result of these findings it would be unwise to develop strategies to block caspase 12 activation. While my results have not ruled out a role for caspase 12 in ER stress induced apoptosis, they have questioned its significance. First, the role of caspase

12 in activating caspase 9 directly may not be significant because the apoptosome is activated in response to ER stress. Therefore, blocking caspase 12 activation would still allow caspase 9 to become activated on the apoptosome and go on to cause neuronal loss. Secondly, I have ruled out the possibility of caspase 12 serving as a link between the ER and the mitochondria to initiate mitochondria mediated apoptosis. Neurons that have been treated with tunicamycin and the pan-caspase inhibitor, zVAD-fmk, remain viable but have released their cytochrome *c* from the mitochondria (data not shown). This suggests that the link between the ER and the mitochondria is not mediated by caspases 12 or any other caspase.

The key finding from this work that is important for developing therapeutics is the understanding of neuronal commitment to die. Blocking upstream of the commitment step will effectively block death and the neurons will recover. On the other hand, blocking downstream of this step will only serve to delay death until another non-apoptotic form of death is initiated. This commitment step appears to occur much earlier in the apoptotic pathway when initiated by ER stress compared to NGF deprivation. During ER stress, commitment requires the function of Bax at the ER and JNK signaling, which can also be activated at the ER (Fig. 2.5) (Nishitoh et al., 2002; Urano et al., 2000b). Therefore, rational strategies should be developed to block activation of Bax or JNK. Interfering with the IRE1/TRAF2/ASK1 complex responsible for activating JNK signaling during ER stress (Nishitoh et al., 2002; Urano et al., 2000b), would likely allow recoverability. Whereas, inhibiting the pathway downstream of these events, such as abolishing cytochrome *c* release, would result in delayed death and is not expected to be a viable strategy for long term survival.

Decreased apoptosome function with skeletal muscle differentiation enables strict XIAP regulation of apoptosis

The studies preformed in chapter three identify that postmitotic myotubes have increased suppression of their apoptotic pathway relative to their mitotic precursors (Fig. 3.1). Differentiated C2C12 cells and primary myotubes were both resistant to cytosolic cytochrome *c* due to the function of endogenous XIAP (Fig. 3.2, 3.3). However, myotubes inhibit caspase activation not by increasing XIAP levels but rather by transcriptional downregulating Apaf-1 levels (Fig. 3.4). Importantly, overexpressing Apaf-1 is sufficient to allow cytosolic cytochrome *c* injection to kill dC2C12 cells (Fig. 3.4). Therefore, Apaf-1 reduction alone is sufficient to set up this differential resistance between mitotic precursors and myotubes.

Based on these data, we propose that following cytochrome *c* release from the mitochondria, the low levels of Apaf-1 present in myotubes results in reduced apoptosome formation and caspase activation. As a consequence, endogenous XIAP levels are sufficient to effectively block this reduced level of caspase activation. However, in mC2C12 cells, high levels of Apaf-1 results in robust apoptosome formation and thus increased caspase activation that cannot be successfully inhibited by endogenous XIAP. As a consequence, these mitotic cells readily undergo apoptosis with cytosolic cytochrome *c* while myotubes do not.

The data shows that in myotubes, the function of XIAP can be overcome to allow for caspase activation. This can occur through one of three identified ways. First, as XIAP-deficient myotubes are completely sensitive to cytosolic cytochrome *c* (Fig. 3.3), a stimulus which is able to degrade or cleave XIAP and release cytochrome *c* would be able to kill

myotubes. In fact, selective XIAP degradation has been seen in neurons undergoing apoptosis in response to nerve growth factor withdrawal (Potts et al., 2003).

Second, dC2C12 cells overexpressing Apaf-1 became sensitive to cytochrome *c* mediated death (Fig. 3.4). Therefore, a death stimulus that increases Apaf-1 expression would be able to overcome XIAP inhibition. Supporting this idea, it has been shown that skeletal muscle undergoing caspase activation due to metabolic deficiencies with ATP-depletion and oxidative stress show an increase in Apaf-1 levels (Ikezoe et al., 2002).

Third, XIAP could be functionally inhibited in response to a death stimulus. This could occur through several different mechanisms including an inactivating posttranslational modification of XIAP or via an endogenous inhibitor of XIAP. While the role of Smac as an IAP inhibitor has been extensively characterized *in vitro* (Chai et al., 2000; Du et al., 2000) the role of endogenous Smac has remained elusive since most cells do not need to inhibit XIAP to die (Harlin et al., 2001; Okada et al., 2002). Here we show that endogenous Smac is required for myotube death initiated by the permeabilization of the mitochondria outer membrane (Fig. 3.5). Interestingly, other than myotubes, the only cell type in which a role for endogenous Smac has been identified is their precursor, myoblasts (Ho et al., 2007). This raises the intriguing possibility that endogenous Smac may play a vital role in this particular myogenic cell lineage but not in other cell types.

Increased apoptotic regulation is common to postmitotic cells and usurped by some cancers

The increased resistance to apoptosis employed by myotubes is strikingly similar to that found in other postmitotic cells, specifically neurons and cardiomyocytes (Potts et al., 2005; Potts et al., 2003; Wright et al., 2004). These findings suggest that regardless of the

function or phenotype of the cell, postmitotic cells share the same features in restricting their ability to undergo apoptosis. By requiring not only cytochrome *c* release but also inhibition of XIAP function in order to activate caspases, endogenous XIAP serves as a safety brake to death. For example, if the mitochondria accidentally release cytochrome *c*, the presence of XIAP would block caspase activation and therefore prevent these cells from undergoing unwanted apoptosis. Arguably, increased resistance to caspase activation is critical to postmitotic cells because of their inability to replicate and their limited regenerative potential. These postmitotic cells also serve vital functions that require their presence for the lifetime of the organism.

On the other hand, not having this resistance to apoptosis in mitotic cells is beneficial to the organism as mitotic cells can give rise to cancer. Indeed, the mechanisms by which the apoptotic pathway is inactivated in many cancers are similar to those seen in postmitotic cells. For example, several melanomas and leukemias restrict their Apaf-1 expression at the transcriptional level in order to block apoptosis (Fu et al., 2003; Furukawa et al., 2005; Soengas et al., 2001). In addition, many chemoresistant cancers have been found to utilize XIAP to effectively block caspase activation (Beauparlant and Shore, 2003).

Therefore an understanding of how postmitotic cells regulate their apoptotic pathway can lead to the development of therapeutic strategies that can evoke death in mitotic cells, such as cancerous cells, but spare postmitotic cells that can not be replaced. In addition, the identification of how postmitotic cells restrict apoptosis may also uncover ways in which cancerous cells may do so as well.

A role for increased regulation of skeletal muscle apoptosis in muscular disease

The potential significance of this XIAP brake can be seen in mitochondrial encephalomyopathies, a group of heterogeneous disorders caused by mutations in either mitochondrial DNA or nuclear genes. These mutations lead to mitochondrial abnormalities, which ultimately result in a decrease in ATP synthesis and increased oxidative stress. Due to the heterogeneous nature of these deficiencies even within the same patient, some muscle fibers appear healthy, some appear to suspend apoptosis and others seem to die with active caspase 3 immunoreactivity (Aure et al., 2006; Ikezoe et al., 2002; Mirabella et al., 2000). Many fibers from mitochondrial encephaolmyopathies show clear Bax upregulation and cytochrome *c* release. However, the number of fibers that show TUNEL staining is much lower (Ikezoe et al., 2002), suggesting that in these fibers cytochrome *c* is released but cannot undergo caspase mediated death. Our data would predict that in these fibers, XIAP is preventing apoptosis despite the mitochondrial release of cytochrome *c*.

To test this prediction, a mouse model of mitochondrial encephalomyopathy could be crossed to a XIAP deficient animal. An increase in the loss of skeletal muscle from the diseased mouse on the XIAP null background would suggest that the increased regulation of apoptosis in skeletal muscle helps protect against unwanted death in the diseased state. In addition, it would imply that XIAP mutation or deficiency could be a risk factor for skeletal muscle degeneration.

Myoblasts utilize a novel alternative mitochondria mediated pathway to activate caspases

My work and that from the Zacksenhaus lab has uncovered that myoblasts are capable of activating caspase 9 and apoptosis in a mitochondria mediated, but cytochrome *c* and Apaf-1 independent manner (Appendix E) (Ho et al., 2004). Unlike all other known mitotic cells, the introduction of cytosolic cytochrome *c* alone was not sufficient to kill

myoblasts (App. Fig. E1). This is not due to the presence of an XIAP break found in myotubes and other postmitotic cells, as the coinjection of Smac and cytochrome *c* was insufficient to kill myoblasts (App. Fig. E1). However, the expression of tBid, which liberates mitochondria proteins (Willis and Adams, 2005), was able to kill (App. Fig. E2), suggesting that the death program in myoblasts requires the mitochondria. This finding is supported by the published result that blocking mitochondria permeabilization with Cyclosporin A, blocks caspase activation and death in myoblasts (Ho et al., 2004). This mitochondria mediated death does not appear to utilize Apaf-1. Apaf-1 deficient myoblasts still die in response to a variety of cytotoxic insults (Ho et al., 2004) as well as with the introduction of tBid (App. Fig. E3). However, it does require caspase 9, as caspase 9 deficient myoblasts are resistant to cytotoxic stimuli (Ho et al., 2004). Further investigation by the Zacksenhaus group has suggested that this is due to decreased levels of XIAP protein in myoblasts and increased levels of Smac available for release from the mitochondria. They propose that upon mitochondria release, Smac levels are sufficient to overcome XIAP levels and allow caspase 9 activation to occur whether Apaf-1 is present or not (Ho et al., 2007).

This alternative pathway is very intriguing but leaves open the question of how caspase 9 becomes activated independently of Apaf-1. It has been suggested that there is a pool of procaspase 9 that resides in the mitochondria (Krajewski et al., 1999). Zacksenhaus suggests that when mitochondria become permeabilized they may release this pool and Smac leading to an increase concentration of procaspase 9 in the cytosol as well as inhibition of XIAP. Without procaspase 9 being antagonized by XIAP, the concentrations may be sufficient to cause caspase 9 autodimerization and activation.

An alternative possibility is that myoblasts contain an additional Apaf-1 like molecule that can scaffold and activate caspase 9. This molecule could be sequestered away in the mitochondria and released upon mitochondria permeabilization or it could be present in the cytosol but activated by a released mitochondrial factor in a manner akin to the activation of Apaf-1 by released cytochrome *c*. There are no sequences in the human genome that have all of the domains found in Apaf-1. However, there are homologues that contain a CARD, a nucleotide binding domain and leucine reach repeats (LRR) instead of WD 40 repeats. Currently these proteins are known to be involved in caspase activation in innate immunity (Ting et al., 2008). Potentially, one of these family members could substitute for Apaf-1 using a ligand other than cytochrome *c*. Whether an Apaf-1 homologue is involved or a completely novel protein, it is likely that this protein would contain a CARD to bind to caspase 9. Therefore, future studies would include looking for caspase 9 CARD binding partners. This could be achieved in a number of ways. Using caspase 9 as bait, binding partners could be identified by yeast-two hybrid or by immunoprecipitation or pulldown followed by mass spectrometry. Once identified, this protein could be knocked down in myoblasts to see if this would block mitochondria mediated death. This interacting protein could also be overexpressed in Apaf-1 deficient MEFs to see if it could reinstate caspase activation in the absence of Apaf-1.

If Apaf-1 is present, why do myoblasts not utilize it to die?

Despite the evidence that Apaf-1 does not participate in myoblast apoptosis, Apaf-1 protein is expressed in myoblasts (Ho et al., 2004). These results led us to wonder why Apaf-1 is not utilized to activate caspases in myoblasts. It is possible that there is some

inactivating posttranslational modification of Apaf-1 in myoblasts which makes Apaf-1 less efficient or incapable of forming a functional apoptosome. Preliminary evidence for this comes from the finding that Apaf-1 is phosphorylated in chronic myelogenous leukemia. This phosphorylation may lead to the impairment of apoptosome formation and resistance to apoptosis seen in this type of cancer (Deming et al., 2004).

Alternatively, Apaf-1 could be completely functional and cytochrome *c* in myoblasts could be modified. Modifications to cytochrome *c* have been shown to drastically affect its ability to activate the apoptosome. Cytochrome *c* lacking its heme group or lacking the nitrosylation of its heme group are ineffective at activating caspases (Martin and Fearnhead, 2002; Martin et al., 2004; Schonhoff et al., 2003).

Why have myoblasts developed an alternative caspase activation pathway?

Overall, the possibility of an alternative Apaf-1 independent pathway of caspase activation is exciting. The phenotype of the Apaf-1 and cytochrome *c* knockin mice (K72A mutation which makes cytochrome *c* incapable of activating the apoptosome) supports the idea of an alternative Apaf-1 independent pathway to apoptosis (Cecconi et al., 1998; Hao et al., 2005; Yoshida et al., 1998). These mice have severe neurodevelopmental phenotypes with overgrowth of the brain that can be expected to occur with a total lack of apoptosis. However, besides the neuronal phenotype, for the most part the mice develop normally. This suggests that there may be Apaf-1 independent apoptotic pathways that can occur during development.

Why myoblasts would have developed a separate pathway is still a mystery. Myoblasts differentiation has been found to involve the activation of caspase 3 (Fernando et al., 2002). Therefore, one possibility is that an alternative pathway arose to activate caspases so that the myoblasts can have one way of activating caspases for executing apoptosis and another for initiating differentiation, without killing themselves. If this were the case, it would be interesting to look to see if the same pathway exists in developing neurons. It has been found that caspases are activated in the growth cone of migrating neurons and are responsible for dendritic pruning during development in *Drosophila* (Kuranaga and Miura, 2007). Therefore, alternative caspase activation pathways may be responsible for activating caspases to carry out non-apoptotic functions.

APPENDIX A

Sympathetic neurons upregulate Apaf-1 in response to ER stress: Mechanism to become competent to die

In chapter two, I established that sympathetic neurons do not bypass the mitochondria to activate caspases. Rather, in response to ER stress they go through the classic Apaf-1 dependent intrinsic apoptotic pathway in order to die. Previously published work from the Deshmukh lab established that sympathetic neurons undergoing the intrinsic pathway of caspase activation must release cytochrome *c* and relieve XIAP inhibition (Deshmukh and Johnson, 1998; Potts et al., 2003; Wright et al., 2004). In response to nerve growth factor deprivation, sympathetic neurons selectively degrade XIAP, which then allows cytochrome *c* release to kill (Potts et al., 2003) (App. Fig. A1). On the other hand, etoposide treated neurons overcome XIAP by upregulating Apaf-1. Increasing Apaf-1 levels increases the level of caspase activation that can occur with the introduction of cytochrome *c*, thus overwhelming endogenous XIAP and allowing death to occur (Vaughn and Deshmukh, 2007). Alternatively, XIAP could be inhibited by a factor such as the endogenous IAP inhibitor Smac.

To determine how XIAP function was overcome in response to lethal doses of the ER stress, sympathetic neurons were treated with tunicamycin (TU) and the levels of XIAP and Apaf-1 were examined at various timepoints. Unlike NGF deprivation, no XIAP degradation was seen with ER stress. However, Apaf-1 protein levels were found to increase (App. Fig. A1). RT-PCR revels that this increase in Apaf-1 occurs at the mRNA level, indicating that Apaf-1 is transcriptionally upregulated in response to ER stress (App. Fig A1).

There are two known transcription factors for Apaf-1, E2F1 and p53 (Fortin et al., 2001; Moroni et al., 2001). In response to DNA damage, p53 is responsible for upregulating Apaf-1 in sympathetic neurons (Vaughn and Deshmukh, 2007). However, experiments utilizing p53 null mice showed that p53 deficient sympathetic neurons were just as sensitive to tunicamycin as neurons from their wildtype littermates (Supp. Fig. 2.3).

Together, these experiments suggest that XIAP is not overcome by degrading XIAP. Instead, like DNA damage, ER stress causes Apaf-1 upregulation, which may overcome XIAP function causing neurons to become competent to die. However, unlike DNA damage, ER stress does not require p53 to upregulate Apaf-1.

The upregulation of Apaf-1 that does not occur by p53 activity could be the result of E2F1 activity. To test this, dominant negative forms of E2F1 or knockdown of E2F1 could be used to see if this would prevent the Apaf-1 upregulation and neuronal death in response to ER stress.

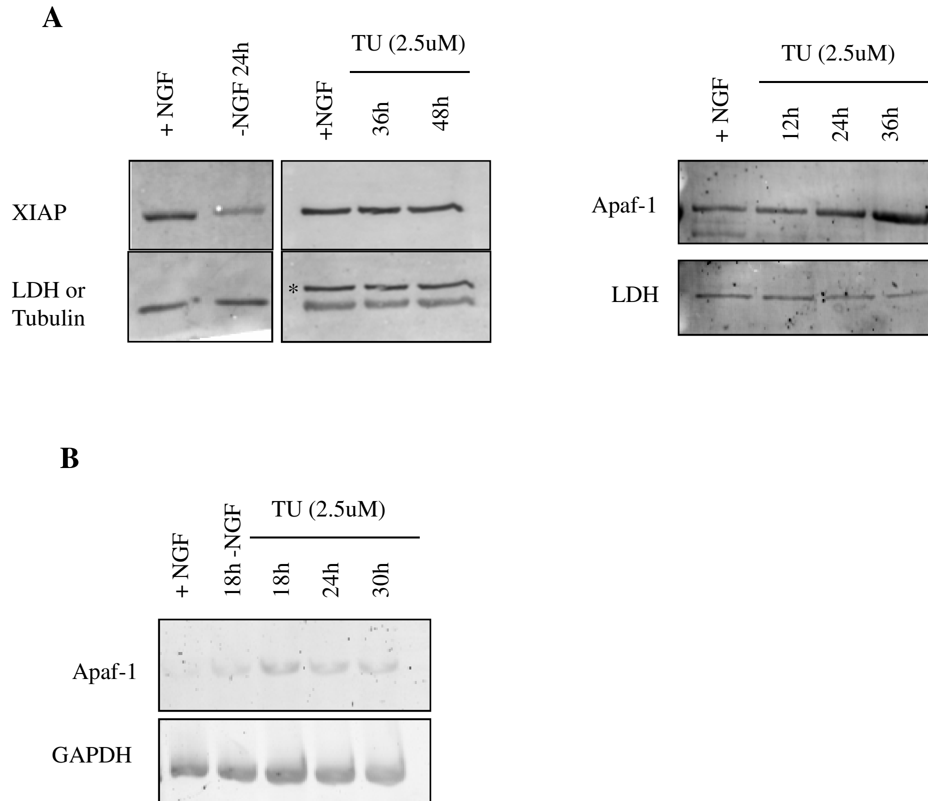
While the results presented so far suggest that Apaf-1 upregulation is responsible for overcoming XIAP function, whether this upregulation is necessary or sufficient has not been established. Studies using a protein translation inhibitor, cycloheximide, are unlikely to be useful since they would not only block Apaf-1 upregulation but also block the protein production required to induce the UPR and thus alleviate the stressor. In addition the use of Apaf-1 deficient neurons to show that the upregulation is required is also not an option. Apaf-1 is required for the death; its absence would block death regardless of whether Apaf-1 upregulation was required or not (Fig. 2.6). It is still possible that XIAP is functionally inhibited by ER stress. One possible candidate for this job is Smac, an endogenous inhibitor

of IAPs. Future experiments will utilize the Smac deficient mice to test whether Smac deficient sympathetic neurons are sensitive to ER stress.

Figure A1: ER stress induced sympathetic neurons maintain XIAP levels and transcriptionally upregulate Apaf-1

Sympathetic neurons were left untreated (+NGF) deprived of NGF (-NGF) or treated with tunicamycin (TU) (2.5uM) for varying lengths of time. **(A)** Levels of the indicated apoptotic proteins were examined by Western blot of whole cell lysates. Tubulin and LDH serve as loading controls. Asterisk labels a non-specific band. **(B)** RT-PCR was carried out with primers for the indicated mRNA. GAPDH serves as a control.

Figure A1



APPENDIX B

Bax deficiency provides long term survival against ER stress, which may involve autophagy

Surprisingly Bax deficient neurons are able to survive in the presence of ER stress for extended periods of time. While wildtype neurons are dead by 48 hours, Bax deficient neurons survive well past 11 days (Fig. 2.4). This survival is especially dramatic considering that protein synthesis has been shut down by the UPR and new transporters, such as glucose transporters, are not being put back onto the cell surface (Chang and Korolev, 1996).

Electron microscopy was used to examine the ultrastructural features of Bax null sympathetic neurons left untreated or treated with tunicamycin for 11 days. Compared to the untreated neurons, prolonged treatment caused dilation of the ER lumen as well as the production of additional electron rich vacuoles (App. Fig. B1). While the identity of these vacuoles has not been established it is possible that they are autophagosomes involved in autophagy.

Autophagy is described as a cell survival mechanism that occurs primarily in response to nutrient starvation. Autophagy provides glycolysis generated ATP to the starved cell by degrading the cells own cellular components (Levine and Yuan, 2005). Autophagy has already been shown to occur in ETNA cells (murine embryonic telencephalic naïve cells) in response to tunicamycin. In ETNA cells, autophagy was found to be responsible for maintaining ATP levels and prolonging cell survival (Ferraro et al., 2008). Therefore, it is possible that Bax-/- neurons are able to survive long term by inducing autophagy.

Future experiments should uncover the existence and importance of autophagy in maintaining survival. To examine existence, formation of autophagosomes can be examined by the conversion of microtubule-associated protein 1 light-chain (LC3)-I to the lipidated form LC3-II by Western blot or by the aggregation of LC3-II *in vivo*. To determine whether autophagy is aiding to the long term survival of ER stress treated neurons, autophagy could be blocked by chemical inhibitors or knocking down vital autophagy genes. If autophagy is important, its inhibition would be expected to allow the death of ER stress treated Bax deficient neurons.

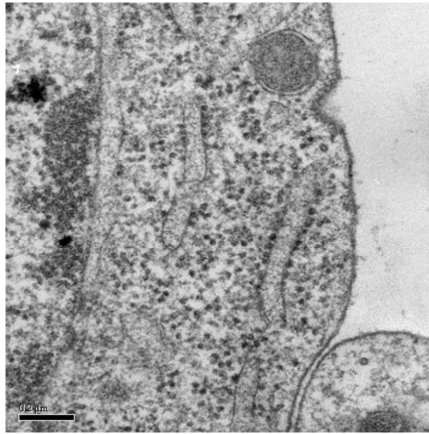
Figure B1: Prolonged ER stress causes changes in the ultrastructural features of Bax deficient sympathetic neurons

Bax deficient (Bax-/-) sympathetic neurons were left untreated or treated with tunicamycin (2.5uM) for 11 days and examined by electron microscopy. Treated neuron show dilated ER (arrows) and unusual electron dense structures (arrow heads).

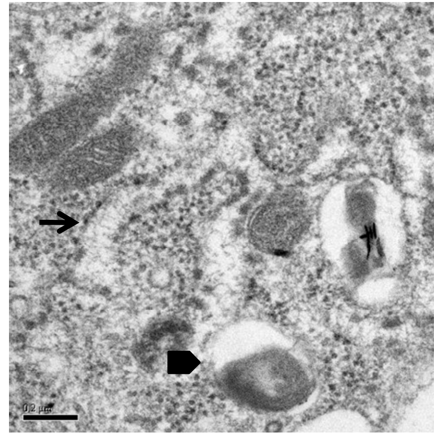
Figure B1

Bax -/- (11 days)

Untreated



TU



APPENDIX C

Transcriptional regulation of decreased Apaf-1 expression in postmitotic myotubes

In chapter three, I established that dC2C12 cells and primary myotubes have decreased Apaf-1 levels at both the mRNA and protein level (Fig 3.4). This decrease directly couples to the increased effectiveness of endogenous XIAP to inhibit caspase activation and death in these myotubes.

In addition to myotubes, other cells that show decreased Apaf-1 levels show increased resistance to apoptosis. These cells include the other postmitotic cells of the body, neurons and cardiomyocytes (Burgess et al., 1999; Potts et al., 2005; Sanchis et al., 2003; Wright et al., 2004) and some chemoresistant tumors (Fu et al., 2003; Furukawa et al., 2005; Soengas et al., 2001). Mature neurons engage silencing chromatin modifications to shut down Apaf-1 transcription. In order to reverse this effect, mature neurons have to undergo chromatin de-repression and E2F1 transcriptional activity (Wright et al., 2007). Some human melanomas and leukemias silence Apaf-1 by methylating the CpG islands of the Apaf-1 promoter. In this case, the use of methylation inhibitors or inhibition of histone deacetylases (HDAC) is sufficient to allow the upregulation of Apaf-1 and render the cancerous cells sensitive to chemotherapeutics (Fu et al., 2003; Furukawa et al., 2005; Soengas et al., 2001).

In this section, I summarize experiments conducted to try to determine the mechanism of transcriptional regulation responsible for the decrease of Apaf-1 in myotubes. To determine if Apaf-1 decrease was due to methylation, the overall level of DNA methylation in mC2C12 cells versus dC2C12 cells was examined. DNA was isolated from cells and subjected to restriction enzyme digestion. Hpa II and Msp I were used because they

are isoschizomers but Hpa II is methylation sensitive while Msp I is not. Decreased digestion of dC2C12 DNA by Hpa II implied that there was significantly more DNA methylation in dC2C12 cells versus mC2C12 cells (App. Fig. C1).

Methylated CpG islands are recognized by proteins responsible for recruiting HDACs, leading to transcriptional repression. This can be reversed by using DNA methylation inhibitors or the histone deacetylase inhibitor tricostatin A (TSA) (Bird and Wolffe, 1999). dC2C12 were treated with 0.25 uM TSA for 24 hours and subsequently microinjected with cytochrome *c*. Unlike untreated dC2C12 cells, TSA treated cells were rendered sensitive to cytochrome *c* (App. Fig. C2). This death was blocked with the addition of the pan-caspase inhibitor zVAD-fmk, suggesting that this death was in fact an apoptotic death. Therefore, inhibiting deacetylation is sufficient to allow cytochrome *c* to kill.

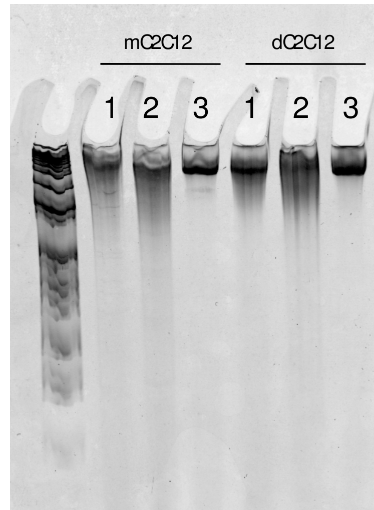
In chapter three, I established that in myotubes Apaf-1 is the limiting component required for cytochrome *c* induced death. Thus, it follows that if TSA is able to restore the ability of cytochrome *c* to induce apoptosis in myotubes it is most likely due to the re-expression of Apaf-1. If this were the case, future experiments probing the protein and mRNA levels would be expected to show an increase in Apaf-1 levels with the addition of TSA.

Additional future experiments would include directly determining if the Apaf-1 promoter was methylated by performing methylation-specific PCR. If the Apaf-1 promoter DNA is methylated, then the addition of methyl transferase inhibitors would be expected to increase Apaf-1 levels and allow cytosolic cytochrome *c* injections to kill.

Figure C1: dC2C12 cell DNA is more methylated than mC2C12 cell DNA

DNA isolated from mC2C12 cells and dC2C12 cells was left undigested or treated with restriction enzymes Hpa II or Msp I. Hpa II is methylation sensitive while Msp I is not.

Figure C1

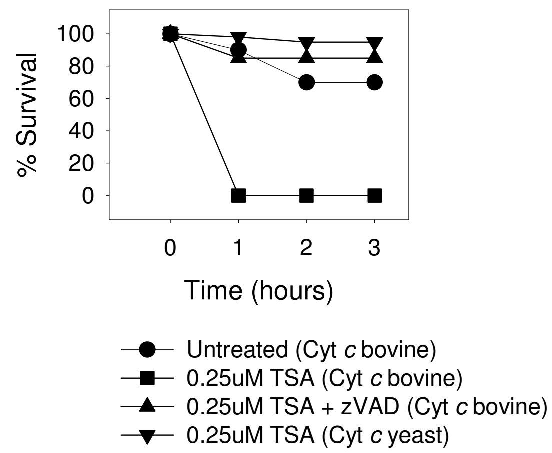


1-Hpa II
2-Msp I (not meth sensitive)
3-no restriction enzyme

Figure C2: HDAC inhibitors induce cytochrome *c* sensitivity in dC2C12 cells

dC2C12 cells were untreated or treated with the HDAC inhibitor TSA (0.25 uM). 24h after treatment, cells were injected with bovine or yeast cytochrome *c* in the presence or absence of the pan-caspase inhibitor zVAD-fmk (50 uM). Survival was assessed by morphology at the indicated times following injection.

Figure C2



APPENDIX D

XIAP function may be overcome by Apaf-1 translational upregulation in response to DNA damage in myotubes

In chapter three, I established that XIAP is efficient at blocking caspase activation in myotubes due to a selective downregulation of Apaf-1. However, there are pathological circumstances including disease and cytotoxic drugs, which cause skeletal muscle to undergo apoptosis (McArdle et al., 1999; Tews, 2002). In addition, I previously established that in order to kill, death stimuli would not only have to release cytochrome *c* from the mitochondria but also overcome XIAP function by one of three means. First, XIAP could be degraded. Second, XIAP could be functionally inhibited or lastly, the levels of Apaf-1 could be upregulated.

To investigate which of these three mechanisms was evoked upon pathological stimuli; dC2C12 cells were treated with varying doses of the DNA damaging agent etoposide and death was accessed by the MTT assay. For all subsequent studies 20 uM etoposide was used. At this concentration, dC2C12 cells died by approximately 120 hours (data not shown). However, at the earlier time points of 48 and 72 hours, dC2C12 cells were largely alive (App. Fig. D1), providing a timeframe in which the effects of etoposide on endogenous XIAP could be explored.

To determine when etoposide caused dC2C12 cells to become competent to die, dC2C12 cells were treated with 20 uM etoposide for varying lengths of time and subsequently injected with cytochrome *c*. Following 24 hours of treatment, dC2C12 cells did not die in response to cytosolic cytochrome *c* microinjection (data not shown). However, by 48 hours of etoposide treatment, dC2C12 cells became sensitive to bovine cytochrome *c*,

and remained unaffected by yeast cytochrome *c* (App. Fig. D2). The pan-caspase inhibitor zVAD-fmk was able to block death implying that the death of these dC2C12 was due to caspase activation and not some other confounding factor. These results suggest that by 48 hours of etoposide treatment, the function of XIAP has been overcome and dC2C12 cells are competent to die.

To determine what was happening to XIAP at this 48 hour timepoint of etoposide treatment the expression of various proteins were examined. The protein and mRNA levels of XIAP did not change between untreated and treated dC2C12 cells (App. Fig. D3). However, Apaf-1 protein levels did increase (App. Fig. D3). Surprisingly, there was not an increase at the mRNA level, suggesting that this increase in Apaf-1 is not likely to be mediated by p53, a DNA damage activated transcription factor known to target Apaf-1 (Fortin et al., 2001; Moroni et al., 2001; Vaughn and Deshmukh, 2007). Together, these results suggest that in response to DNA damage, myotubes do not degrade XIAP but rather translationally upregulate Apaf-1 to overcome the function of endogenous XIAP and allow caspase activation.

While the results presented so far show that Apaf-1 is upregulated whether this upregulation is necessary or sufficient to overcome XIAP has not been established. Studies using cycloheximide to block Apaf-1 translation have not been useful as cycloheximide alone is toxic to dC2C12 cells (data not shown). These experiments have also not ruled out the possibility that XIAP is functionally inhibited upon DNA damage. One possible candidate for this job is Smac, an endogenous inhibitor of IAPs. Future experiments will utilize the Smac deficient mice to test whether Smac deficient myotubes are sensitive to etoposide.

Figure D1: Dose response of etoposide induced death in dC2C12 cells

dC2C12 cells were treated with the indicated doses of etoposide for either 48 or 72 hours. Survival was assessed by MTT assay. Percent survival was normalized to untreated dC2C12 cells.

Figure D1

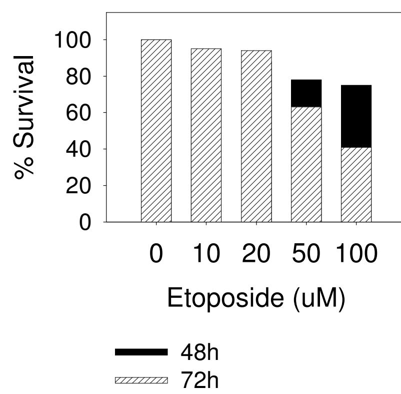


Figure D2: Etoposide treatment permits cytochrome *c* to kill dC2C12 cells

dC2C12 cells were treated with etoposide (20 uM) with or without the pan-caspase inhibitor zVAD-fmk (50 uM). Following 48 hours of treatment, dC2C12 were microinjected with bovine or yeast cytochrome *c* (25 ug/ul). Cell survival was accessed by morphology at the indicated times following injection.

Figure D2

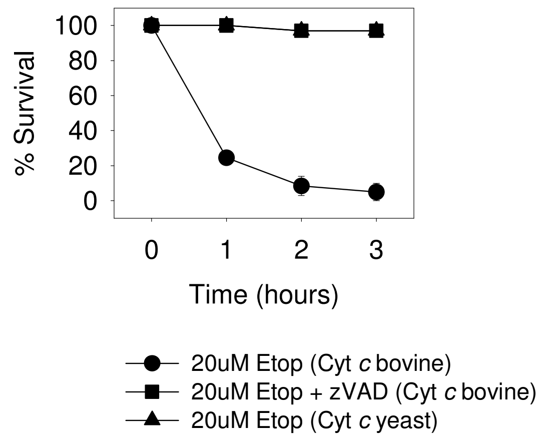
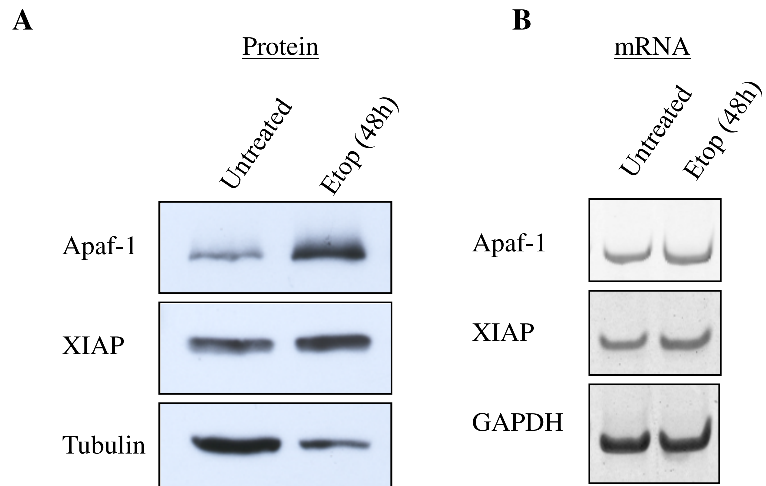


Figure D3: XIAP levels remain constant but Apaf-1 protein levels increase with etoposide treatment of dC2C12 cells

dC2C12 cells were left untreated or treated with etoposide (20 uM) for 48 hours. **(A)** Levels of the indicated apoptotic proteins were examined by Western blot of whole cell lysate. Tubulin serves as a loading control. **(B)** RT-PCR was carried out with primers for the indicated mRNA. GAPDH serves as a control.

Figure D3



APPENDIX E

Mitochondria mediated, Apaf-1 independent pathway of caspase activation in primary myoblasts

Downstream of the mitochondria, all of the components necessary to activate apoptosis, namely Apaf-1, procaspase 9 and caspase 3 are already present in the cytosol. Therefore, the release of cytochrome *c* into the cytosol is the key initiating factor. As a result, the injection of cytosolic cytochrome *c* into individual cells or the addition of cytochrome *c* to cytosolic lysates is sufficient to activate caspases in most cells (Liu et al., 1996). As expected, mitotic C2C12 (mC2C12) cells were found to be sensitive to cytochrome c (Fig. 3.1). Surprisingly, primary mitotic myoblasts were resistant to cytosolic cytochrome *c* microinjection (App. Fig. E1).

In primary myotubes, cytochrome *c* resistance is due to the efficiency of XIAP to inhibit caspase activation. When primary myoblasts were coinjected with cytochrome *c* and Smac they were found to be resistant, suggesting that their resistance was not due the function of IAPs (App. Fig. E1).

tBid was used to determine whether the release of mitochondrial contents was sufficient to induce myoblast apoptosis. tBid is a member of the proapoptotic BH3-only family of Bcl-2 proteins that acts directly through Bax and Bak to cause mitochondria permeabilization and the release of cytochrome *c*, Smac and other mitochondrial factors (Cory and Adams, 2002). Isolated myoblasts were microinjected with plasmids carrying GFP and tBid and the number of cells expressing GFP were counted five hours after injection. The number of GFP positive cells that were still alive 24 hours after injection was used to determine the percent survival. There was a complete loss of tBid injected myoblasts

(App. Fig. E2). However, tBid injected cells could be saved by the addition of the pan-caspase inhibitor zVAD-fmk. This suggests that the death seen in tBid injected myoblasts was due to caspase activation and not simply damage done to the mitochondria. Taken together, the data implies that there is mitochondria mediated death in myoblasts that may be cytochrome *c* independent.

Like wildtype cells, Apaf-1 deficient myoblasts are also completely sensitive to tBid microinjection (App. Fig. E2). This finding is in agreement with a publication which found Apaf-1 deficient, but not caspases 9 deficient, myoblasts undergo apoptosis in response to toxic stimuli (Ho et al., 2004). This work implies that primary myoblasts utilize a unique mitochondria mediated Apaf-1 independent pathway to activate caspases 9. This poses the intriguing question of how caspases 9 becomes activated in myoblasts.

Caspase 9 activation is known to be the result of induced proximity and conformational change. Therefore, it is possible that another adapter protein is present in myoblasts which acts like Apaf-1 to scaffold and activate caspase 9.

In the future, the lab hopes to identify the factor which activates caspase 9 in myoblasts. To address this, we plan to identify caspase 9 binding partners using pulldown or immunoprecipitation experiments followed by mass spectrometry. Alternatively, we could use yeast-two hybrid to identify this protein. Once identified, we would design and implement experiments to identify its localization, activation and the mechanism by which it activates caspase 9.

Figure E1: Primary myoblasts are resistant to cytochrome *c* and Smac induced death

Isolated primary myoblasts were microinjected with yeast or bovine cytochrome *c* (25 ug/uL) with or without the IAP inhibitor Smac (1 ug/ul). Survival was assessed by morphology at the indicated times following injection.

Figure E1

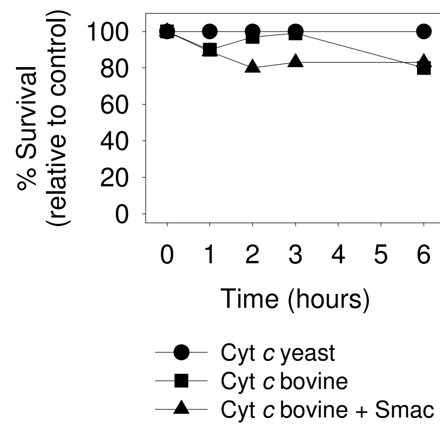
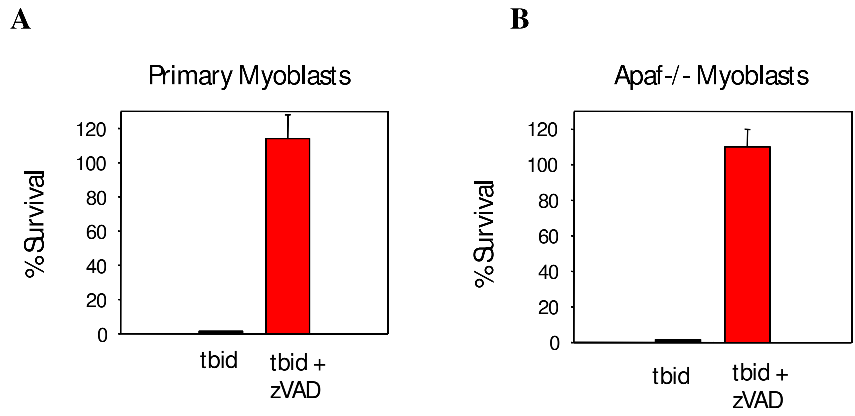


Figure E2: Wildtype and Apaf-1 deficient primary myoblasts are sensitive to tBid induced death

(A) Wildtype or **(B)** Apaf-1 deficient primary myoblasts were microinjected with plasmids for GFP and the BH3-only protein tBid in the presence or absence of the pan-caspase inhibitor zVAD-fmk (50 uM). GFP expressing cells were counted five hours following injection. The number of GFP positive cells that were still alive 24 hours after injection was used to determine the percent survival. Survival was assessed by morphology.

Figure E2



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